幹細胞技術の
現状と展望

No 6

胚葉をこえた多分化能と自己細胞移植治療への可能性

Insights into auto-transplantation: the unexpected discovery of transdifferentiation systems in bone marrow stromal cells

出澤真理

Summary

骨髄間質細胞は分化転換能をもつ細胞である．この細胞にサイトカイン処理やNotch導入を順序だてて行なうことにより，非常に高い効率で神経細胞やシュワン細胞，骨格筋細胞を選択的に誘導することができる．また，これらの細胞を神経変性疾患モデル・筋変性疾患モデルへ移植することにより，その有効性が確認された．この系を用いた，自らの細胞による再生医療，すなわち，"自己細胞移植治療"の可能性を考察する．

Key words

●間葉系幹細胞　●分化転換　●パーキンソン病
●筋ジストロフィー　●細胞移植

Mari Dezawa
京都大学大学院医学研究科 生体構造医学講座機能微細形態学
E-mail：dezawa@anat2.med.kyoto-u.ac.jp

はじめに

ES細胞や組織幹細胞から目的とする細胞を誘導して治療に用いる移植医療は，有効な治療法がないさまざまな疾病において期待されている．とりわけ重篤な病気である，筋萎縮性側索硬化症，筋ジストロフィー，重篤な糖尿病，脊髄損傷などは，神経細胞や筋肉細胞が脱落する疾患であることから，これらの疾患を対象として失われた細胞を補充する細胞移植治療法が有効な手段のひとつと考えられ，一日も早い確立が切望されている．

あらゆる細胞に分化する可能性をもつES細胞や，目的とする細胞への分化能がある程度担保されている組織幹細胞から細胞供給を行なう研究はさかんに行なわれているが，筆者は，まったく別のアプローチから研究を進めてきた．すなわち，患者自身から分化転換能をもつ細胞を採取し，それを目的とする細胞に分化させて個体に戻すシステムを確立したい，というのがそもそもの動機である．1997年から1999年にかけて，Prockopらのグループから，骨髄間質細胞（bone marrow stromal cells）に関する重要な発表があった[1]．それは，骨髄間質細胞はさまざまな細胞に分化転換する可能性がある，というものであった．大学院生のころからシュワン細胞移植による中枢神経軸索の再生機構を研究していた筆者は，シュワン細胞は中枢再生に非常に有効であるが，細胞を採取するためには新たに患者の末梢神経を損傷しなくてはならず，また，細胞数の確保もできないというジレンマを感

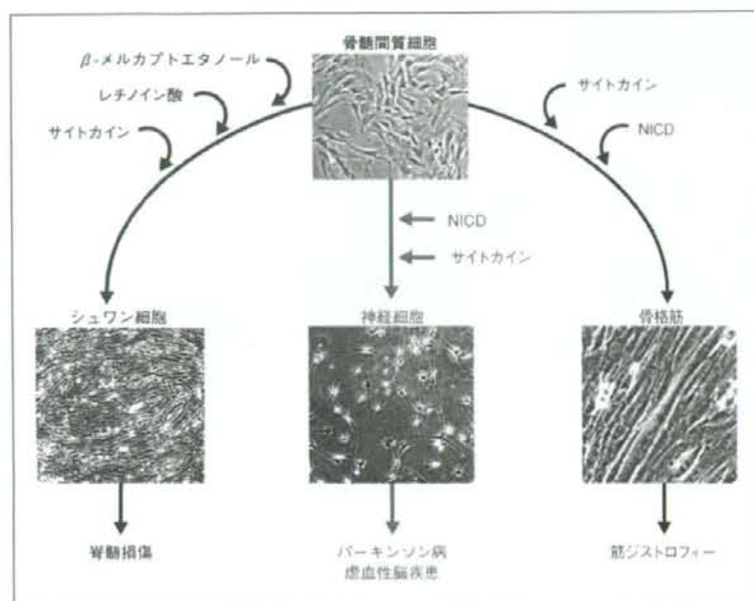

図1　骨髄間質細胞からの特異的な誘導システム

サイトカインや構成活性型であるNotchの細胞内ドメイン(NICD)の導入などを組み合わせることによって，シュワン細胞，神経細胞，骨格筋細胞が選択的に誘導される．

じていた．そこで，骨髄間質細胞からシュワン細胞の性質をもつ細胞を大量に誘導することができれば，脊髄損傷において"自己細胞による再生"が可能になる，と考えたのが研究のスタートである．早速シュワン細胞を誘導するための試行錯誤を開始した．

人生と実験は似た部分がある．もくろみや計画が予想どおりの展開をするわけではない．ときには思わぬ結果におわることもある．その多くは，もくろみから外れた単なる失敗であろうが，しかし，視点を変えてみれば失敗と片付けられているなかにとんでもないお宝（副産物）が隠れていることがある．例にもれず，筆者らの実験は誤算の連続であったが，結果としてシュワン細胞を誘導する方法が開発された．同時に，誤算の連続が思わぬ副産物をもたらした．得られた副産物は神経誘導と骨格筋誘導である．いまでは，副産物というよりこちらがむしろ主産物になっている（**図1**）．

1 骨髄間質細胞とは

骨髄には，造血系細胞のほかに間葉系の骨髄間質細胞がある．造血系細胞は浮遊細胞であるので培養皿には接着しないが，骨髄間質細胞は接着する．骨髄穿刺で採取した骨髄液を培養すると，この性質の違いによって骨髄間質細胞を接着性細胞として容易に採取することが可能である．ここで念頭におかなくてはならないのは，このようにして採取された骨髄間質細胞は均質な細胞群ではなく，ヘテロな集団であるということである．実際にフローサイトメトリー(FACS)解析を行なうと，間葉系のマーカーとされているCD29やCD90などはほとんどの細胞に発現しているが，造血系のマーカーであるCD34やc-Kitなども数パーセントではあるが混在している．

骨髄間質細胞は増殖力が非常に旺盛であり，短期間で移植できるだけの細胞数を確保することが可能である．たとえば，数十mlの骨髄液を培養すれば数週間で1000万個の細胞を得ることができる．なによりも患者自身の細胞を利用できるので，倫理問題のハードルが低く，免疫拒絶の問題も回避できるなど，利点の多い細胞である．

骨髄間質細胞には，骨・軟骨・脂肪・心筋などに分化する能力があることが以前より示唆されてきた[2,3]．また，骨髄間質細胞を静脈注射などの方法で生体に投与すると，

さまざまな臓器に分布し，肝臓や神経系細胞などをはじめ多様な細胞に分化転換すると報告されている．しかし，残念ながらその率は低い．さらに，培養下で成長因子や薬剤，メチル化剤などを投与して特定の細胞に分化させ，生体に移植しようという試みもされてきた．神経誘導に関しては，還元剤や成長因子だけで誘導されるという報告がある一方[4,5]，これらの神経細胞は生体で機能しうるという確証が示されておらず，機能的な神経に分化していないのではないかという否定的な報告がされている[6]．

II シュワン細胞の誘導

同じ神経系であっても末梢神経と中枢神経ではグリア細胞の構成が大きく異なっており，このことが損傷を受けたあとの再生能力にかかわっている．中枢神経はアストロサイトやオリゴデンドロサイトがグリア環境をつくっているが，組織が損傷を受けると，これらの細胞は神経軸索の伸長を積極的に阻害する作用をもつ[7]．一方，末梢神経を構成するグリア細胞はシュワン細胞であり，その重要な機能はミエリンを形成し跳躍伝導を可能にすることである．末梢神経が損傷を受けるとシュワン細胞は増殖を開始し，多岐にわたるサイトカインや成長因子を産生する．そして，これによって神経線維の再伸長を誘導し，ミエリンを再形成して跳躍伝導の回復をもたらす[7]．シュワン細胞は中枢神経系に移植されても同様の作用をもち，軸索再伸長の足場を提供してミエリンも形成できるので，跳躍伝導の回復も見込まれ，目的にかなった細胞である．

当初，骨髄間質細胞からシュワン細胞を誘導するにあたりかなり試行錯誤したが，最終的に行き着いた発想は，細胞になんらかの因子を加えて未分化状態に戻し，ついで，シュワン細胞の発生を制御する因子を投与すれば誘導できるのではないかというものであった．この着眼はうまくいき，まず，無血清培地で還元剤β-メルカプトエタノールを24時間投与し，つづいて，10%ウシ胎仔血清を添加したαMEM培地にレチノイン酸を加えて3日間培養して，最後に，塩基性線維芽細胞増殖因子（bFGF），細胞内cAMP上昇作用をもつフォルスコリン（forskolin），ニューレグリン（neuregulin），および，血小板由来増殖因子（PDGF），10%ウシ胎仔血清を含むαMEM培地で培養することによって97%近くの効率で選択的にシュワン細胞を誘導することができた（図1）．誘導された細胞はシュワン細胞の形態にきわめて類似したものであり，シュワン細胞のマーカーである，P0，p75，O4，GFAP，S-100などを発現し，Krox20などの転写因子の上昇もみられた[8]．

III 末梢神経および脊髄損傷モデルへの移植

誘導された細胞がシュワン細胞様であっても，生体で軸索再生を誘導しミエリン再建による跳躍伝導を可能にしなくては，真に細胞移植治療への要件を満たすとはいえない．このことを検証するため，誘導したシュワン細胞をマトリゲルとともに物質透過性チューブに充填して移植片を作製し，成体ラットの坐骨神経に1.2 cmほどの欠損をつくり架橋移植した．半年後，移植片内への旺盛な神経線維の再生を認め，移植細胞によるミエリン形成，移植片の遠位部への再生線維の伸長と神経筋接合の再建，そして，神経伝導速度の回復と歩行機能の改善を認めた[9]．

また，ラット脊髄損傷モデルへの移植においても軸索伸長と機能回復を認めている．脊髄の第7胸髄の一節分を完全に切除し，上記と同様の移植片で上下の脊髄を架橋すると，7カ月後には移植片内に組織が形成されて上下の脊髄組織と連結し，さらに，移植片内への神経線維の伸長，脊髄機能を示すBBBスコアの改善を認めた[10]．

これらの結果より，骨髄間質細胞から誘導した細胞はシュワン細胞としての形質とともに，軸索再生誘導能というもっとも重要な機能をもちあわせたものであることが証明された．

IV 神経細胞の誘導

Notchは発生や分化を制御することが知られており，幹細胞の維持，神経や骨格筋の分化制御など多くの機能をもつ[11]．筆者は当初，先述のように脊髄再生の目的でシュワン細胞を誘導する研究に取り組んでいた．Notchは神経発生においてはグリア誘導因子として作用することが知られていたので，さまざまな細胞に分化する能力をもつ骨髄間質細胞にNotchを導入し，ついで，サイトカイ

ンを投与することによりシュワン細胞が誘導されるのではないかと期待して実験した．ところが驚いたことに，ごくわずかではあるが神経細胞が誘導されたのである．この発見は"意外な副産物"であったが，骨髄間質細胞は患者自身から大量に採れるので，この現象を再現しブラッシュアップすれば自己細胞由来の神経細胞を大量に得ることにつながるのではと考えて検証を進めた．そして，最終的に，図2に示すようなシステムを見いだした[12]．

構成活性型であるNotchの細胞内ドメイン（Notch1 intracellular domain；NICD）をコードする遺伝子をpCI-neoプラスミドサイトメガロウイルスプロモーター下流に組み込み，リポフェクションで導入し選択すると，骨髄間質細胞は神経幹細胞あるいは神経前駆細胞のマーカーであるネスチン（nestin），GLAST，3-ホスホグリセリン脱水素酵素（3-PGDH），Sox2などを発現するようになる．プロモーター解析においてもこれらの活性上昇がみられた．これらのことから，骨髄間質細胞はNICDの導入によって神経幹細胞/神経前駆細胞の性質の一部を獲得したと推定した[12]．

Notchを導入した細胞から効率的に神経細胞を誘導するため，さまざまなサイトカインや栄養因子を投与したところ，bFGF，forskolin，毛様体神経栄養因子（CNTF）の3種を同時に投与することによって，非常に高い効率（96％前後）で神経細胞誘導がひき起こされることを見いだした．誘導された神経細胞は，Brd-U取り込み実験から最終分裂を終えた神経細胞であることが確認され，さらに，パッチクランプにおいて内向きNa^+電流，すなわち，活動電位を認めたことから機能的な神経細胞であると考えられた．また特記すべきことに，種々の検討を重ねた結果，この細胞群にはグリア細胞が含まれていないことがわかった．これらの結果より，このシステムにより骨髄間質細胞から機能的な神経細胞が選択的に誘導されたと結論した[12]．なお，当初導入したNICDは細胞の分裂とともに消退し，神経分化を終えた時点では，ほとんど検出されなかった．

なぜ，神経細胞だけが選択的に誘導されたのか．これは非常に不思議なことである．骨髄間質細胞はもともと神経分化とグリア分化の両方のポテンシャルをもっているようで，NeuroD，Math1，Mash1，ニューロゲニン（neurogenin）など神経分化を促進する前神経遺伝子（proneural gene）と，グリア分化にかかわるSTAT1/3，Hes1/5の両方を発現している．ただし，転写活性を調べると前者のほうが圧倒的に強い．NICDを導入し，ひき続きサイトカイン処理するとこれらの因子がどのように変化するのか調べたところ，STAT1/3，Hes1/5の発現が順次抑えられるのに対して，前神経遺伝子の発現は続き，転写活性も増強していた．このことが神経細胞の選択的誘導とかかわりがあるものと思われる[12]．

Ⅴ 神経変性モデル動物への移植

骨髄間質細胞から誘導された神経細胞を，中大脳動脈

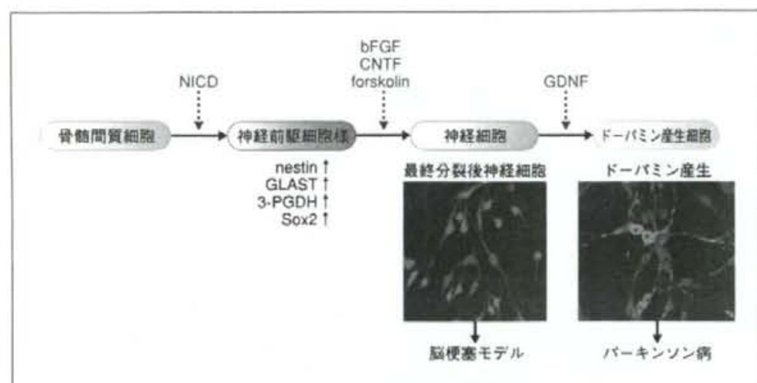

図2　神経誘導の概要
骨髄間質細胞に構成活性型であるNotch細胞内ドメイン（NICD）を導入すると，神経幹細胞/神経前駆細胞様に分化し，nestin，GLAST，3-ホスホグリセリン脱水素酵素（3-PGDH）などのマーカーの発現が上昇する．これらの細胞にbFGFなどのサイトカイン処理を加えると，最終分裂を終えた神経細胞に分化する．これらの細胞は脳梗塞モデルにおいて有効性が認められた．誘導した神経細胞にGDNFを投与するとドーパミン産生細胞への分化が促進され，パーキンソン病モデルにおいてその効果が確認されている．

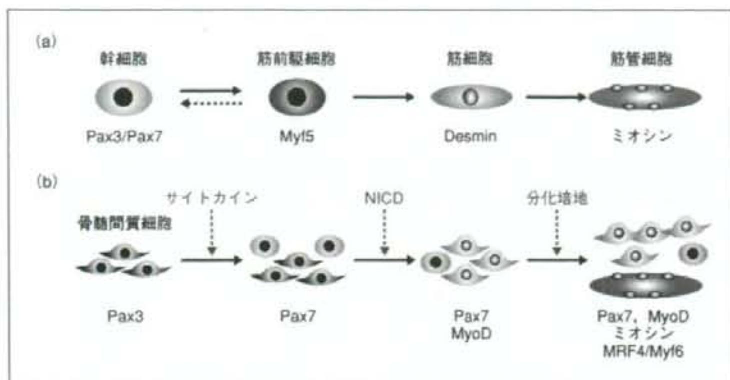

図3　筋発生と骨格筋誘導系との比較

(a) 筋発生の各段階で発現される代表的な因子.
(b) 骨髄間質細胞からの骨格筋誘導系. 筋発生とは同じ経過をたどるわけではないが, 類似性がある.

虚血再還流によって作製したラット脳梗塞モデルへ移植し, その効果を観察した. 誘導した神経細胞を, 脳梗塞モデル作製後7日目の虚血領域の大脳皮質3カ所に5万個ほど移植したところ, 移植後4週間で平衡運動や知覚機能のみならず, 学習認知機能(モリス水迷路試験)の顕著な改善も認められた. 残念ながら梗塞領域の縮小は認められなかったが, GFPで標識された移植細胞は, 皮質あるいは海馬領域に神経マーカー陽性の細胞として生着していた. とくに海馬には多くの移植細胞があったことから, 認知機能の改善との関連を推定している[13].

誘導された神経細胞は, 形態的にも機能的にも神経細胞の特性を有しており, ラット脳梗塞モデルへの移植でも有効性が認められたが, 実は, この段階の神経細胞は特定の神経伝達物質を産生しておらず, ドーパミン神経のマーカーであるチロシン水酸化酵素陽性細胞も3～4%前後であった. パーキンソン病モデルへの応用においてはドーパミン産生細胞が必要である. そこで, 誘導した神経細胞に中脳ドーパミン神経の発生において作用することが知られているグリア細胞株由来神経栄養因子(glial-cell line derived neurotrophic factor; GDNF)を投与したところ, チロシン水酸化酵素陽性率が30～40%に上昇した. さらに, 培養上清中のK^+濃度を上昇させて脱分極を誘発するとドーパミンが放出されることを, 高速液体クロマトグラフィー(HPLC)において確認している. これらの神経細胞を6-ヒドロキシドーパミン投与によって作製したパーキンソン病モデルラットの線状体に10万個移植したところ, HPLCで脳内でのドーパミン産生能の上昇が確認され, また, アポモルフィン投与によって誘発される異常回転運動の顕著な改善を認めた. これらの結果は, 骨髄間質細胞から生体で機能しうる神経細胞を誘導できることを示している[12].

Ⅵ 筋肉細胞の誘導法

神経誘導の実験中に, 対照実験のつもりでNICDとサイトカインの投与順序を逆転させてみたところ, 意外にも骨格筋細胞が誘導される所見を得た. 最初に目にした像は, 長細い細胞に2, 3個の核が含まれる小さな多核の細胞でしかなかったが, 生体内に多核の細胞というのはそうそうあるものではない. 血小板をつくる巨核球や骨形成にかかわる破骨細胞は確かに多核であるが, これらは球形の細胞である. 長細い細胞である場合, 考えられるのは骨格筋ということになる. 筆者は当然ながらこの意外な結果に驚いたが, この方法を検証することによって骨髄間質細胞から骨格筋を有効につくり出すシステムにつながると考えた. そして, 最終的に, 図3のような誘導方法を導き出した[14].

骨髄間質細胞を特定の密度でまき, bFGF, forskolin, ニューレグリン, および, PDGFを含んだ培地で3日間培養する. この段階で筋肉発生の初期に認められるPax7が発現しており, 骨髄間質細胞は筋前駆細胞様に分化したと考えた. この細胞にNICDを導入すると筋細胞へと分化し, MyoD, ミオゲニン(myogenin)などの骨格筋特有のマーカーの発現が認められる. さらに, 分化培地(2%ウマ血清を含むDMEM培地, あるいは, ITS培地)に切り替えることにより, 一部の細胞が成熟した多核の筋管

細胞に分化し，ミオシン重鎖，骨格筋ミオシン，トロポニンなどの細胞骨格蛋白質とともに，成熟マーカーとして知られているMRF4/Myf6も発現するようになる．この分化した最終産物には，増殖可能な①単核の筋芽細胞（MyoD陽性），②骨格筋の幹細胞である筋衛星細胞（Pax7陽性），そして，③成熟した多核の骨格筋細胞，の3種類の骨格筋系譜の細胞が含まれていた．さらに，増殖可能な①筋芽細胞，および，②筋衛星細胞をクローン培養することにより，誘導過程で混在していた骨格筋に分化する能力をもたない細胞を除去し，骨格筋系細胞のみを純化して大量に増殖させることに成功した[14]．

VII 骨格筋への移植

クローン培養によって純化したヒト由来の骨格筋系細胞が実際に生体で機能することを確認するため，カルジオトキシン（cardiotoxin）によって変性させたラット前脛骨筋に免疫抑制剤投与下で移植した．移植細胞はGFPで標識し，10^6個の細胞を変性筋肉組織に局所注入，ないしは，尾静脈からの静脈注射により投与した．いずれの投与方法でも2週後には移植細胞は変性筋組織に生着し，ヒトジストロフィンを発現していた．また，局所注入（37％）のほうが静脈投与（22％）に比べてやや高い生着率を示していた．筋ジストロフィーのモデルマウスであるmdxヌードマウスに移植しても，ほぼ同様の結果が確認された[14]．

筋肉が変性・崩壊すると，幹細胞である筋衛星細胞が増殖して筋組織を再生するが，本来はわずかしか培養できない筋衛星細胞を骨髄間質細胞から大量に誘導することができた．筋衛星細胞は筋細胞が崩壊すると増殖刺激を受けて増殖を開始し，筋細胞に分化すると同時に一部が筋衛星細胞として残るので，くり返し組織再生に寄与することができる（図4a）．筆者らは，骨髄間質細胞から誘導した筋衛星細胞が同様の性質を示すかどうかを，以下のように検討した．最初の移植から4週後に移植細胞の生着を組織生検によって確認し（筋管細胞の多くは辺縁核で成熟した筋管であることを確認した），再び細胞を移植することなくカルジオトキシンを投与して2度目の筋変性を誘導した．移植された細胞から形成された多核の筋管細胞は筋変性によって崩壊し，再び筋細胞になることはできない．しかし，もし，移植された細胞の一部が筋衛星細胞の性質を保持していれば，筋細胞変性に伴って増殖誘導が起こり，筋管細胞を形成することができる．そこで，2度目の筋変性を誘導し，2週後に筋組織を調べ

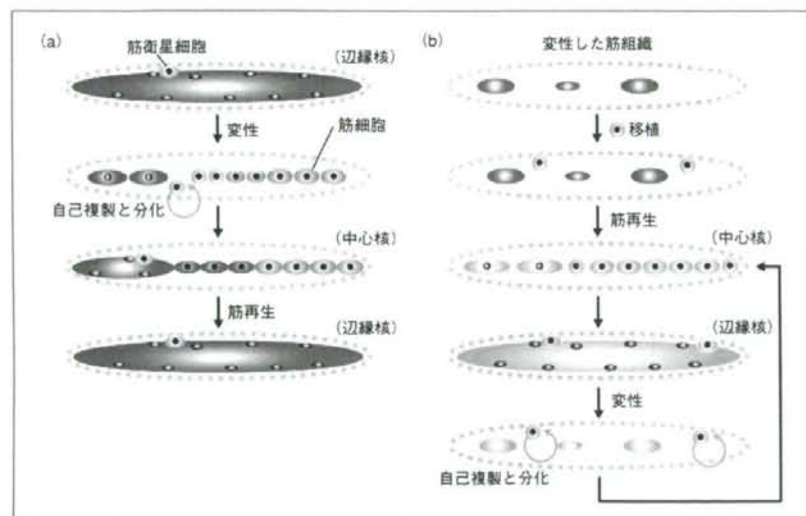

図4 正常筋組織での再生と骨髄間質細胞から誘導された筋衛星細胞による筋再生

(a) 正常筋組織での再生．筋肉が変性すると，幹細胞である筋衛星細胞が増殖して筋組織を再生する．再生途中では，まず中心核の成熟過程にある筋管細胞がつくられ，ついで辺縁核の筋管（筋線維）へと成熟する．(b) 骨髄間質細胞から誘導された筋衛星細胞による筋再生．変性した筋組織に誘導したGFP陽性の筋衛星細胞を移植すると，筋組織が再生される．このの ち，再び細胞を移植することなく2度目の筋変性を誘導すると，移植した筋衛星細胞の増殖誘導が起こり，中心核の筋管細胞，ついで，辺縁核の筋線維への再生がみられる．

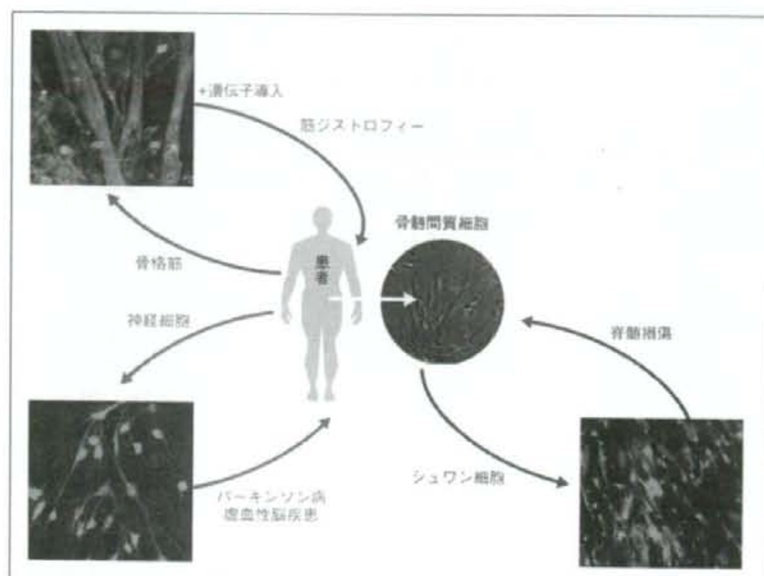

図5　骨髄間質細胞を用いた自己細胞移植治療の概念図

たところ，最初に移植したヒト由来のGFP陽性の細胞から分化した"中心核の筋管細胞"の再生を確認することができた（図4b）．筋衛星細胞から筋管細胞が形成される過程では，まず，中心核の成熟過程にある筋管細胞がつくられ，ついで，辺縁核の筋管（筋線維）へと成熟する．中心核の筋管細胞が観察されたことは，2度目の筋変性後に筋衛星細胞から新たに形成された筋管細胞であることを示している（図4）．すなわち，この方法で誘導された筋衛星細胞を移植すれば，2度，3度と筋肉が崩壊することがあっても，生着した筋衛星細胞が増殖して崩壊した筋肉を補うことが可能である[14]．

さて，筋ジストロフィーの治療においては，移植された正常な機能をもつ筋管細胞は長期にわたって生着し機能することが期待できるが，1回の移植ですべての細胞を置換することは困難である．残念ながら，残った患者自身の筋肉細胞は，日々，変性・崩壊が進行すると推定される．ところが，この方法では移植された正常な筋衛星細胞がくり返し増殖刺激を受け，増殖と分化をくり返すと推定される．すなわち，徐々に異常な筋管細胞から正常な筋管細胞への置き換えが起こることが期待できる．この性質は筋ジストロフィーの治療においては大きな利点である．

Ⅷ 神経・筋分化誘導におけるNotchの機能

骨格筋の発生においては，Notchは筋芽細胞の分化を抑制すると報告されている[15]．今回の研究結果では，骨髄間質細胞にNotchを導入し，選択的に骨格筋細胞を誘導しているが，これまで知られている神経や筋肉発生におけるNotchの作用からすれば，逆の作用をもたらしたようにみえる．従来，Notchの下流ではHes1，Hes5が機能し，細胞分化を抑制していると考えられてきたが，今回のシステムでは，NICDによりHes1，Hes5の誘導は認められない．この違いは，Notch刺激を受けとる細胞内環境の違いによっていると推定され，おそらく，骨髄間質細胞には発生過程の細胞とは異なる特有のシステムが存在することを示唆している．また，細胞の状況によってはNotchが異なる機能を発揮する可能性があること，すなわち，Notchにはこれまで知られていない新たな機能があると推定される．

Ⅸ 骨髄間質細胞の広い可能性

骨髄間質細胞には広くいろいろな細胞に分化するポテンシャルがあり，これまで骨，軟骨，脂肪に分化すると報告されている．筆者らの開発した，神経，骨格筋，シュワン細胞への誘導系は，選択的で効率的な誘導システムであるが，骨髄間葉系細胞からはこのほかに，心筋，肝細胞，インスリン産生細胞，気道系上皮細胞が誘導されると報告されている[3,16,17]．いずれも選択的，あるいは，それに近い誘導系で高い効率を示しており，その有効性が今後ますます期待されると思われる．骨髄間質細胞は，多分化能においてはES細胞には匹敵しないまでも，かなり広い多分化能を有している有望な細胞であることが示唆されている．重要なことは，骨髄間質細胞が生体内において仮に自発的に分化転換を起こすとしても，その効率はきわめて低く，細胞移植治療に活用できるものではないということである．胚葉をこえた分化転換をひき起こし，目的とする細胞を一定量以上得るためには，順序だてた分化誘導操作を着実に行なう必要がある．

おわりに

骨髄間質細胞は骨髄移植の際にすでに移植されている細胞であり，安全かつ容易に採取可能で，旺盛な増殖力をもっている．患者自身（本人の細胞は免疫拒絶がなくよい細胞であるが，遺伝性疾患を対象とした場合，たとえば，筋ジストロフィーではジストロフィン遺伝子など機能を欠失している遺伝子を補う必要がある）や家族からの採取も可能であり，既存の骨髄バンクの登録者から提供を受ける方法もある．とくに，患者自身の細胞を用いた場合には，免疫拒絶のない自らの細胞を用いた理想的な細胞移植治療，すなわち，"自己細胞移植治療"が可能となる（図5）．よって，骨髄間質細胞から神経細胞，骨格筋細胞を誘導する方法を確立したことは，臨床応用の観点から大きな利点があると考えられる．今回，脊髄損傷，脳梗塞，パーキンソン病，筋変性モデル動物に移植し有効性が確認されたことから，神経変性疾患・筋変性疾患への応用が期待される（図5）．しかし，実際に治療に応用するためには，安全性，有効性など解決しなければならない多くの課題が残されている．ES細胞，各種の組織の幹細胞から分化誘導した細胞の活用にも多くの期待が寄せられており，骨髄間質細胞も含めて，それぞれ長所を活かされるべきである．また，最終的に安全で有効な細胞を樹立し，確信をもって移植医療に活用するためには，これらの細胞の性質や分化誘導の分子機構を解明する必要がある．

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出澤真理

略歴： 1989年 千葉大学医学部 卒業，内科研修医を経て，1995年 千葉大学大学院医学研究科博士課程 修了，同年 千葉大学医学部解剖学教室 助手，1997年 千葉大学医学部眼科学教室 助手，2000年 横浜市立大学医学部解剖学教室 講師，2003年より京都大学大学院医学研究科 助教授．

研究テーマ： 間葉系細胞の分化転換と細胞移植治療への応用．

抱負： 結論がわかっているのであれば学生実習である．研究はわからないからやるのである．人生も結末がわかっていたら，生きていておもしろくない．それと同じで，なにが起こるか予測不能だからこそ研究はスリルに満ちている．

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Cellular and Molecular Life Sciences

Review

Insights into autotransplantation: the unexpected discovery of specific induction systems in bone marrow stromal cells

M. Dezawa

Department of Anatomy and Neurobiology, Kyoto University Graduate School of Medicine, Yoshidakonoe-cho, Sakyo-ku, 606-8501 Kyoto (Japan), Fax: +81 75 751 7286, e-mail:dezawa@anat2.med.kyoto-u.ac.jp

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Abstract. Many kinds of cells, including embryonic stem cells and tissue stem cells, have been considered candidates for transplantation therapy for neuro- and muscle-degenerative diseases. Bone marrow stromal cells (MSCs) also have great potential as therapeutic agents since they are easily isolated and can be expanded from patients without serious ethical or technical problems. Recently, new methods for the highly efficient and specific induction of functional neurons and skeletal muscle cells have been developed for MSCs. These induced cells were transplanted into animal models of stroke, Parkinson's disease and muscle degeneration, resulting in the successful integration of transplanted cells and improvement in the behavior of the transplanted animals. Here I describe the discovery of these induction systems and focus on the potential use of MSC-derived cells for 'auto-cell transplantation therapy' in neuro- and muscle-degenerative diseases.

Keywords. Mesenchymal cell, transdifferentiation, regenerative medicine, cell therapy, transplantation, Schwann cell, neuronal differentiation, myogenic differentiation.

Introduction

Neurodegenerative diseases, such as Parkinson's disease and brain ischemia, and muscle-degenerative diseases, such as muscular dystrophy, are responsible for a decline in neuronal and muscular function which often limits the life span. While transplantation of liver, kidney, and bone marrow has already been performed on thousands of patients, transplantation of the nervous system and general muscle tissue has faced many limitations. Effective therapeutic strategies still need to be developed. In the central nervous system (CNS), where neurons become post-mitotic after birth, neural cell transplantation is one potential treatment of such neurologic disorders. As for muscles, satellite cells are considered stem cells in adult muscle tissue, although the difficulty isolating a sufficient number of pure satellite cells has precluded their use in cell-based tissue repair [1, 2] Furthermore, there is a need to establish cell therapies using healthy donors since muscle dystrophies are inheritable diseases.

Recently, embryonic stem (ES) cells and tissue stem cells have aroused a great deal of interest because of their potential for treating degenerative diseases. ES cells are known to differentiate into various kinds of cells including neurons and skeletal muscle cells, either by spontaneous differentiation or following certain induction methods [3–5].

Tissue-specific stem cells have been identified in various tissues at more advanced developmental stages. Neural precursors and/or progenitors have been identified in developing and adult CNS tissues [6–10]. These cells have the ability to self-renew and the potential to differentiate into neurons, astrocytes and oligodendrocytes. For neuronal cell replacement, transplantation of neural stem cells (NSCs) has been attempted in a wide range of animal models of diseases and injuries such as Parkinson's disease, Huntington's disease, stroke, and spinal cord injury,

and functional improvement has often been reported [11–15]. Stem cells and satellite cells isolated from adult and prenatal muscle tissue [16–19] and myogenic stem cells from bone marrow [20, 21] are considered to be sources of cell replacement, and several attempts have been made to ameliorate muscle degeneration by transplantation of these muscle stem cells [20]. Although tissue stem cells have great potential, they face limitations inherent in procurement from fetal tissue, including problems of histocompatibility and ethical concerns.

Bone marrow contains a category of nonhematopoietic cells that can be cultivated *in vitro* as plastic adherent cells, namely bone marrow stromal cells (MSCs) [22]. MSCs are mesenchymal elements that normally provide structural and functional support for hematopoiesis and express mesenchymal markers but lack hematopoietic surface markers [23, 24]. The great benefit of MSCs is that they are easily accessible through aspiration of the bone marrow from patients. This strategy avoids ethical issues, enabling us to use them for 'auto-cell transplantation therapy'. They are also easily expanded on a large scale; for example, 20–100 ml of bone marrow aspirate provides 10^7 cells within 2–3 weeks, a plentiful number for transplantation.

At the present time, the benefits of MSCs for transplantation therapy are twofold. First, the transient trophic effect of MSCs can delay cell death and restore the tissues [25–29] and, second, the multipotency of MSCs gives rise to 'cells with a purpose' for cell-based transplantation therapy.

According to a hierarchical paradigm, MSCs differentiate into mesenchymal lineage cells such as osteocytes, chondrocytes and adipocytes [22, 30, 31]. Recently, however, the unorthodox plasticity of MSCs has been described, as they have the ability to cross oligolineage boundaries which were previously thought to be impenetrable. In fact, it has been suggested that various kinds of cells are inducible from MSCs both *in vivo* and *in vitro*. The possibility of MSC plasticity and transdifferentiation was initially described in *in vivo* experiments, where transplanted donor bone marrow-derived cells differentiated into glial cells in the recipient brain [32]. In the case of muscle, infused bone marrow cells integrated into host muscles and supported regeneration [20]. While these studies suggested the plasticity of MSCs because of the expression of donor markers and cell-specific markers, the clonality and functions of these transdifferentiated cells were not clearly evaluated in some cases. Moreover, there has been the suspicion that these phenomena are based on cell fusion or spontaneous transdifferentiation at a very low frequency [33, 34].

Apart from these *in vivo* experiments, there have been several *in vitro* attempts to induce MSCs into purposeful cells such as cardiomyocytes with cardiac muscle properties, hepatocytes, insulin-producing cells, and airway epithelial cells. However, some of these reports had a low induction efficiency [35–38]. Nevertheless, the potential of MSCs to transdifferentiate from mesenchymal lineages to other lineages is now of great interest. It is clear that MSCs will represent good candidates for practical cell-based therapy if their differentiation into target cells can be controlled with high efficiency and purity.

Recently, a method was developed which systematically induced neurons, skeletal muscle cells (Fig. 1) and Schwann cells from human and rat MSCs on a therapeutic scale [39–41]. This review describes the discovery of systemic induction, the properties of induced cells, and finally their potential, advantages, and disadvantages for clinical application in neurodegenerative and muscle-degenerative diseases. Schwann cells, peripheral glia known to support axonal regeneration both in the peripheral nervous system (PNS) and CNS, are also inducible from human and rodent MSCs [39, 42, 43]. MSC-derived Schwann cells elicited axonal regeneration and functional recovery in spinal cord injury. The utility of these induced Schwann cells has been reviewed elsewhere [44, 45].

Systems for inducing neurons and skeletal muscle cells from MSCs; the fruit of unexpected discovery

Specific induction of neurons from MSCs

Recently, my research team established a new method to induce neurons systematically from human and rat MSCs. Highly efficient and specific induction of post-mitotic, functional neuronal cells, without glial differentiation, can be achieved by gene transfer of Notch1 intracellular domain (NICD) followed by the administration of certain trophic factors [40] (Fig. 1). However, all these findings are the fruit of an unexpected discovery.

The initial goal of this MSC study was to develop an efficient Schwann cell induction system from MSCs for application to spinal cord injury. A series of experiments demonstrated that transplanted Schwann cells can delay nerve cell death and promote regeneration of nerve fibers and functional recovery when supplied to the damaged spinal cord [46]. However, it is difficult to obtain a sufficient amount of Schwann cells. To cultivate Schwann cells for autologus transplantation in humans, for example, another PNS must be sacrificed. Furthermore, there are other technical difficulties in harvesting and expanding Schwann cells from PNS. Therefore, it would be more desirable to establish cells with Schwann cell characteristics from sources other than the PNS that are easily accessible and capable of rapid expansion. MSCs were thought to be a good candidate.

As described previously, induction of Schwann cells was finally established using a reducing reagent, retinoic acid, and trophic factors related to Schwann cell development [44, 45]. However, I first tried to induce Schwann cells

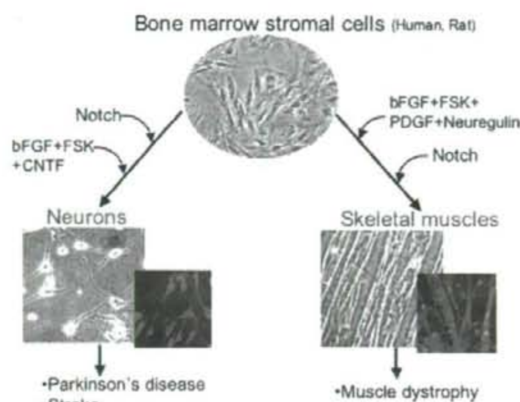

Figure 1. Schematic diagram of the induction system for neurons and skeletal muscle cells. Neurons are induced by Notch intracellular domain gene transfer followed by administration of trophic factors basic fibroblast growth factor (bFGF), forskolin (FSK) and ciliary neurotrophic factor (CNTF). The final population consisted mostly of neurons immunopositive for neuronal markers such as neurofilament. Skeletal muscle cells could be obtained by the reverse treatment, namely trophic factor treatment with bFGF, FSK, Platelet-derived growth factor (PDGF) and neuregulin, followed by Notch gene transfer.

from MSCs by Notch transfection. The Notch gene encodes a 300-kDa single transmembrane cell surface receptor protein that is activated by Delta/Serrate/Lag-1 ligands presented by neighboring cells [47]. Upon ligand binding, the intracellular portion of the Notch receptor is cleaved and enters the nucleus, where it influences the expression of numerous transcription factors related to progenitor pool maintenance, cell fate, and, in the case of the nervous system, terminal specification as glial cells [47–49]. In fact, a series of studies have shown that when Notch signaling is activated, astrocytes and Schwann cells differentiate from NSCs and neural crest stem cells, respectively [48, 49]. Initially, it was expected that MSCs would shift from mesenchymal to Schwann cell characteristics by Notch introduction when combined with administration of trophic factors related to Schwann cell development, such as basic fibroblast growth factor (bFGF), ciliary neurotrophic factor (CNTF) and forskolin (FSK), known to upregulate intracellular cyclic AMP [50]. After such treatment, however, it was very surprising to see a small population of neuron-like cells induced in the final product. The experiment was repeated and the original method was improved to establish the neuronal induction system from MSCs and to examine the properties of induced cells.

The mouse NICD cDNA was subcloned into pCI-neo, a cytomegalovirus (CMV) promoter-containing mammalian expression vector, and transfected into human and rat MSCs by lipofection followed by G418 selection [40]. After transfection with NICD, the MSCs substantially upregulated markers related to NSCs and/or neuronal progenitor cells (NPCs), such as the glutamate transporter GLAST, 3-phosphoglycerate dehydrogenase (3-PGDH) and nestin [51, 52]. This suggested that MSCs may acquire some of the characteristics of NSCs/NPCs when NICD is introduced (Fig. 2).

Next, cells were subcultured once (60–70% confluence) with administration of the trophic factors bFGF, FSK, and CNTF for 5 days, which resulted in a highly efficient and specific induction of cells with neuronal characteristics (Fig. 2) [40]. It was crucial that the cell density of NICD-transfected MSCs be reduced by subculture just before the administration of trophic factors. Some cells already started to extend neurite-like processes 6 h after trophic factor administration. However, if the cell density was too high, neurites attached to the neighboring cells soon after their extension, thereby retracting their neurites and preventing the differentiation of a neuron-like morphology. Therefore, adequate intercellular distance and timing of trophic factor stimulation are crucial for the MSCs to become neurons. Nontransfected as well as control vector-transfected MSCs could not be induced to neurons by trophic factors, indicating that NICD transfection is necessary for MSCs to acquire neuronal potential [40].

These MSC-derived neuronal cells (MSC-Ns) extended neurite-like processes with abundant varicosities and expressed neuronal markers such as MAP-2ab, neurofilament-M, and beta-tubulin isotype3. Approximately 96% of cells were immunopositive for MAP-2ab, although nearly 2% of nestin-positive cells could also be recognized. MSC-Ns did not proliferate when subcultured after trypsin treatment. Indeed, Brd-U incorporation performed 5 days after trophic factor administration showed

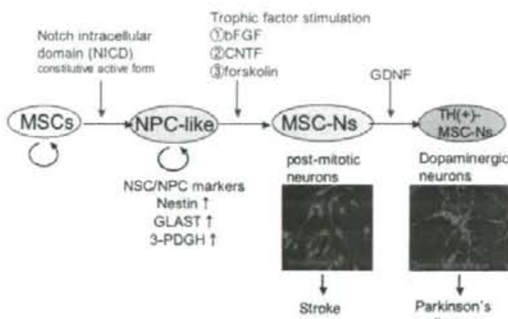

Figure 2. An outline of the neuronal induction system. After NICD transfection, MSCs become similar to NSCs/NPCs (NPC-like), since they express nestin, GLAST and 3-PGDH. After trophic factor stimulation, cells became post-mitotic neurons (MSC-Ns) expressing neuronal markers such as neurofilament. These neurons are effective in the stroke rat model. After administration of GDNF, post-mitotic neurons became dopamine-producing cells [TH(+)-MSC-Ns], useful in the Parkinson's disease model.

that few MAP-2ab-positive cells incorporated Brd-U. In addition, less than 1% of MAP-2ab-positive cells were immunoreactive to an intrinsic proliferation associated marker, Ki67, suggesting that the majority of MSC-Ns were post-mitotic [40].

MSC-Ns were evaluated physiologically using the voltage clamp method. Seven days after trophic factor induction, an outwardly rectified K^+ current was elicited by positive voltage steps in MSC-Ns, which was dramatically higher than in untreated MSCs. Concomitantly, resting membrane potential was lowered. However, the voltage-gated fast sodium currents, which represent functional neuron characteristics, could not be observed up to 14 days after trophic factor induction, suggesting that although MSC-Ns exhibit a neuron-like morphology and express several neuronal markers, they are not fully mature neurons but rather are in a process of maturation. Neurotrophins such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) which were administered to MSC-Ns to promote their maturation, resulted in the generation of sodium currents and action potentials in the small population of MSC-Ns. These results indicated that, just after trophic factor induction, MSC-Ns are neuronal cells in a premature state and can be induced to become functionally mature neurons with further administration of neurotrophins [40].

The final population of MSC-Ns are devoid of glial development. In fact, few cells positive for glial fibrillary acidic protein (GFAP, a marker for astrocytes), galactocerebroside, or O4 (markers for oligodendrocytes) were detected in the final MSC-N population by immunocytochemistry, Western blot or RT-PCR [40]. NSCs/NPCs are known to differentiate into GFAP-positive glial cells when the gliogenic factors Hes1/5 and STAT1/3 are activated, while they differentiate into neuronal cells with activation of proneural genes Mash1, Math1, and neurogenin

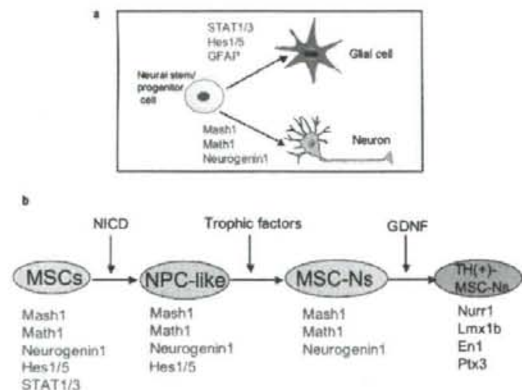

Figure 3. Neurogenic and gliogenic factors in neuronal induction. (*a*) Summary of neurogenic factors and gliogenic factors in conventional neural development. (*b*) Expression of factors during the neuronal induction system.

(Fig. 3a) [53–55]. To examine the induction event from MSCs to MSC-Ns, the expression of those genes was examined by RT-PCR. MSCs initially expressed both neurogenic (Mash1, Math1, and neurogenin1) and gliogenic factors (Hes1/5 and STAT1/3), but during the induction procedure, gliogenic factors were sequentially inhibited and thus finally converged on neuronal factors (Fig. 3b). In fact, STAT1/3 was suppressed after the introduction of NICD and, following trophic factor administration, suppressed Hes1/5 expression (Fig. 3b) [40].

While it was quite accidental, this method was found to induce functional post-mitotic neurons without glial cells from MSCs. Identification of the molecular mechanism played by NICD in the neuronal induction in MSCs is underway The application of MSC-Ns to stroke and Parkinson's disease is discussed further on in this review.

Specific induction of skeletal muscle cells from MSCs

During the experiment of neural induction, I reversed the order of treatment in the control experiment (Fig. 1). Again, the surpising phenomenon of muscle differentiation could be recognized in the culture dish. The induction experiment was repeated, and finally a new method to systematically and efficiently induce skeletal muscle lineage cells with high purity from a large population of MSCs was established (Fig. 1) [41].

Human and rat MSCs were first treated with the trophic factors bFGF, FSK, PDGF, and neuregulin for 3 days and then transfected with an NICD expression plasmid by lipofection followed by G418 selection, and allowed to recover to 100% confluency (Fig. 4). At this stage, a large majority of MSCs developed into mononucleated myogenic cells expressing MyoD and myogenin, while a small population of Pax7(+) satellite cells also existed. Cells were then supplied with a differentiation medium of either 2% horse serum, insulin-transferrin-selenite (ITS)-serum-free medium, or the supernatant of the original untreated MSCs [41], and the final muscle lineage population (termed MSC-Ms) was acquired. MSC-Ms contained three kinds of muscle lineage cells (Fig. 4). The first population included post-mitotic multinucleated myotubes, which expressed myogenin, Myf6/MRF4 (a marker for mature skeletal muscle), and contractile proteins of skeletal myosin, myosin heavy chain, and troponin, all related to skeletal muscle characteristics. In fact, some multinucleated cells exhibited spontaneous contraction *in vitro*. They were also positive for p21, a marker for post-mitotic muscle lineage cells. The second group comprised mononucleated myoblasts which expressed MyoD and myogenin. The third group was composed of satellite cells which were immunopositive for Pax7 and c-MetR, both markers for muscle satellite cells [41].

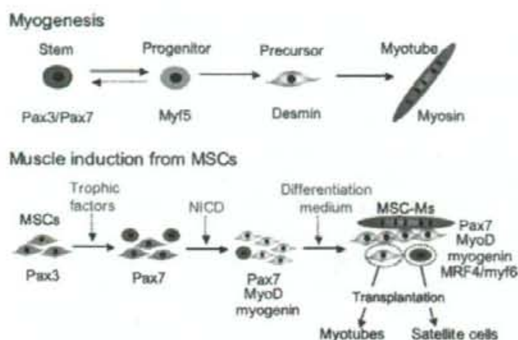

Figure 4. Factors related to myogenesis and the muscle induction system. There is some similarity between conventional myogenesis and the muscle induction system. MSCs generate Pax7-positive precursor cells after trophic factor stimulation and, after NICD transfection, induce MyoD- and myogenin-positive myoblasts. Myoblasts fuse to form multinucleated myotubes in differentiation medium, expressing the marker of maturity, MRF4/myf6. Single myoblast (yellow) and satellite (green) cells were subjected to clonal culture. Clonally isolated myoblasts differentiated into myotubes after transplantation, and clonally isolated satellite cells integrated as muscle stem cells which continued to contribute to muscle regeneration in the host muscle tissue.

However, it was critical to determine if these MSC-derived neuronal and skeletal muscle cells could integrate into host tissue and function as genuine neurons and muscle cells. The effectiveness of these induced cells was verified by a transplantation experiment using animal models of stroke, Parkinson's disease and muscle dystrophy.

Application of MSC-Ns to a stroke model

MSC-Ns were transplanted into the infarction area in a left middle cerebral artery occlusion (MCAO) rat model. The MCAO procedure was somewhat modified in our study, and circling to the right and adduction of the right forelimb when lifted up by the tail were used as signs of successful left MCAO. Seven days after reperfusion, MSC-Ns were transplanted into the nonnecrotic brain parenchyma by stereotaxical injection into the left cerebrum at three locations. The total number of transplanted cells was approximately 40,000–50,000. The control group received only PBS without cell transplantation [56].

MSC-Ns-transplanted rats showed significant recovery, compared with controls, of gross vestibulomotor function (beam balance test), sensorimotor function (limb-placing test), and cognitive function (Morris water maze test) ($p < 0.01$) after 28 days. Histologically, there was no statistical difference in the mean infarct volume between MSC-Ns-transplanted and the control group ($p > 0.05$). However, green-fluorescent protein (GFP)-labeled transplanted cells migrated from the injection site into the ischemic boundary area and integrated mainly into the hippocampus and extended neuritis. Most transplanted cells expressed the neuronal markers neurofilament, MAP-2ab, and beta3-tubulin, while very few cells were positive for GFAP. The reason why cognitive function showed significant recovery may partly be due to the integration of MSC-Ns into the hippocampus [56].

These results showed that MSC-Ns are effective in the amelioration of the rat stroke model. The potential of other kinds of stem cells, such as NSCs and umbilical cord blood cells, in stroke has been reported [57, 58]. These reports indicate that only a small fraction of NSC (1–3% of the grafted cells survived and 3–9% expressed NeuN) or human umbilical cord blood cell (1–2% of injected cells survived and 2–3% were positive for NeuN and MAP-2) populations are expected to integrate into the host brain and differentiate into neurons. Our study showed that approximately 30–45% of MSC-Ns survived in the host brain 1 month after the transplantation and a large fraction expressed the neuronal markers. Thus, the specific induction of neuronal cells from MSCs has great potential in cell transplantation therapy for stroke.

Application of MSC-Ns to the Parkinson's disease model

For Parkinson's disease, transplantation of dopaminergic neurons is believed to be effective; however, cells committed to the expression of certain transmitters account for lower ratios in MSC-Ns [40]. For example, the percentage of tyrosine hydroxylase (TH)-positive cells was approximately 4%, and that of other transmitters such as acetylcholine, glutamate, and substance P fell within a range of 1–3%. As glial cell line-derived neurotrophic factor (GDNF) is known to be involved in the generation and development of midbrain dopaminergic neurons [59], it was administered to MSC-Ns and finally resulted in nearly 40% of MSC-Ns becoming TH-positive cells (Fig. 2). Furthermore, other dopaminergic markers, Nurr-1, Lmx1b, En1, and Ptx3, were elevated (Fig. 3b). The production of dopamine by these TH-positive cells was confirmed by high-performance liquid chromatography (HPLC); high-potassium medium was administered to the culture and subjected to HPLC using a reverse-phase column and an electrochemical detector system, showing that these cells released dopamine to the culture medium in response to high-K^+ depolarizing stimuli. These results indicate that functional dopamine-producing neuronal cells could be induced effectively from MSCs [40].

To explore the ability of induced dopaminergic neurons to survive and function in the host brain, both rat and human cells were transplanted separately into the striatum in a rat model of Parkinson's disease. Unilateral administration of 6-hydroxy dopamine (6-OHDA) into the

medial forebrain bundle is known to selectively destroy dopaminergic neurons in the substantia nigra, leading to quantifiable rotational behavior and providing a useful and commonly used model of Parkinson's disease [60]. Apomorphine-induced rotational behavior (mean rotation index = the mean rotation number in post-/pre-grafting) was examined every 2 weeks up to 10 weeks following cell implantation. 1×10^5 cells were grafted into the ipsilateral striatum. The control group received no grafting after 6-OHDA administration, which provoked a rotational bias away from the lesioned side which persisted, whereas rats grafted with TH-MSC-N rat-induced dopaminergic neurons demonstrated substantial recovery from rotation behavior up to 10 weeks ($p < 0.01$). The mean rotation index was 1.3 ± 0.1 in the control group and 0.3 ± 0.1 in induced dopaminergic neuron-transplanted rats. In addition, nonpharmacological behavior tests, the adjusting step test and paw-reaching test, were performed. Four and 6 weeks after grafting, these rats showed significant improvement in both step adjustment and paw-reaching tests ($p < 0.01$). Immunohistochemically, grafted GFP-labeled, induced dopaminergic neurons were found to migrate and extend beyond the injected site, and approximately 30% of cells remained in the striatum 10 weeks after transplantation. TH-positive processes extended to the outside of the implantation zone. The grafted striatum showed migration of GFP-positive transplanted cells that expressed the markers of neurofilament, TH and dopamine transporter (DAT). Among GFP-labeled cells, TH- and DAT-positive cells were approximately 45% and 30%, respectively. In contrast, most of the GFP-labeled cells were negative for GFAP and O4, consistent with the *in vitro* data that none of the induced cells were positive for these glial markers. Grafted animals were followed up to 16 weeks and no tumor formation was observed in the brain [40].

Human induced dopaminergic neurons were similarly transplanted into the striatum of 6-OHDA-lesioned rats. Animals were immunosuppressed with FK 506 daily, and rotational behavior was recorded 4 weeks after cell transplantation. Grafting resulted in significant improvement in rotational behavior [40].

In summary, the additional administration of GDNF to MSC-Ns can efficiently induce TH-positive, dopamine-producing cells, and functional improvement could be achieved when grafted in a rodent model of Parkinson's disease.

Application of MSC-Ms to a muscle-degenerative disease model

As induced multinucleated myotubes in MSC-Ms are already post-mitotic, single cells of MyoD-positive myoblasts and Pax7-positive satellite cells were subjected to clonal culture (clonal MSC-Ms) to exclude non-muscle cells, and were transplanted into muscle-degenerative disease models [41].

Human clonal-MSC-Ms were transplanted into immunosuppressed rats whose gastrocnemius muscles were damaged with cardiotoxin pre-treatment. Cells were labeled with a GFP-encoding retrovirus and then transplanted by local injection (l.i.) into muscles or by intravenous injection (i.v.). Two weeks after transplantation, GFP-labeled cells incorporated into newly formed immature myofibers exhibited centrally located nuclei in both l.i.- and i.v.-treated animals. Four weeks after transplantation, GFP-positive myofibers exhibited mature characteristics with peripheral nuclei just beneath the plasma membrane. Functional differentiation of grafted human cells was also confirmed by the detection of human dystrophin in GFP-labeled myofibers, indicating that clonal MSC-Ms are able to incorporate into damaged muscles and contribute to regenerating myofiber formation, regardless of the transplantation method [41].

Clonal MSC-Ms contained Pax7-positive satellite cells which integrated into the satellite cell position after transplantation, namely the plasma membrane and the basal lamina in between [61]. In general, muscle satellite cells are known to contribute to the regeneration of myofiber formation upon muscle damage [1]. To confirm the contribution of transplanted satellite cells as *in vivo* satellite cells to muscle regeneration, the following experiment was performed. Four weeks after the initial transplantation of human clonal MSC-Ms i.v., cardiotoxin was re-administered into the same muscles without additional transplantation. Two weeks after the second cardiotoxin treatment (6 weeks after initial transplantation), many regenerating GFP-positive myofibers with centrally located nuclei were observed. This implies that, upon transplantation of clonal MSC-Ms to the muscles of patients, those retained as satellite cells should be able to contribute to future muscle regeneration [41].

Transplantation of muscle lineage cells is a potential therapeutic approach for muscle degenerative disorders such as Duchenne muscular dystrophy (DMD), a severe, progressive, muscle-wasting disease that results from a mutation in the dystrophin gene. The mdx-mouse, an animal model for DMD, was used for this experiment. The mdx-mouse is characterized by the absence of the muscle membrane-associated protein, dystrophin. Human clonal MSC-Ms were transplanted into cardiotoxin-pre-treated muscles of mdx-nude mice. Immunohistochemistry revealed the incorporation of transplanted cells into newly formed myofibers which expressed human dystrophin [41].

Cell transplantation therapy also offers hope for the treatment of intractable muscle degenerative disorders. Indeed, ES cells, stem cells derived from adult and prenatal muscle tissues, and myogenic stem cells from bone marrow are powerful candidates for transplantation therapy

[16–19, 62]. Compared to these muscle stem cell systems, the MSC system offers several important advantages. This induction system does not depend on a rare stem cell population, but can utilize the general population of adherent MSCs, which can be easily isolated and expanded. Thus functional skeletal muscle cells can be obtained within a reasonable time course on a therapeutic scale. In the case of MSCs derived from inherited muscle dystrophy patients, genetic manipulation is possible after the isolation and expansion of MSCs. At present, there are no effective therapeutic approaches for muscle dystrophy. Hopefully, this MSC differentiation system may contribute substantially to eventual cell-based therapies for muscle disease.

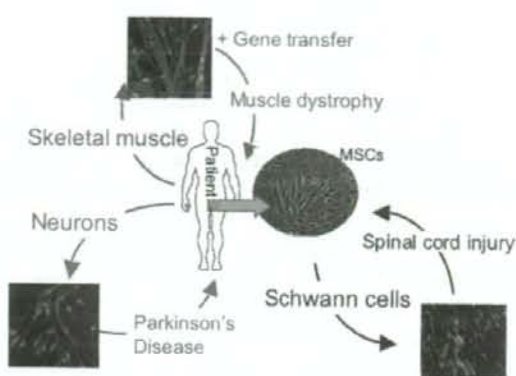

Figure 5. Schematic diagram of an 'auto-cell transplantation system' using MSCs. Neurons, Schwann cells, and skeletal muscle cells induced from patient's MSCs are transplanted back to their owner. Such a self-regenerative system avoids ethical issues and immunorejection.

Conclusions

MSCs provide hopeful possibilities for clinical application, since they can efficiently expand *in vitro* and a therapeutic scale of induced cells are available. In addition, transplantation of MSC-derived cells should pose fewer ethical problems than stem cells, since bone marrow transplantation has already been widely performed. As MSCs are easily obtained from patients or marrow banks, autologous transplantation of induced cells or transplantation of induced cells with the same HLA subtype from a healthy donor may minimize the risks of rejection. Needless to say, the bone marrow should at least be 'normal and healthy' for transplantation.

Even though transplantation of untreated MSCs is partly supportive in various kinds of degenerative models, probably due to trophic supply, it is desirable to develop a systematic induction system to obtain large amounts of purposeful cells, from the viewpoint of cell-based therapy. Obviously, induced cells must be confirmed to be morphologically and physiologically functional. Moreover, the practical application to human degenerative diseases depends on the ability to control their differentiation with high efficiency and purity into functional cells. As mentioned, 10^7 MSCs can be harvested from 20–100 ml of bone marrow aspirate within 2–3 weeks. If an induction procedure takes the shortest and most perfect course, 10^7 MSCs give rise to nearly 10^7 neurons within 3 weeks and 10^7 skeletal muscle cells within 5 weeks, taking into account the time necessary for NICD introduction, G418 selection, and trophic factor administration. Therefore, these induction systems may be useful, since large amounts of purposeful cells can be obtained from the patient's bone marrow for transplantation therapy within a reasonable time course.

However, there are several problems that need to be solved in the future. First, while there have been few reports of tumor formation after transplantation of untreated MSCs, further studies are needed to ensure the safety and efficacy of manipulated MSCs over a long period, using primates and nude-mice/rats. In fact, recent reports have raised the possibility of transformation in the long-term cultivation of MSCs [63, 64]. Second, as the potential differentiation may differ with age, individual, race, and sex, each of these characteristics must be examined in the future. Finally, MSCs have been shown to be heterogeneous in terms of growth kinetics, morphology, phenotype, and plasticity. With the development of specific markers and detailed characterization of heterogeneous, generally adherent MSCs, their properties and plasticity can be studied and defined with more accuracy.

Notch-Hes signaling is known to inhibit neuronal and myogenic differentiation in conventional development [47]. However, in our system, NICD introduction accelerated the induction of neuronal and skeletal muscle cells from MSCs. Although our results appear inconsistent with previous work, they do not refute the known role of Notch-Hes signals during normal development. In our previous report, JAK/STAT inhibitor administration and constitutive active STAT1/3 transfection revealed that downregulation of STATs was tightly associated with NICD-mediated neuronal induction in MSCs, whereas Hes, downstream of Notch, was not involved in the induction event [40]. Skeletal muscle induction was also revealed to be independent of Hes1/5 and the conventional Notch signaling pathway [41]. Thus, our results suggest distinct cellular responses to Notch signals; for example, the repertoire of second messengers and active factors in MSCs may well be different from conventional neural stem cells and myogenic precursor cells, or the susceptibility of MSCs to the Notch signal is probably different from that of known neuronal and myogenic precursor cells. Thus, further studies are needed to identify the factor involved in this phenomenon.

Since MSCs can be obtained from patients, it is possible to establish an 'auto-cell transplantation system' using MSCs (Fig. 5). To realize this ideal, it is necessary to develop the regulatory system of differentiating MSCs into cells with a purpose. Our method would be a possible way to regulate MSC differentiation into functional Schwann cells, neurons and skeletal muscle cells which will be applicable to neuro- and muscle-degenerative diseases.

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Bone Marrow Stromal Cells Generate Muscle Cells and Repair Muscle Degeneration

Mari Dezawa,[1*] Hiroto Ishikawa,[1] Yutaka Itokazu,[1] Tomoyuki Yoshihara,[1] Mikio Hoshino,[2] Shin-ichi Takeda,[3] Chizuka Ide,[1] Yo-ichi Nabeshima[2]

Bone marrow stromal cells (MSCs) have great potential as therapeutic agents. We report a method for inducing skeletal muscle lineage cells from human and rat general adherent MSCs with an efficiency of 89%. Induced cells differentiated into muscle fibers upon transplantation into degenerated muscles of rats and mdx-nude mice. The induced population contained Pax7-positive cells that contributed to subsequent regeneration of muscle upon repetitive damage without additional transplantation of cells. These MSCs represent a more ready supply of myogenic cells than do the rare myogenic stem cells normally found in muscle and bone marrow.

Cell transplantation therapy offers hope for the treatment of intractable muscle degenerative disorders. Embryonic stem (ES) cells and stem cells derived from muscle have been considered as candidates for transplantation therapy (*1–7*). Although they have great potential, they face limitations inherent in procurement from fetal tissue, including problems relating to histocompatibility and ethical concerns. Although muscle stem cells and satellite cells can be isolated from adult and prenatal tissues (*2*, *4–6*), the number of cells that can be harvested may be limited. Bone marrow is another source of myogenic stem cells (*3*, *8*); however, because the stem cell population is very small, the problem of inadequate tissue supply for therapeutic scale again arises.

Because bone marrow stromal cells (MSCs) are easy to isolate and expand rapidly from patients without leading to major ethical and technical problems, they have great potential as therapeutic agents. However, despite their potential for use in cell transplantation therapy, practical application to human muscle degenerative diseases depends on the ability to control their differentiation into functional skeletal muscle cells with high efficiency and purity. Recently we reported that efficient induction of neurons, without glial differentiation, from human and rat MSCs could be achieved by Notch1 intracellular domain (NICD) gene transfer and administration of certain trophic factors (*9*). Further addition of glial cell line–derived neurotrophic factor (GDNF) effectively induced dopamine-producing cells and resulted in functional recovery when those cells were grafted into the brains of Parkinson's disease model rats (*9*). Here we report a method to systematically and efficiently induce skeletal muscle lineage cells

[1]Department of Anatomy and Neurobiology, [2]Department of Pathology and Tumor Biology, Kyoto University Graduate School of Medicine, Yoshidakonoe-cho, Sakyo-ku, Kyoto, 606-8501 Japan. [3]Department of Molecular Therapy, National Center of Neurology and Psychiatry, Kodaira, 187-8502 Tokyo, Japan.

*To whom correspondence should be addressed. E-mail: dezawa@anat2.med.kyoto-u.ac.jp

with high purity from a large population of adherent MSCs, rather than from a rare subpopulation of myogenic stem cells contained in the bone marrow. The induced population effectively differentiated into mature myotubes with some cells persisting as Pax7-positive satellite cells that continued to function in host muscle to restore degenerating muscles in the absence of repeated transplantations. Because our induction system uses a large population of adherent MSCs, which can be easily isolated and expanded, functional skeletal muscle cells including satellite cells can be obtained on a therapeutic scale in a short time period.

General adherent MSCs were established as described [(*10*), Note1]. After three passages, induction was initiated. The induction procedure and corresponding phase contrast images taken at each step are shown (Fig. 1, A and B). Human and rat MSCs plated at a set cell density ([(*10*), Note1] were treated with basic fibroblast growth factor (bFGF), forskolin (FSK; known to up-regulate intracellular cyclic adenosine 3′,5′-monophosphate), platelet-derived growth factor-AA (PDGF), and neuregulin for 3 days (cells at this stage are referred to as C-MSCs). The C-MSCs were then transfected with an NICD expression plasmid by lipofection followed by G418 selection and allowed to recover to 100% confluency (referred to as CN-MSCs). Although MyoD expression was detected in CN-MSCs (Fig. 2J), the frequency of spontaneous cell fusion (the fusion index) was very low ["percentage nuclei incorporated in myotubes (*11*)" was <0.1%] in both rat and human CN-MSCs 5 days after cells reached 100% confluency. To confirm the potential of CN-MSCs to differentiate into multinucleated myotubes, we supplied cells with either 2% horse serum or ITS (insulin-transferrin-selenite) serum-free medium, both of which promote differentiation of myoblasts to myotubes (*11*, *12*). The fusion index was ~24% at 5 days after administration of 2% horse serum or 12% by ITS serum-free medium (Fig. 1A). A much higher production of differentiated myotubes was observed based on the appearance of a muscle phenotype that mainly arose from the spontaneous differentiation of original MSCs (*13*). Because horse serum is not appropriate for clinical usage, and cell survival and myotube formation were unsatisfactory in ITS serum-free medium, we searched for alternative conditions. We found that the supernatant of the original MSCs was also an effective inducer, with a fusion index of about 20% at 5 days after administration and plateauing at ~40% 14 days after induction (Fig. 1C). In the following experiments, we used MSC supernatants for the fusion induction and refer to CN-MSCs treated with supernatant of MSCs as M-MSCs (muscle-MSCs). Rat CN-MSCs and M-MSCs displayed the same features as human MSC-derived cells. Some multinucleated cells in both rat and human M-MSCs exhibited spontaneous contraction in vitro. Furthermore, these multinucleated cells expressed MyoD, myogenin (Fig. 2, A and B), skeletal myosin (Fig. 2F), myosin heavy chain (MHC) (Fig. 2, A, B, and D), and troponin (Fig. 2E), exhibiting skeletal myotube characteristics (*11*). The multinucleated cells appeared postmitotic as determined by p21 immunostaining (Fig. 2C, arrows) and 5-bromo-2′-deoxyuridine (BrdU) incorporation (Fig. 2D) (*12*). In addition to multinucleated cells and MyoD-positive mononucleated cells, cells immunopositive for Pax7 (Fig. 2F, arrows) and c-MetR (Fig. 2E, arrows), both markers for muscle satellite cells (*14*, *15*), were detected. These data suggest that M-MSCs consist of skeletal muscle lineage cells.

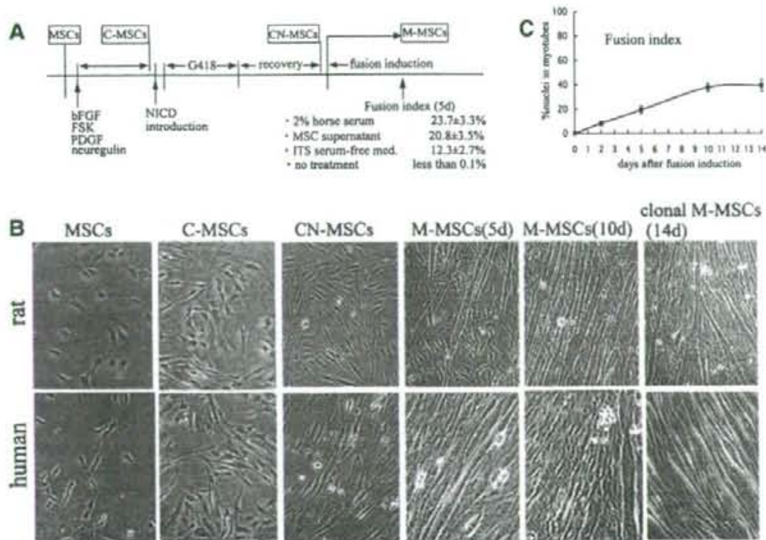

Fig. 1. Induction of skeletal muscle lineage cells. **(A)** Schematic diagram of the induction process. When human CN-MSCs reached 100% confluency, fusion induction was initiated. Fusion indexes were estimated after 5 days in human M-MSCs. For the cytokine treatment, omission of bFGF resulted in a major reduction of the fusion index in human M-MSCs (5 days; 0.5 ± 0.1%). Singular omission of Neuregulin, PDGF, or FSK singly resulted in fusion indexes of 1.8 ± 0.6%, 2.1 ± 0.4%, and 2.5 ± 0.7%, respectively. **(B)** Phase contrast microscopy of rat and human cells at each step and of clonal-M-MSCs (14 days). **(C)** Fusion indexes of human M-MSCs upon administration of human MSC supernatant.

Although most M-MSCs seemed to consist of skeletal muscle lineage cells, the possible existence of nonmuscle elements could not be neglected. We therefore subjected human and rat M-MSCs to single-cell clonal culturing (clonal-M-MSCs) and showed that ~89% of viable clones formed multinucleated cells at 14 days in vitro (Fig. 1B). Our results indicated that a large majority of proliferation-competent cells in M-MSCs possess myogenic potential. Clonal-M-MSCs were also shown to develop into MHC, skeletal myosin and MyoD-expressing multinucleated cells, MyoD-positive mononucleated cells, and Pax7-positive mononucleated cells as observed in their parental M-MSC population (Fig. 2, G and H). The ratios of MyoD-, myogenin-, and Pax7-positive cells to the total clonal-M-MSC cell number are shown in Fig. 2I.

To understand the induction events leading from MSCs to M-MSCs, we investigated the expression of genes related to myogenesis in these cells by means of reverse transcription–polymerase chain reaction (RT-PCR) (Fig. 2J). In MSCs, Pax3, Six1, and Six4 were detected, whereas Pax7, MyoD, and myogenin were not. In C-MSCs, Pax3 was down-regulated, whereas Pax7 expression was detected [(*10*), Note 2], which persisted in CN-MSCs and M-MSCs. Expression of MyoD and myogenin was found in CN-MSCs and M-MSCs. These results were confirmed by Western blot analyses (Fig. 2K). Myf6/MRF4, a marker for mature skeletal muscle (*16*), was detectable only in M-MSCs (Fig. 2J). Whereas expression of Six1 and Six4 persisted in M-MSCs, another myogenic factor, myf5, was not detected in any MSC-derived cells (Fig. 2J). This induction process mimicked some aspects of conventional skeletal muscle development in that Pax3, Pax7, MyoD, Myogenin, and Myf6/MRF4, all of which are related to muscle development (*11*, *12*, *14*, *16*), could be detected in a sequential manner. However, because the characteristics of MSCs used in this induction system are different from those of the conventional myogenic progenitor cells, it is possible that some of the mechanisms might differ, especially in the initial step in which MSCs are converted to MyoD-positive CN-MSCs. For this initial step, cytokine pre-

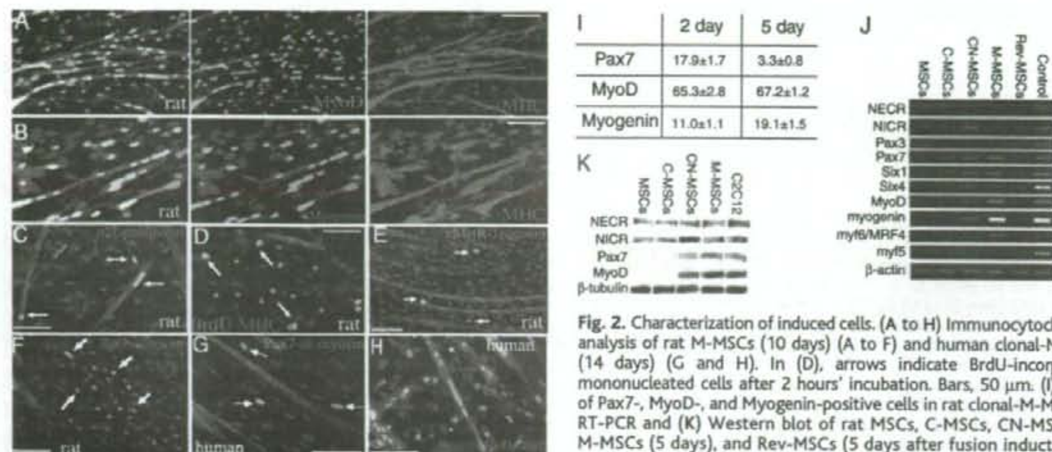

Fig. 2. Characterization of induced cells. (A to H) Immunocytochemical analysis of rat M-MSCs (10 days) (A to F) and human clonal-M-MSCs (14 days) (G and H). In (D), arrows indicate BrdU-incorporated mononucleated cells after 2 hours' incubation. Bars, 50 μm. (I) Ratios of Pax7-, MyoD-, and Myogenin-positive cells in rat clonal-M-MSCs. (J) RT-PCR and (K) Western blot of rat MSCs, C-MSCs, CN-MSCs and M-MSCs (5 days), and Rev-MSCs (5 days after fusion induction). In RT-PCR, the positive control (Control) is C2C12 cells, except for Pax3, which used ES cells. Notch extracellular region (NECR; corresponding to endogenous Notch) and intracellular region (NICR; corresponding to endogenous plus exogenous Notch) were detected in MSCs, suggesting that MSCs are endogenously expressing a small amount of Notch. After transfection with an NICD expression plasmid (CN-MSCs), NICR was up-regulated. The down-regulation of NECR in Rev-MSCs corresponds to the neuronal induction data in our previous report; when MSCs are first transfected with NICD, endogenous expression of Notch is down-regulated (9). β-tubulin was used as a loading control.

treatment and the subsequent NICD transfection are critical for MSC-derived cells to acquire competence for myogenic induction. Indeed, when we reversed the order of cytokine treatment and NICD transfection, muscle-lineage markers were not detected (Fig. 2J; Rev-MSCs), nor were multinucleated cells observed (*17*). The expression profiles of Notch and Hes genes during myogenic induction processes and effects of Notch/Hes signaling in the muscle induction system are described in (*10*), Note 3. Furthermore, we induced re-expression of NICD in CN-MSCs and estimated its effects on myogenic differentiation by analyzing the expression of MyoD and the fusion induction [(*10*), Note 3].

Bone marrow contains a small population of myogenic stem cells known to express c-Kit, CD45 and CD34 (*2–7*). However, the major population of MSCs is negative to these markers [(*10*), Note 1]. To exclude the possibility that the production of muscle-lineage cells was due to the vast proliferation of myogenic stem cells contained in MSCs, we isolated human MSCs negative for c-Kit, CD45, and CD34 by fluorescence-activated cell sorting (FACS) and subjected them to the induction process (Fig. 3A). We confirmed that isolated cells could also be driven to become muscle-lineage cells as efficiently as the unsorted MSCs. The data from rat MSCs were essentially identical to those from human MSCs. Thus, in our system, it appears that the major population of MSCs, rather than a small fraction of bone marrow–derived myogenic stem cells, contributes to the production of muscle lineage cells.

We next tested the differentiation of clonal-M-MSCs in vivo by transplantation into animals. Human clonal-M-MSCs were labeled by means of a green fluorescent protein (GFP)–encoding retrovirus and then transplanted by local injection (L.I.) into muscles or by intravenous injection (I.V.) into immunosuppressed rats whose gastrocnemius muscles were damaged with cardiotoxin pretreatment (*18*). Two weeks after transplantation, GFP-labeled clonal-M-MSCs incorporated into newly formed immature myofibers, and most of the GFP-positive myofibers exhibited centrally located nuclei in both L.I.- (*17*) and I.V.- (Fig. 3, B and D) treated animals. The incorporation ratios of human and rat GFP-positive cells at 2 weeks are indicated in (*10*), Note 4. Four weeks after transplantation, 60 to 70% of the GFP-positive myofibers exhibited mature characteristics with peripheral nuclei just beneath the plasma membrane (Fig. 3, E to G). Functional differentiation of grafted human clonal-M-MSCs was also confirmed by the detection of human dystrophin in GFP-labeled myofibers (Fig. 4A). In both L.I.- and I.V.-treated animals (4 weeks after injection), GFP-labeled human-derived cells were not detected in the host brain, heart, liver, kidney, and nondamaged muscles (*17*), suggesting that transplanted cells incorporate only into the damaged tissues. However, in the lung, a small number of rat and human GFP-positive cells were detected in the I.V.-treated animals (4 weeks), but not in the L.I.-treated animals. These findings indicate that clonal-M-MSCs are able to incorporate into damaged muscles and contribute to regenerating myofiber formation, regardless of the transplantation method.

In addition, some of the transplanted cells were observed between the plasma membrane and laminin-positive basal lamina that surround distinct myofibers (Fig. 3I). Because these cells expressed the satellite cell marker Pax7 (*14*) (Fig. 3H), they might be retained as satellite cells and/or developed into satellite cells in the host muscle. The ratios of transplanted Pax7/GFP-positive cells within total Pax7-positive satellite cells (transplanted and host satellite cells) are described in (*10*), Note 4. It is believed that muscle satellite cells contribute to regenerating myofiber formation upon muscle damage (*19*). We examined whether the transplanted satellite-like cells were able to function as satellite cells in vivo. Four weeks after transplantation of human clonal-M-MSCs (I.V.), cardiotoxin was readministered into the same muscles without additional transplantation just after the muscles were biopsied. The biopsies confirmed that 60 to 70% of GFP-positive myotubes displayed peripheral nuclei (Fig. 4A). Two weeks after the second cardiotoxin treatment (6 weeks after initial transplantation), we observed many regenerating GFP-positive myofibers with centrally located nuclei (Fig. 4B), and $16.5 \pm 4.7\%$ (mean $\pm$ SD; $n = 4$) of myofibers in the damaged area were GFP-positive. These results suggest that the Pax7-positive cells retained in the host muscle function as satellite cells, contributing to muscle repair. This implies that, upon transplantation of clonal-M-MSCs to muscles of patients, cells retained as satellite cells in clonal M-MSCs should be able to continue to contribute to future muscle regeneration. Similar characteristics were observed with rat clonal-M-MSCs (*17*).

Transplantation of muscle-lineage cells offers a potential therapeutic approach for the treatment of muscle degenerative disorders such as Duchenne muscular dystrophy. We therefore locally injected GFP-labeled human clonal-M-MSCs into cardiotoxin-pretreated