厚生労働科学研究費補助金（ヒトゲノム・再生医療等研究事業）
分担研究報告書

骨格筋に対するAAVベクターの安全性の検討

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研究要旨

Duchenne 型筋ジストロフィー（DMD）はジストロフィン欠損によって起こる進行性の筋変性疾患である。その遺伝子治療法を確立するために，我々はAAVベクターを用いたマイクロジストロフィン遺伝子の骨格筋への導入実験を行ってきた。*mdx* マウスでは良好な治療成績を得たので，霊長類を用いて，AAVベクターの有効性と安全性をさらに詳細に検討した。本研究では，従来よく用いられてきたAAV-type 2ベクターにLacZ遺伝子又はマイクロジストロフィン遺伝子を組み込んだものをカニクイザル骨格筋へ導入し，その骨格筋組織と血清を経時的にサンプリングし，導入遺伝子の発現効率，導入遺伝子産物に対する免疫応答を検索し，AAVベクターによる治療が安全であるか検討した。

A．研究目的

アデノ随伴ウイルス（AAV）ベクターは骨格筋への遺伝子導入に適した特性を備えている。我々は，Duchenne 型筋ジストロフィー（DMD）に対する遺伝子治療法を確立するために，AAVベクターを用いたマイクロジストロフィン遺伝子の骨格筋への導入実験を行ってきた。ジストロフィンを欠損する *mdx* マウスにおいてマイクロジストロフィンは長期に発現し，張力も回復していた。しかし，AAVベクターとマイクロジストロフィン遺伝子を臨床応用する前に，よりヒトに近い動物でAAVベクターの導入に伴う細胞毒性／免疫応答を検討し，AAVベクターの有効性と安全性を詳細に検討する必要がある。本研究では，サル骨格筋へAAVベクターを用いてLacZ遺伝子又はマイクロジストロフィン遺伝子を導入し，骨格筋組織と血清を経時的に解析することにより，導入遺伝子の発現効率，導入遺伝子産物に対する免疫応答を検索し，AAVベクターがDMD治療に応用できるか検討する。

B．研究方法

カニクイザルの左右の上腕筋及び前脛骨筋の計4箇所にAAVベクター（3箇所）とPBS（1箇所）を直接注入する。コントロールとしては，導入遺伝子を発現しないpromoter-less AAV vectorを投与する。LacZ遺伝子組換えAAVベクター投与群及びマイクロジストロフィン遺伝子組換えAAVベクター投与群の2群は3頭ずつを設け，コントロール群は2頭を用いる。なお，使用個体の雌雄は問わない。導入1及び2週後に筋組織の生検を，4週後に安楽死後のサンプリングをそれぞれ行い，同時に各時点で採血も実施する。ベクターの投与及び採血は塩酸ケタミン，生検はイソフルランによる麻酔下で行う。サンプリングは，ペントバルビタールナトリウム深麻酔下に放血死させた後に実施する。ジストロフィンの発現をウエスタンブロット法及び免疫組織化学染色法を用いて解析し，β-ガラクトシダーゼの発現について組織化学染色法を用いて解析する。β-ガラクトシダーゼ及びマイクロジストロフィンに対する血清抗体価の測定をELISAまたはウエスタンブ

ロット法で行う。

C．研究成果

最初に，最も効率よく遺伝子が導入できかつ免疫反応が許容可能な AAV ベクターの投与量を確認するために，カニクイザル（3 頭）の両側の上腕二頭筋と前脛骨筋の計 4 カ所に，LacZ 遺伝子組み換え AAV ベクターの濃度をそれぞれ 10^{11}vg，10^{12} vg，10^{13} vg と段階的に変えて投与し，1，2，4 週後に生検を行った。切片作成後 HE 染色を行い，β-gal 染色を施し，injection の有無とβ-gal の発現を確認した。β-gal に対する血清抗体価の測定は ELISA 法を用いて行った。その結果，10^{11} vg を投与したサルでは 1，2，4 週後ともβ-gal の発現は認められず，抗体価の上昇もなかった。10^{12} vg を投与したサルでは 2 週目に最も強いβ-gal の発現が筋周膜に沿って認められ，4 週後には抗体価の上昇とともに，細胞浸潤などの免疫反応が認められた。β-gal 発現細胞の濃淡および細胞浸潤などから筋注部位は容易に同定可能であった。10^{13} vg を投与したサルでは，1 週後からβ-gal の発現が筋周膜に沿って認められたが，すでに細胞浸潤が認められ，4 週後にはかなり広範にβ-gal 発現細胞の周囲を中心に細胞浸潤が認められ，一部に壊死や間質の線維化が認められた。以上より，10^{11} vg では導入遺伝子の発現が認められず，10^{13} vg では遺伝子発現効率はよいが，細胞浸潤などの免疫反応が強いため，10^{12} vg の投与濃度がもっとも望ましいと考えられた。

次に，10^{12} vg の LacZ 遺伝子組換え AAV ベクターを投与した場合の，遺伝子発現と免疫反応の経時的変化を調べるために，もう 1 頭のサルに 10^{12} vg の LacZ 遺伝子組換え AAV ベクターを投与し，4, 8, 16 週後に生検を行った。その結果，4 週目にはβ-gal の発現が認められたが，8 週後にはβ-gal の発現はなく，細胞浸潤が広範に認められた。16 週後には発現はなく，細胞浸潤は経度残存していたが，8 週後より改善していた。血清のβ-gal 抗体価は 4 週にも上昇していたが，8 週後にさらに上昇が認められた。

実験中に血液生化学的検査を定期的に施行したが，肝・腎障害，貧血など検査値の異常や他の全身状他の悪化などは認められなかった。

D．考察

今後，筋生検による導入遺伝子の発現解析，ウイルスベクターゲノムの有無（感染効率）を定量的かつシステマチックに解析する方法の確立が重要である。また，最近開発された新しい血清型 AAV ベクターには，免疫反応を惹起しにくいもの，血流にのって，広範な骨格筋組織に遺伝子導入可能なものが報告されている。今回は AAV type2 を用いたが，新しい血清型のベクターで同様の検討を行う事も意義深いと思われる。

E．結論

霊長類による AAV ベクターの安全性の検討は，AAV ベクターによる遺伝子治療の開発に重要である。今後も個体数を増やし，効率と安全性に関して検討を進める必要がある。

F．健康危険情報

なし

G．研究発表

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H. 知的所有権の出願・登録状況

準備中

厚生労働科学研究費補助金（ヒトゲノム・再生医療等研究事業）
分担研究報告書

縁取り空胞型筋変性の発生機序に関する研究

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研究要旨

1. 縁取り空胞変性には，核内に 20nm のフィラメント様封入体を入れ，アミロイドタンパクが沈着するタイプ（縁取り空胞型ミオパチー，封入体筋炎）と強い核の変性，それに続く筋原線維の変性をみるが，アミロイドの沈着のないタイプ（Marinesco-Sjögren 症候群），そのほか（眼咽頭筋ジストロフィー）に分類された。
2. *hmutGNE*Tg-*GNE*（-/-）マウスは，DMRV 患者で見られる筋病理所見を再現しており，世界で初めて縁取り空胞型ミオパチーのモデルマウス作製に成功した。

A．研究目的

筋線維変性過程の代表的なものは，筋ジストロフィーに代表される筋線維壊死である。次に多くみられるのが縁取り空胞(rimmed vacuoles: RV)型筋変性である。両者の筋変性過程，変性後の再生は全く異なっている。両者を比較，検討することは，筋疾患の病因・病態を考える上にきわめて重要である。

RV は遠位型ミオパチー(DMRV)，封入体筋炎，眼咽頭型筋ジストロフィー，Marinesco-Sjögren（MSS）症候群など数多くの疾患で認められる[1]。今回は RV 形成について，まず上記各疾患での変性過程の相違を検討した。

DMRV はシアル酸生合成の律速段階酵素 UDP-GlcNAc 2-epimerase/ManNAc kinase をコードする *GNE* 遺伝子の機能喪失型変異による疾患である[2]。罹患筋の病理観察ではリソソーム酵素活性を示す縁取り空胞の形成に加え，筋線維の大小不同，核内封入体形成，βアミロイドタンパク質の沈着などの特徴が見られる。しかし，*GNE* 変異からこれらの病理像や筋萎縮に至るプロセスは全く不明である。本疾患の病態解析と治療法開発を目的に，DMRV のモデル動物として変異 *GNE* のみを発現するマウスを作製し，その表現型を解析した。

B．研究方法

対象：

①DMRV，封入体筋炎：各 10 例，眼咽頭型筋ジストロフィー：5 例，MSS：10 例の生検筋を対象とした。生検に当たっては，患者から生検，研究用使用の許可を書類（国立精神・神経センター倫理委員会承認）にて得たものを使用した。生検筋には組織化学的染色，免疫組織化学的染色，電子顕微鏡的検索を行った。

②胎生致死である *GNE* 遺伝子ノックアウトマウス遺伝形質(*GNE-/-*)と，本邦 DMRV 患者で最も頻度の高いヒト V572L 変異 *GNE* を発現するトランスジェニックマウス(*hmutGNETg*)の掛け合わせにより，ヒト V572L 変異 *GNE* のみを発現するマウス（hmutGNETg-GNE-/-）を作製した。動物の作成，解析は神経研究所疾病研究第一部（西野一三部長，May C. V. Malicdan 研究員）の協同研究によって行われた。

C．研究結果

①DMRV，封入体筋炎では変性線維の核内に約 20 nm の tubulo-filamentous な封入体を入れていた。核の変性が強い場所ではアミロイド様物質の沈着，筋原線維の変性，自己貪食空胞がみられた。これらの疾患では，核の変性が一次的な意味をもつと考えられた。MSS は強い核の変性（空胞化など）をみ，さらに筋原線維の変性をみたがアミロイド様物質の沈着は認められなかった。眼咽頭型筋ジストロフィーでは，核内の封入体は 8-10 nm と細く，アミロイドの沈着はみられなかった。

②*hmutGNETg-GNE-/-*は，やや出生率が劣るが，生下時の外観および発達は，野生型とほぼ同様であった。前肢筋力測定では，30 週齢以降で，野生型より低値を示した。また，30 週齢以降，血清 CK 値が上昇していた。筋力低下と血清 CK 値上昇は，進行性であった。30 週齢マウスでは，血清・骨格筋ともに，シアル酸量が低下していた。骨格筋の病理解析では筋線維の大小不同が観察され，また，38 週齢で筋線維内にβアミロイドタンパク質の蓄積が観察された。さらに，50 週齢以上のマウスの腓腹筋，大腿四頭筋では縁取り空胞が観察された。また，リソソーム膜タンパク質，ポリユビキチン，筋鞘膜タンパク質の筋線維内での強い免疫反応も観察された。電子顕微鏡的には RV の特徴とされる自己貪食空胞，ミエリン小体の出現とともに，アミロイド構造の出現も確認できた。

D．結論

RV 型筋変性も一様ではなく，種々のタイプがあることが分かった。いずれも，核の変化を伴っており，今後核の変化が何を意味するのか，注目されるであろう。とくに DMRV ではシアル酸生合成の律速段階酵素 UDP-GlcNAc 2-epimerase/ManNAc kinase をコードする *GNE* 遺伝子の機能喪失型変異による疾患であることが分かっている。この *GNE* 遺伝子産物は核内にも存在することが明らかにされている。酵素異常がどのようにして，核に変化をきたし，筋原線維の変性，自己貪食機転，RV 形成に関与するのか，不明な点が多い。まだ解析は十分でないが，*hmutGNETg-GNE-/-*マウスは前述の疑問に答えを与えてくれるモデルマウスと評価出来る。このマウスでは RV 形成と同じく，むしろ先んじてアミロイド形成がみられる。RV のアミロイド沈着は，タンパク変性の二次的な結果であると簡単に処理できない。MSS は著明な核の変化と，筋原線維の変化をみるがアミロイド沈着はみられない。MSS では *SIL1* という小胞体機能の調節因子をコードする遺伝子に変異がみられている [3]。この遺伝子変異と RV 形成の関係も今後明らかにされねばならない。

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E．健康危険情報

なし

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G. 知的所有権の出願・登録状況

なし

厚生労働科学研究費補助金（ヒトゲノム・再生医療等研究事業）
分担研究報告書

骨格筋再生時の炎症・免疫学的反応の解析

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研究要旨
骨格筋が再生する際におこる炎症反応の意義を明らかにするために，筋再生時に浸潤する炎症性細胞の役割について解析した。その結果，骨格筋での線維化の亢進は，マクロファージ由来の線維化抑制因子の産生低下が原因であることが示唆された。

A．研究目的

骨格筋が再生する際，筋肉内に種々の炎症性細胞の浸潤が認められる。本研究では，炎症性細胞のなかでもマクロファージの筋再生に及ぼす影響について検討し，その役割を明らかにすることにより，筋ジストロフィーの病態改善のための新しい手法を確立することを目的とする。

あわせて，筋ジストロフィーに伴い認められる脂肪細胞の出現様式，および骨格筋組織の構築に必須の細胞外基質膜構成分子メロシンの産生にあずかる細胞の同定を試みた。

B．研究方法

1. 正常マウス前脛骨筋にカルジオトキシン（CTX）を投与し，骨格筋の再生を誘導した。
2. 再生筋からマクロファージを高純度に精製し，線維化に関連する遺伝子について遺伝子チップ解析した。
3. 線維化に関連する遺伝子について，定量的 RT-PCR 法で遺伝子発現の変動を，また分子については免疫組織化学的に調べた。
4. マクロファージ不在下での筋再生時に脂肪細胞がどの程度出現してくるかを組織化学的に検討した。
5. メロシン欠損マウスへの筋細胞移植を試み，主たるメロシン産生細胞の同定を試みた。

C．研究成果

マウス骨格筋に CTX を投与し，72 時間目の筋から浸潤単核細胞を採取した。またここから FACS でマクロファージを精製するため，F4/80 陽性分画を採取した。一方対照とするマクロファージは，正常腹腔内から同様の方法で精製した。その後両者から mRNA を得，Affymetrix GeneChip 解析し，線維化に関連する分子群を調べた。

その結果，線維形成に関わる因子として，CTGF が興味ある変動を示すことがわかった。CTGF は，CFS98 抗体の投与（マクロファージ浸潤を抑制）＋CTX 誘導筋再生 7 日目に著明に上昇していた。またコラーゲン産生量も，CTGF 発現とパラレルな関係にあった。このことは，マクロファージ不在下でおこる線維形成には，CTGF が重要な役割を果たしていることが強く示唆された。

一方，マクロファージ不在下では筋再生は正常にはおこらず，線維の増生とともに，脂肪細胞が出現してくることがわかった。そこで筋前駆細胞を特異的モノクロナル抗体 SM/C-2.6 で精製し，増殖条件下で維持した後 single cell manipulation 法でクローニングした。クローン化細胞を脂肪細胞分化誘導条件で培養すると，程度の差はあるもの

の全てのクローンで，脂肪細胞が出現した。

さらにメロシン欠損型（dy^{3k}）マウスの治療法を探るため，SM/C-2.6 で精製した筋衛星細胞を骨格筋内に移植し，基底膜のメロシン発現を調べた。また SM/C-2.6 陽性分画，陰性分画のうち，いずれがメロシンを発現するか，定量的 RT-PCR 法で確認した。その結果，メロシンは骨格筋中の非筋系細胞が産生している可能性が強く示唆された。

D．考察

骨格筋の再生時には，秩序だった細胞浸潤とそれに連動した筋再生が起こる。昨年度は，強力なマクロファージ機能障害モデルを用いて，この現象を確認した。本年度は，マクロファージ機能が障害された条件下で認められる線維形成の誘導にあずかる分子の探索を試み，CTGF がその強力な候補遺伝子であることを見出した。線維形成は，骨格筋の再生不良の問題にとどまらず，生体のさまざまな組織の構築・再構築に重要な役割を果たし，線維化を防ぐことができれば、筋再生にのみではない多くの疾患治療・予防に役立つ。今後 CTGF 以外の分子群との関連性を追及していきたい。

筋衛星細胞由来細胞が脂肪細胞に分化する能力を持っていることがわかった。このことは，筋ジストロフィーの発症後期では，骨格筋中に多くのコラーゲン線維が認められると同時に，多くの脂肪細胞浸潤が知られている。本研究では，マクロファージ機能の低下がこうした原因の一つである可能性を示すことができた。マクロファージ機能の改善が筋ジストロフィーの進行を抑制する可能性について今後検討したい。

メロシン欠損型筋ジストロフィーモデルマウスでは，骨髄移植法はほとんど効果がないことを確認してきた。むしろ骨格筋から得た単核細胞の重要性について検討してきたが，メロシン産生にあずかる細胞種の同定は不明であった。今回 SM/C-2.6 を指標に，メロシン産生が筋衛星細胞に由来するかどうかの確認作業を進め，筋系譜以外の細胞種がメロシン産生にあずかっている可能性を示唆することができた。今後，より効率の良い移植法の検索を目指し，メロシン産生細胞の種類を明確にする予定である。

E．結論

1. マクロファージ機能を障害してみとめられる繊維形成に，CTGF が重要な役割を果たしていることがわかった。
2. 筋衛星細胞が脂肪細胞に分化する能力を有していることがわかった。
3. メロシンは，骨格筋中の非筋系細胞が産生している可能性が強く示唆された。したがってメロシン欠損型筋ジストロフィーの細胞移植の手法には，従来とは異なったアプローチが必要である。

F．健康危険情報

なし

G．研究発表

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H. 知的所有権の出願・登録状況

なし

研究成果の刊行に関する一覧表

雑誌

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Functional heterogeneity of side population cells in skeletal muscle

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Abstract

Skeletal muscle regeneration has been exclusively attributed to myogenic precursors, satellite cells. A stem cell-rich fraction referred to as side population (SP) cells also resides in skeletal muscle, but its roles in muscle regeneration remain unclear. We found that muscle SP cells could be subdivided into three sub-fractions using CD31 and CD45 markers. The majority of SP cells in normal non-regenerating muscle expressed CD31 and had endothelial characteristics. However, $CD31^-CD45^-$ SP cells, which are a minor subpopulation in normal muscle, actively proliferated upon muscle injury and expressed not only several regulatory genes for muscle regeneration but also some mesenchymal lineage markers. $CD31^-CD45^-$ SP cells showed the greatest myogenic potential among three SP sub-fractions, but indeed revealed mesenchymal potentials in vitro. These SP cells preferentially differentiated into myofibers after intramuscular transplantation in vivo. Our results revealed the heterogeneity of muscle SP cells and suggest that $CD31^-CD45^-$ SP cells participate in muscle regeneration.

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Keywords: Side population cells; Muscle regeneration; Mesenchymal differentiation; Transplantation

Adult skeletal muscles have a remarkable ability to regenerate following muscle damage. This regeneration has been attributed to satellite cells that reside between the sarcolemma and the basal lamina. Satellite cells are quiescent mononucleated cells in normal conditions, however, in response to muscle damage, they become activated, proliferate, and then exit the cell cycle either to renew the quiescent satellite cell pool or to differentiate into mature myofibers. Thus, they have been considered to be the myogenic precursor cells that give rise to myoblasts and the sole source of adult myogenic cells [1].

In 1998, Ferrari et al. [2] have demonstrated for the first time that bone marrow (BM)-derived cells contribute to the skeletal muscle after BM transplantation. Side population (SP) cells were first identified in bone marrow based on the ability to exclude Hoechst 33342 dye as an enriched fraction of hematopoietic stem cells (HSCs) [3], later, it has been reported that they also participate in muscle regeneration [4]. Studies using whole BM cells showed that BM-derived mononucleated cells display several characteristics of satellite cells, suggesting that donor-derived BM cells contribute to muscle fibers in a stepwise biological progression [5,6]. However, using single HSC transplantation experiment, Camargo et al. [7] suggested that cells committed to the myeloid lineage contribute to muscle through fusion event. Therefore, multiple mechanisms underlay contribution of BM-derived cells to skeletal muscle regeneration.

SP cells have been also identified in skeletal muscle [4]. Muscle SP cells cannot only reconstitute the hematopoietic system of lethally irradiated mice [4,8], but also differentiate into skeletal muscle cells [4,9]. Furthermore, they have been reported to participate in vascular regeneration [10]. Several lines of evidence suggest that muscle SP cells are a cell population distinct from satellite cells [9,11–13]. While muscle SP cells possess these attractive

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features, they have been reported to be heterogeneous population. In fact, muscle SP cells contain both $CD45^+$ and $CD45^-$ cells, and hematopoietic potential has been exclusively found in $CD45^+$ fraction [8,9]. As regards the myogenic potential, both $CD45^+$ and $CD45^-$ fractions have been shown to differentiate into skeletal muscle cells [9,14], but there is no comparative study dealing with subpopulation of muscle SP cells during muscle regeneration.

In the present study, we have further divided muscle SP cells into three sub-fractions using CD31 and CD45, examined the properties of each sub-fraction, and identified a novel subpopulation ($CD31^-CD45^-$ SP cells) that showed the greatest myogenic potential both in vitro and in vivo. These results provide a new insight for stem cell-based therapy of muscular dystrophy.

Materials and methods

Animals. All procedures using experimental animals were approved by the Experimental Animal Care and Use Committee at the National Institute of Neuroscience. Eight- to ten-week-old C57BL/6 mice were purchased from Nihon CLEA (Japan). GFP Tg mice were provided by Dr. M. Okabe (Osaka University) and used in cell transplantation experiments. NOD/*scid* mice provided by the Institute for Experimental Animals, Japan, were used as recipients.

To induce muscle regeneration, 100 μl of CTX (10 μM in saline, Wako Chemicals) was injected into the tibialis anterior (TA) muscle with a 29-gauge needle. In FACS analysis experiments, CTX was injected into TA (50 μl), gastrocnemius (50 μl), and quadriceps femoris muscles (25 μl).

BM transplantation was performed as previously described [14]. Mice were subjected to analysis 12 weeks after transplantation.

Antibodies. Mouse Bcrp-1 cDNA was provided by Dr. A.H. Schinkel [15]. A DNA fragment corresponding to cytoplasmic domain of Bcrp1, amino acids 300–337, was fused to GST in a pGEX-4T-2 vector (Amersham Biosciences), and the fusion protein was used to immunize rabbits. The serum obtained was affinity-purified. Other antibodies used in these studies are listed in Table S1.

Cell preparation and FACS analysis. Muscle-derived mononucleated cells were prepared from C57BL/6 mice, GFP Tg mice, or GFP-BM transplanted mice as previously described [14]. Hoechst staining was performed as described by Goodell et al. (http//www.bcm.tmc.edu/genetherapy/goodell/new_site/protocols.html). Cells were re-suspended at 10^6 cells per ml in DMEM (Invitrogen) containing 2% FBS (Trace Biosciences), 10 mM Hepes, and 5 μg/ml Hoechst 33342 (Sigma), and incubated for 90 min at 37 °C in the presence or the absence of 50 μM verapamil (Sigma). During incubation, cells were mixed 3–4 times. For analysis of Ac-LDL uptake, 10 μg/ml DiI-labeled Ac-LDL (Biomedical Technologies) was added. After antibody staining, cells were re-suspended in PBS containing 2.5% FBS and 2 μg/ml propidium iodide (PI) (BD PharMingen). Cell sorting was performed on a FACS VantageSE flow cytometer (BD Biosciences). Debris and dead cells were excluded by forward scatter, side scatter, and PI gating. Cell viability after staining and sorting was comparable to that previously reported [14].

RNA extraction and RT-PCR. Total RNA was extracted from 1×10^4 FACS sorted cells by using a RNeasy Micro Kit (Qiagen) and then reverse transcribed into cDNA by using TaqMan Reverse Transcription Reagents (Roche). The PCRs were performed with 1 μl cDNA product under the following cycling conditions: 94 °C for 3 min followed by 40 cycles of amplification (94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s) with a final incubation at 72 °C for 5 min. Specific primer sequences used for PCR are available on request.

Cell culture. SP cells were cultured alone with growth medium (GM); DMEM containing 20% FBS and 2.5 ng/ml bFGF (Invitrogen) in chamber slides (Nalge Nunc) coated with Matrigel (BD Biosciences) for 3–5 days. For osteogenic differentiation, the medium was changed to a differentiation medium (DM), 5% horse serum in DMEM supplemented with or without 500 ng/ml recombinant human BMP2 (R&D Systems), and cultured for 4–6 days. For adipogenic differentiation, cells were exposed to 3 cycles of 3 days of adipogenic induction medium (Cambrex Bioscience) followed by 1 day of adipogenic maintenance medium (Cambrex Bioscience) and then cells were maintained for five more days in the adipogenic maintenance medium. Alkaline phosphatase (AP) was stained using Sigma kit #85 according to the manufacturer's instructions. To stain lipids, cells were fixed in 10% formalin, rinsed in water and then 60% isopropanol, stained with Oil red O in 60% isopropanol, and rinsed in water. For myogenic differentiation, muSP-31, muSP-45, or muSP-DN purified from GFP Tg mice were co-cultured with myoblasts prepared from C57BL/6 mice as previously described [16,17] in GM. DM was supplied 3–5 days after starting co-culture.

Osteogenic activity and myotube-forming activity were determined by the following formulas: osteogenic activity = [the number of AP^+ cells in seven randomly selected fields (corresponding to one-tenth of the whole area of the well)]/(the number of seeded cells) and myotube-forming activity = (the number of GFP^+ myotubes in seven randomly selected fields)/(the number of seeded cells). In order to measure the extent of adipogenic differentiation, stained oil droplets were extracted for 5 min with 100 μl of 4% Nonidet P-40 in isopropanol, and the absorbance of the dye-triglyceride complex was measured at 520 nm [18]; then, adipogenic activity was determined by the following formula: (the absorbance at 520 nm)/(the number of seeded cells).

Intramuscular transplantation experiments. muSP-DN or muSP-31 cells were purified from GFP Tg mice and were injected directly into the TA muscles of NOD/*scid* mice. One day before transplantation, host TA muscles were treated with CTX. The number of transplanted cells is indicated in Table 1. Three weeks after transplantation, TA muscles were excised and fixed in 4% PFA for 30 min, immersed sequentially in 10% sucrose/PBS and 20% sucrose/PBS, and frozen in isopentane cooled with liquid nitrogen.

Immunohistochemistry. FACS sorted cells were collected by Cytospin3 (ThermoShandon). Cells were fixed with 4% PFA for 5 min. Frozen muscle tissues were sectioned using a cryostat. Specimens were blocked with 5% goat serum (Cedarlane) in PBS for 15 min and incubated with primary antibodies at 4 °C overnight, followed by secondary staining. Stained cells were mounted in Vectashield with DAPI (Vector) and photographed using a fluorescence microscope IX70 (OLYMPUS) equipped with a QuantixTM air-cooled CCD camera (Photometrics) and IP Lab software (Scanalytics Inc.). Stained muscle sections were counterstained with TOTO-3 (1:5000; Molecular Probes), then mounted in Vectashield (Vector), and observed under the confocal laser scanning microscope system TCSSP (Leica).

Statistics. Values were expressed as means ± SD or ± SEM. Statistical significance was assessed by Student's *t* test. In comparison of more than two groups, one-way analysis of variance (ANOVA) followed by the Fisher's PLSD was used. A probability of less than 5% ($P < 0.05$) or 1% ($P < 0.01$) was considered statistically significant.

Table 1
Appearance of GFP^+ myofibers after intramuscular transplantation

Cell type	Experiment No.	Number of injected cells/TA muscle	Number of GFP^+ myofibers/TA muscle
muSP-DN cells	Ex. 1	1.7×10^3	14
	Ex. 2	2.5×10^3	9
	Ex. 3	2.5×10^3	0
muSP-31 cells	Ex. 1	1.6×10^4	3
	Ex. 2	1.6×10^4	0
	Ex. 3	1.6×10^4	0

Results

Most muscle SP cells are found in a subset of capillary or vein endothelial cells in non-regenerating skeletal muscle

We identified verapamil-sensitive SP cells in skeletal muscle after Hoechst staining (Fig. 1A) and analyzed the expression of several markers on them. The majority of muscle SP cells were $CD31^+$, usually recognized as a marker of endothelial cells (Figs. 1B–E), and negative for a pan-hematopoietic marker, CD45 (Fig. 1B). More than half of muscle SP cells were $CD34^+$, and $Sca\text{-}1^+$ cells comprised 90% of muscle SP cells (Figs. 1C and D). Compared to FACS profiles of whole-muscle-derived cells, SP cells were enriched in $Sca\text{-}1^+$ cells (Fig. S1). More than 85% of muscle SP cells were $CD31^+$ and took up acetylated low-density lipoprotein (Ac-LDL), a functional marker for endothelial cells and macrophages (Fig. 1E). These results indicate that most muscle SP cells have endothelial characteristics. Only cells in the main population (MP) were found to be $Pax7^+$, indicating that SP cells do not include muscle satellite cells (data not shown).

To examine the localization of muscle SP cells, we generated a rabbit polyclonal anti-mouse Bcrp1 antibody, because it has been reported that Bcrp1 is the major determinant of the SP phenotype [19]. Our antibody clearly recognized Bcrp1 expression in liver, small intestine, and kidney, as previously reported (Fig. S2) [20,21]. We confirmed that Bcrp1 antibody recognizes more than 80% of SP cells and less than 3% of MP cells collected by cytospin (Figs. 1F and G). In skeletal muscle, $Bcrp1^+$ cells were found outside the muscle basal lamina (Fig. 1H), which clearly distinguished $Bcrp1^+$ cells from satellite cells. Next, Bcrp1 expression in the vascular system was investigated. CD31 staining identified all endothelia from larger vessels to capillaries in muscle sections. Intriguingly, Bcrp1 was expressed by CD31-expressing endothelial cells, and its expression was preferentially observed on a subpopulation of capillary endothelium (Figs. 1I–K) and venous endothelium surrounded by thin vessel walls, as revealed by α-smooth muscle actin (αSMA) expression (Figs. 1L–N). These results, together with the results of FACS analysis, strongly suggest that the majority of muscle SP cells are a subset of endothelial cells present in capillaries or veins in non-regenerating skeletal muscle.

Behavior of muscle SP cells during muscle regeneration

We next examined the kinetics of SP cells during muscle regeneration induced by injection of cardiotoxin (CTX). After CTX injection, the total number of mononuclear cells per muscle weight gradually increased, with a peak at day 3. The number of SP cells also increased and reached its peak at day 3 (Fig. 2A). Muscle SP cells could be divided into three subpopulations based on CD31 and CD45 expression: $CD31^+CD45^-$ SP cells (designated muSP-31 cells), $CD31^-CD45^+$ SP cells (muSP-45 cells), and $CD31^-CD45^-$ SP cells (muSP-DN cells). muSP-31 cells and muSP-DN cells distributed throughout the SP tail, but muSP-45 cells were located close to the shoulder (data not shown). The majority of muscle SP cells in untreated muscle were muSP-31 cells (Fig. 1B). During regeneration, however, muSP-45 cells and muSP-DN cells increased in both their ratios and their numbers (Figs. 2B and C). Although $CD45^+$ cells were abundant in whole muscle-derived cells during regeneration and most of them were F4/80 antigen-positive mature macrophages, SP cells did not contain any mature inflammatory cells, as previously reported (data not shown) [14].

To clarify the origin of each subpopulation of SP cells, BM transplantation experiments were performed. We confirmed that muSP-45 cells were mobilized from bone marrow as previously reported (Figs. 3A and B) [14]. In contrast, both $CD45^-$ SP fractions are residents of skeletal muscle (Figs. 3A and B), consistent with the results reported by Rivier et al. [22].

Next, to determine whether each subpopulation of SP cells proliferates in damaged muscle, cells were stained with Ki67 antibody. Most muSP-45 cells (Figs. 3C and D) and muSP-31 cells (Figs. 3G and H) prepared from regenerating muscle were negative for Ki67, suggesting that the proliferation activities of these two fractions were low. On the other hand, about 60% of muSP-DN cells were positive for Ki67 (Figs. 3E and F), indicating that muSP-DN cells actively proliferated during muscle regeneration.

We next examined Bcrp1 expression on three sub-fractionated SP cells and found that only muSP-31 cells were Bcrp1-positive (Fig. 3K). These results suggest that some ABC transporters other than Bcrp1 are responsible for the phenotype of $CD31^-$ SP cells.

Gene expression of muscle SP cells during muscle regeneration

Our analysis revealed that each subpopulation of SP cells showed distinct kinetics during muscle regeneration. To better understand the traits of muscle SP cells, we analyzed gene expression during muscle regeneration. Three subpopulations of SP cells (in following experiments, muSP-45 cells from untreated muscle were omitted because of their low yield) or MP cells were collected from each time point during muscle regeneration, and RT-PCR was performed. We chose several myogenic (*Pax3*, *Pax7*, and *myf5*), endothelial (*Tie2*, *Flk1*, and *vWF*), and mesodermal-mesenchymal-associated (*αSMA*, *PPARγ*, *Runx2*, *PDGFRα*, and *PDGFRβ*) genes to clarify lineage characteristics of the target cells. We also examined expression of genes of developmental regulators (*msx1*, *Frizzled4* (*Fzd4*), *Patched1* (*Ptc1*), and *BMPR1A*), angiogenic factors (*angiopoietin-1* (*ang1*) and *VEGF*), and TGF-β superfamily antagonists (*follistatin* and *DAN*). muSP-DN cells from untreated muscles expressed only *PDGFRβ*, *Ptc1*, *ang1*, *follistatin*, and *DAN* (Fig. 4, cont, lane 1). Neither myogenic nor other lineage-specific markers could be detected in

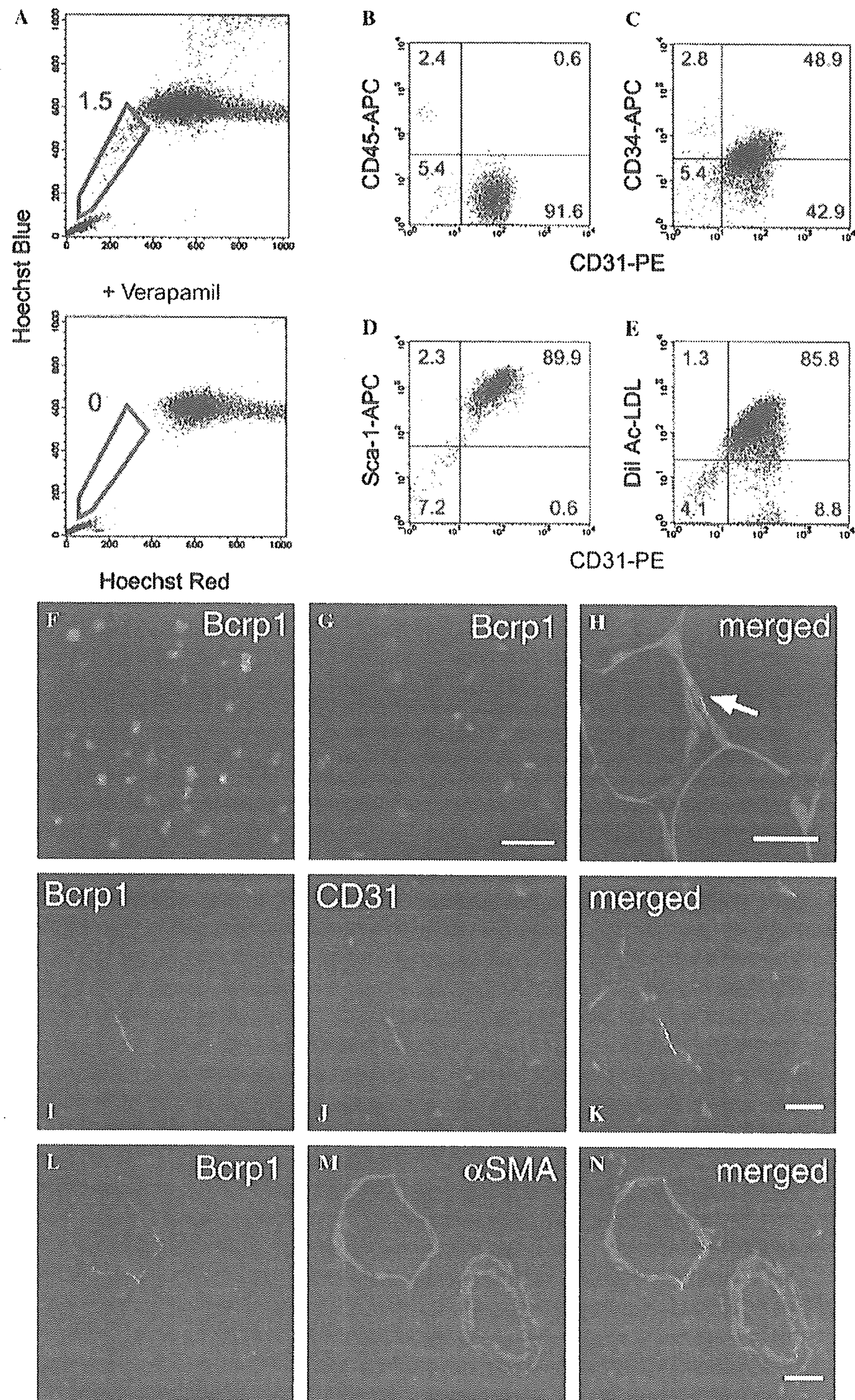

Fig. 1. Characterization of skeletal muscle SP cells. (A) Flow cytometric analysis of muscle-derived mononucleated cells after Hoechst 33342 staining with (lower panel) or without Verapamil (upper panel). The numbers indicate the percentage of SP cells (blue pentagons) in all mononucleated cells. (B–E) The expression of CD45 (B), CD34 (C), Sca-1 (D), and DiI-Ac-LDL uptake (E), and CD31 (B–E) on muscle SP cells. The percentage of cells in each quadrant is shown in the panel. (F,G) Immunofluorescent staining for Bcrp1 (green) and DAPI counterstaining (blue) of freshly sorted SP (F) and MP (G) cells. Immunofluorescent staining for Bcrp1 (green) and laminin α2 chain (red) (H), Bcrp1 (green) and CD31 (red) (I–K), and Bcrp1 (green) and α-smooth muscle actin (red) (L–N). TOTO-3 nuclear staining is shown in merged images (blue in H, K, and N). Bcrp1-positive cells are located outside the basal lamina (arrow), and they are partially overlapped with endothelial cells of capillary (I–K) and vein (L–N). Bars: 50 μm in (F,G), 20 μm in (H–N).

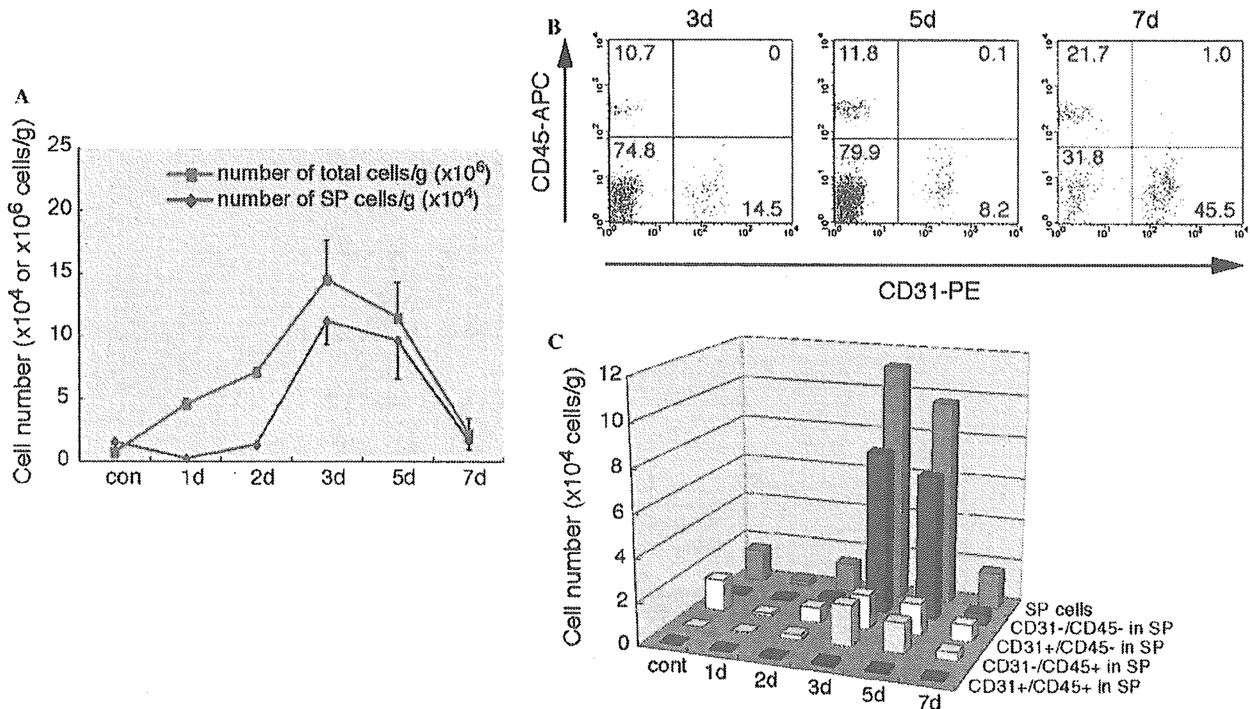

Fig. 2. Behavior of subpopulations of SP cells during muscle regeneration. (A) At 1 day (1d), 2 days (2d), 3 days (3d), 5 days (5d), and 7 days (7d) after CTX injection, the number of total cells (pink line) and SP cells (blue line) per gram of muscle weight was quantified. (B) At 3 days (3d), 5 days (5d), and 7 days (7d) after CTX injection, muscle SP cells prepared from regenerating muscle were analyzed for CD31 and CD45 expression. (C) Cell numbers in subpopulations of SP cells. muSP-45 cells (light blue bar) and muSP-DN cells (dark red bar) were significantly increased in number during muscle regeneration. Values (A,C) are the average of three independent experiments. Error bars represent SD.

this population indicating that muSP-DN cells do not contain cells committed to the lineages tested. At day 3 after CTX injection, muSP-DN cells began to express developmental regulator genes (Fig. 4, 3d, lane 1), and then at day 5, they also began to express several other lineage-specific genes (*Tie2*, *αSMA*, *PPARγ*, and *Runx2*). Angiogenic factors and TGF-β superfamily antagonists were also strongly expressed at this time point (Fig. 4, 5d, lane 1). In contrast, muSP-31 cells continuously expressed all three endothelial genes analyzed throughout the regeneration process (Fig. 4, lane 2). Expression of mature endothelial marker, such as *vWF*, suggests that muSP-31 cells represent committed endothelial cells. muSP-45 cells expressed only low levels of *αSMA*, *PDGFRβ*, and *follistatin* at day 5 after CTX injection (Fig. 4, lane 3). Myogenic markers, *Pax7* and *myf5*, were detected only in the MP fraction (Fig. 4, MP) indicating that myogenic cells are completely sorted into the MP fraction even during the process of muscle regeneration.

Differentiation potential of muscle SP cells for mesenchymal lineages

muSP-DN cells showed a unique gene expression pattern during muscle regeneration process: they began to express several mesenchymal genes at a late phase of muscle regeneration. Therefore, we examined the mesenchymal potentials of muscle SP subpopulations. muSP-DN cells from untreated muscle readily gave rise to alkaline phosphatase (AP)-positive cells when cultured in the presence of bone morphogenetic protein 2 (BMP2) (Figs. 5A and C). With adipogenic induction, they also differentiated into adipocytes containing numerous lipid droplets in the cytoplasm (Figs. 5A and D). Reflecting the results of gene expression analysis, muSP-DN cells from regenerating muscle more efficiently differentiated into osteogenic cells and adipocytes than those from untreated muscle did (Figs. 5B–D). Unexpectedly, muSP-DN cells from regenerating muscle also differentiated into adipocytes without adipogenic induction (Figs. 5B and D), suggesting that they are susceptible to adipogenesis under our culture condition. In contrast, muSP-31 cells did not possess these differentiation potentials (Figs. 5A–D). Nor did muSP-45 cells, which were dramatically mobilized from BM into regenerating muscle (Figs. 5B–D). The attribute of differentiation potential is therefore a feature of muSP-DN.

Myogenic potential of muscle SP cells in vitro

We next evaluated the myogenic potential of muscle SP cells in vitro. When SP cells were cultured alone, they never differentiated into skeletal muscle cells (data not shown). Each subpopulation of SP cells was prepared from GFP Tg mice and co-cultured with wild type (WT) primary

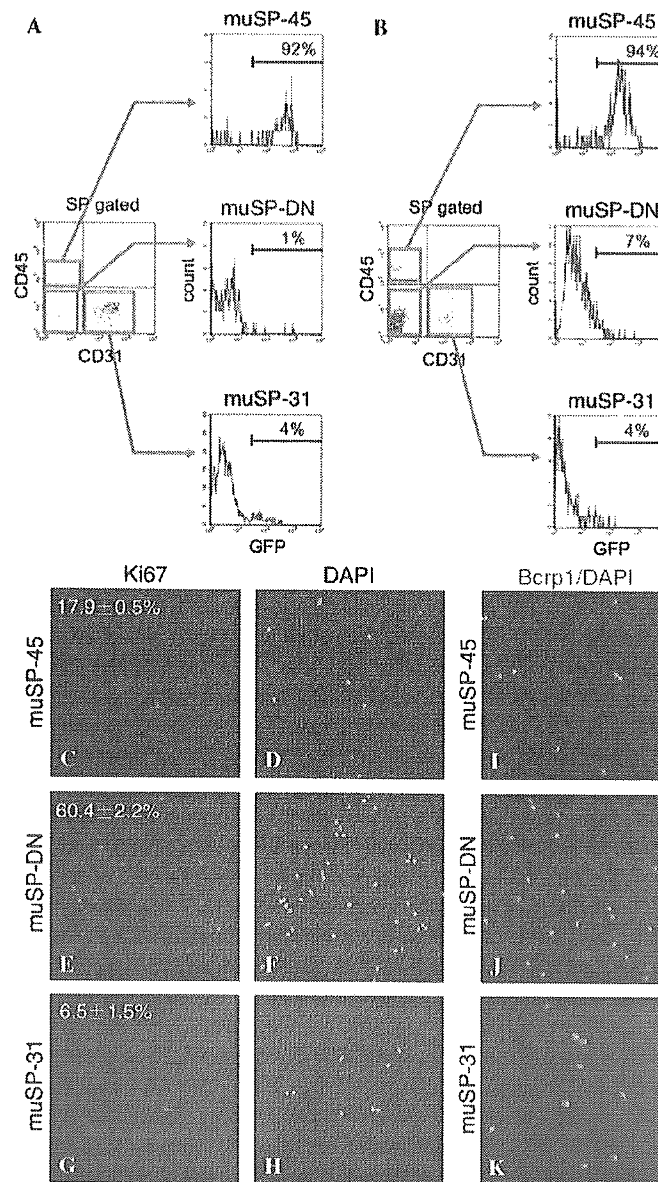

Fig. 3. Origin, proliferative activity, and Bcrp1 expression of subpopulations of muscle SP cells. (A,B) C57BL/6 mice were transplanted with whole BM from GFP Tg mice, and 3 months later, SP cells from untreated muscle (A) or regenerating muscle (3 days after CTX injection) (B) were further analyzed for CD31, CD45, and GFP expression. Note that $CD45^-$ SP cells (middle and lower panels) are almost all negative for GFP, indicating that they do not originate from BM. In contrast, more than 90% of muSP-45 cells were GFP^+ (upper panels). (C–H) Ki67 expression (green) and nuclei stained with DAPI (blue) on muSP-45 (C,D), muSP-DN (E,F), and muSP-31 (G,H) cells. The percentages of Ki67-positive cells were expressed as means ± SD of three independent experiments. muSP-45 (I), muSP-DN (J), and muSP-31 (K) were sorted from regenerating muscle and stained for Bcrp1 (green) and nuclei (blue). Only muSP-31 cells were stained positive for Bcrp1 (K). Bar: 50 μm.

myoblasts derived from satellite cells. muSP-DN cells from untreated muscle rapidly proliferated in vitro as observed in regenerating muscle (Fig. 2C). On the contrary, muSP-31 cells hardly expanded. After 2–3 weeks co-culture, both muSP-DN cells and muSP-31 cells differentiated not only into multinucleated myotubes co-expressing GFP and sarcomeric-α-actinin (Figs. 5E–G, only muSP-DN culture is shown) but also mononucleated myocytes (shown in insets). The frequency of mononucleated myocytes was too low to quantify, but existence of these cells suggests that myogenic differentiation of SP cells could occur without fusion. Strikingly, the myotube-forming activity (the frequency of GFP^+ myotubes, see Materials and methods for details) of muSP-DN cells was approximately 10-fold that of muSP-31 cells (Fig. 5H, lane for cont. 0.026 ± 0.007 vs 0.002 ± 0.001). In the experiments using SP cells from regenerating muscle at 3 days after CTX injection, muSP-DN cells showed the highest

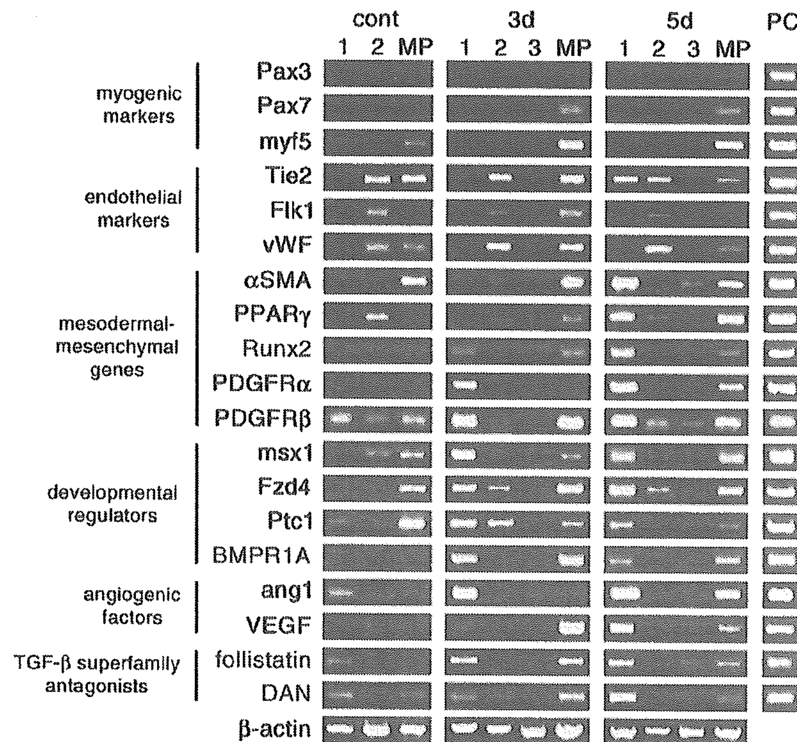

Fig. 4. Gene expression profiles of muscle SP cells during muscle regeneration. muSP-DN (lane 1), muSP-31 (lane 2), muSP-45 (lane 3), or MP cells were collected from untreated (cont) and regenerating muscle at 3 days (3d) or 5 days (5d) after CTX injection, and RT-PCR was performed against the indicated genes. Total embryo extract (E13) was used as a positive control (PC). β-actin was amplified to confirm that the quantities of mRNA were equal.

myotube-forming activity, although each SP subpopulation did form myotubes co-expressing GFP and sarcomeric-α-actinin (Fig. 5H, lane for CTX3d). This clearly demonstrates that muSP-DN cells have the highest myogenic potential among SP sub-fractions in vitro. For comparison, we quantified the myotube-forming activity of satellite cell-derived myoblasts. The value was 0.09 ± 0.01, indicating that myogenic activity of myoblasts is much higher than that of muSP-DN cells.

Myogenic potential of muscle SP cells in vivo

To evaluate the myogenic potential of muscle SP cells in vivo, we performed transplantation experiments. muSP-DN or muSP-31 cells from untreated muscle of GFP Tg mice were directly transplanted into CTX-treated TA muscles of immunodeficient NOD/*scid* mice. Three weeks after transplantation, muSP-DN cells had generated myofibers more efficiently than muSP-31 cells (Figs. 6A and B, and Table 1), indicating that muSP-DN cells had relatively higher myogenic potential in vivo as well as in vitro. Contrary to our expectation, muSP-DN cells formed no GFP-positive adipocytes after transplantation.

Discussion

Muscle SP cells have been suggested to be multipotent and can contribute to skeletal muscle regeneration [4,9,10,23]. However, most of these studies dealt with whole muscle SP cells as one functional unit. We subdivided, for the first time, muscle SP cells using CD31 and CD45 markers and revealed functional heterogeneity of muscle SP cells. $CD31^{+}CD45^{-}$ SP cells (muSP-31 cells) are a main subpopulation in non-regenerating muscle, but $CD31^{-}CD45^{-}$ SP cells (muSP-DN cells) which represent a minor subpopulation in non-regenerating muscle have the greatest differentiation potentials and become predominant subpopulation of SP cells upon muscle injury.

Differentiation potential of muscle SP cells

Phenotypic and immunohistochemical analysis suggested that muSP-31 cells are a subset of endothelial cells of capillaries and veins. They poorly proliferate after injury or in in vitro culture, and their differentiation potentials are limited both in vitro and in vivo.

$CD45^{+}$ muscle SP cells (muSP-45 cells) were shown to have both hematopoietic and myogenic potentials, and hematopoietic potential of muscle-derived cells was exclusively found in this fraction [8,9]. We previously reported the contribution of muSP-45 cells to muscle regeneration [14]. In this study, we identified novel subpopulation that possesses much higher myogenic potential than muSP-45, muSP-DN.

muSP-DN cells showed the highest differentiation potential of all the mesenchymal lineages tested among

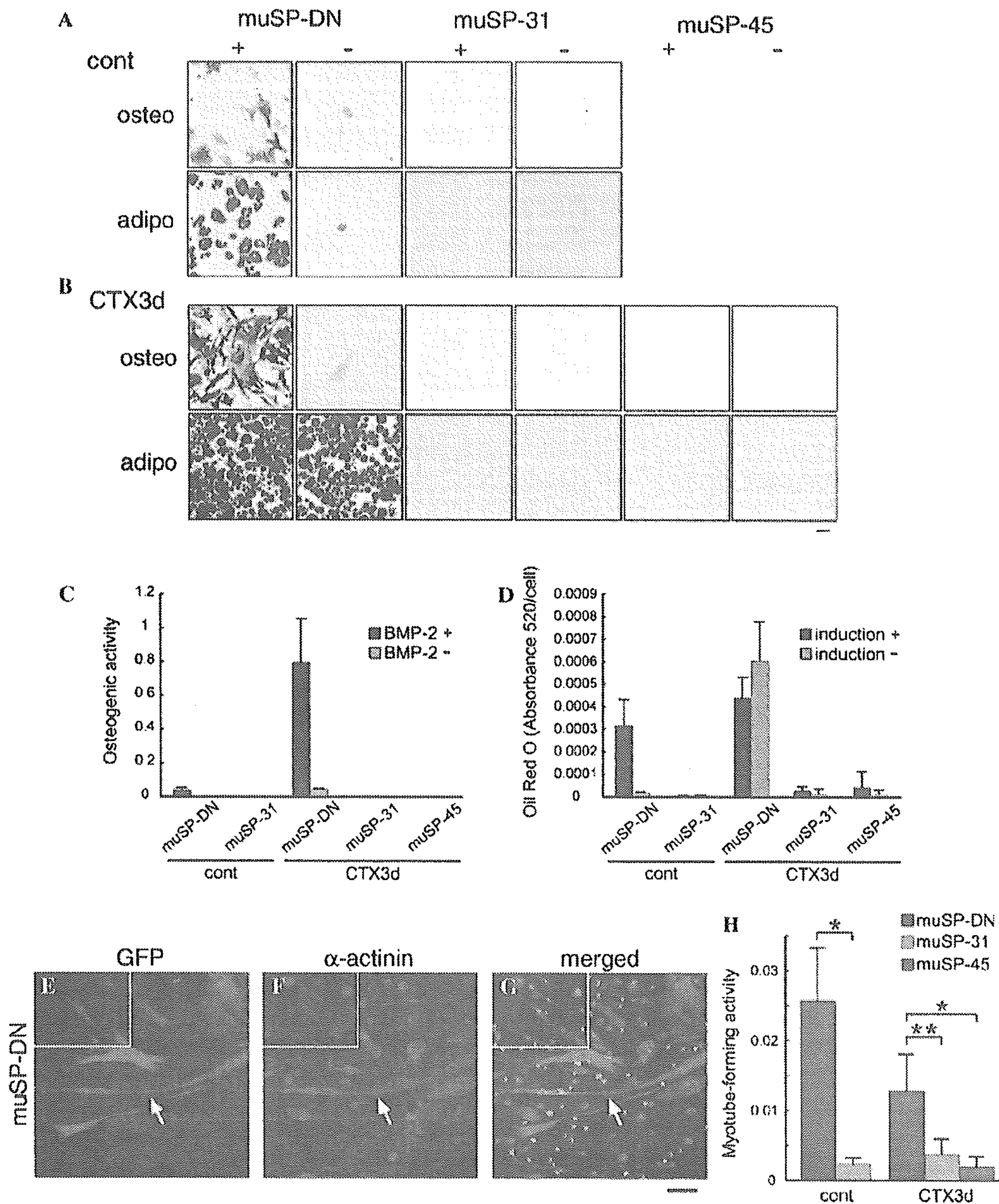

Fig. 5. muSP-DN cells differentiate into osteogenic cells, adipocytes, and skeletal muscle cells. (A,B) Three subpopulations of SP cells prepared from untreated (A) or regenerating (B) muscle were induced to differentiate into osteogenic or adipogenic cells. Uninduced cells (−) and induced cells (+) were then examined for alkaline phosphatase expression (osteo) or oil deposits (adipo). Bar: 50 μm. (C,D) Osteogenic (C) and adipogenic (D) activities of subsets of SP cells prepared from control (cont) or regenerating muscle at 3 days after CTX injection (CTX3d) were quantified. Values are the average of three independent experiments. Error bars represent SD. (E–G) Co-culture of muscle SP cells with myoblasts. muSP-DN cells from GFP Tg mice were sorted and co-cultured with WT primary myoblasts in differentiation medium. Cells were stained with anti-GFP (green) and anti-sarcomeric α-actinin (red) antibodies. Nuclear staining with DAPI (blue) is shown in merged images (G). Insets show GFP^+ mononucleated myocyte. Bar: 50 μm. (H) Myotube-forming activities of muSP-DN cells (red bars), muSP-31 cells (blue bars), and muSP-45 cells (green bar) are shown. Each subpopulation was prepared from untreated (cont) or CTX-treated regenerating muscle (CTX3d). Values are the average of three independent experiments. Error bars represent SD. $^*P < 0.01$, $^{**}P < 0.05$.

SP subpopulations. They were negative for lineage-specific markers under the non-regenerating condition, but after muscle injury or in in vitro expansion, they actively proliferated and were readily induced to express several mesenchymal genes. Their differentiation potential seems to be restricted to mesenchymal lineages because we did not detect hematopoietic colonies derived from muSP-DN cells in vitro and muSP-DN cells failed to rescue the lethally irradiated mice (data not shown). These observations indicate that muSP-DN cells are enriched for primitive mesenchymal cells. This notion is further supported by gene expression pattern of muSP-DN cells. muSP-DN cells

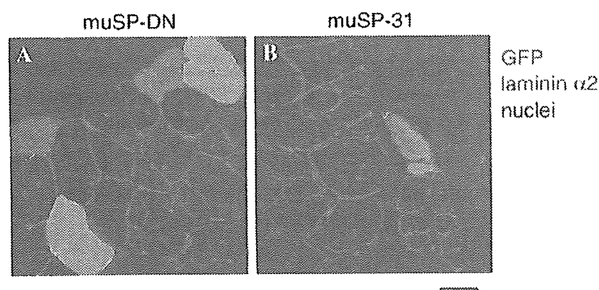

Fig. 6. muSP-DN cells participate in myofiber formation during muscle regeneration. (A,B) muSP-DN (A), muSP-31 (B) were transplanted into CTX-injected NOD/*scid* muscles. Each subpopulation was prepared from untreated muscle of GFP Tg mice. Muscle sections were stained with anti-GFP (green) and anti-laminin α2 (red) antibodies 3 weeks after transplantation. More GFP-positive myofibers were detected in muSP-DN-transplanted muscles (A) than in muscles transplanted with muSP-31 cells (B). Bar: 40 μm.

specifically expressed *ang1* under the non-regenerating condition and during the early phase of regeneration (Fig. 4, lane 1, cont or 3d). Perivascular cells, such as pericytes, express ang1 [24,25], and several groups suggest that multipotent mesenchymal stem cells may be derived from pericytes [26–28]. A recent report demonstrated that vascular mural precursor cells are negative for endothelial markers but positive for Tie2 and smooth muscle cell markers [29]. Likewise, muSP-DN cells were negative for *Flk1* and *vWF* throughout the regeneration process (Fig. 4, lane 1), but began to express *Tie2* and *αSMA* during late phases of regeneration (Fig. 4, lane 1, 5d). Given the similarity between muSP-DN cells and those reported perivascular primitive cells, muSP-DN cells would represent perivascular primitive mesenchymal cells in skeletal muscle.

Roles of muscle SP cells in muscle regeneration

muSP-DN cells actively proliferated and significantly increased in number upon muscle injury. The precise fate of muSP-DN cells has remained to be determined, since the number of muSP-DN cells returned to normal level at late stage of muscle regeneration.

We noted that angiogenic factors and TGF-β superfamily antagonists were strongly expressed in muSP-DN cells during muscle regeneration. Previous reports showed that $Ptc1^+$ interstitial mesenchymal cells in muscle produce angiogenic factors, including *ang1*, and promote muscle regeneration after ischemia [30,31]. Some members of the TGF-β superfamily, such as myostatin and TGF-β1, are known to act as negative regulators of myogenesis [32,33]. Inversely, one of the TGF-β superfamily antagonists, follistatin, has been reported to promote myoblast recruitment and fusion [34]. Therefore, muSP-DN cells might promote muscle regeneration by producing regeneration-regulating factors.

muSP-DN cells preferentially differentiate into myogenic cells after intramuscular transplantation, implying that normal muscle environment facilitates myogenic differentiation of muSP-DN cells. However, we revealed that muSP-DN cells have a high tendency to differentiate into osteogenic or adipogenic cells in vitro. Therefore, it is possible that muSP-DN cells differentiate into osteogenic or adipogenic cells in some pathological conditions such as Duchenne muscular dystrophy [35,36]. Recent finding that microvascular pericytes can differentiate into adipocytes [37] further supports the notion that muSP-DN cells might be implicated in pathological changes.

In conclusion, we identified novel subpopulation of muscle SP cells, $CD31^-CD45^-$ SP cells, which possesses capacity of mesenchymal differentiation in vitro and reveals myogenic differentiation potential in vivo. Our findings might provide new insights that may well be useful in understanding adult skeletal muscle regeneration and in designing therapeutic strategies of muscular dystrophy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.01.037.

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