(2) ES 細胞の既報に基づき、マウス及びヒト iPS 細胞より、SP-C（サーファクタント）産生性の肺胞上皮細胞の誘導を確認した。SP-C プロモータへの薬剤耐性遺伝子の導入により、ヒト iPS 細胞から SP-C 産生性細胞を選択的に誘導する方法を確立した。気道上皮細胞に関しても、気液界面法などを用いることにより、iPS 細胞から誘導可能であることを確認した。

D．考察

肺を含む内胚葉系の分化誘導効率は、他の胚葉に比べ低いのが現状である。今後は上記薬剤耐性遺伝子導入法等を活用しながら効率的に肺細胞を誘導する方法の確立が必要となる。

E．結論

難治性肺疾患の iPS バンク化を検討した。同時に iPS 細胞から肺細胞の分化誘導法を確立し、今後の疾患フェノタイプの検討に応用する予定である。

F．研究発表

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G．知的財産権の出願・登録状況（予定も含む）

1．特許取得：該当なし

2．実用新案登録：該当なし

3．その他：該当なし

Ⅳ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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V. 研究成果の刊行物・印刷物

特集　臨床遺伝学の進歩と日常診療

【遺伝性疾患の新しい治療と今後期待される治療研究】

iPS細胞と遺伝性疾患

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【遺伝性疾患の新しい治療と今後期待される治療研究】

iPS細胞と遺伝性疾患

中畑龍俊

キーワード●疾患特異的iPS細胞，遺伝性疾患，疾患モデル，創薬

はじめに

Induced pluripotent stem cell（iPS細胞）のもつ最も画期的な臨床的側面は，さまざまな疾患の患者から疾患特異的iPS細胞を樹立できることである．この細胞を用いて，遺伝性疾患の病態解析，新規治療法の開発などへの応用が期待されている．京都大学ではDuchenne型筋ジストロフィー患者を第1例目として，疾患特異的iPS細胞の作製が2008年6月より開始された．その後，多くの疾患患者からiPS細胞が樹立され，さまざまな解析が始まっている．

iPS細胞の発見

2006年，京都大学の山中教授らはマウス線維芽細胞にたった4つの転写因子遺伝子を導入することにより，embryonic stem cell（ES細胞）と比べても遜色がない能力をもつiPS細胞の樹立に成功し，世界中に大きな衝撃を与えた[1]．翌年，彼らはヒトiPS細胞の樹立にも成功した[2]．

iPS細胞はES細胞同様，ほぼ無限の自己複製能とさまざまな細胞への多分化能を併せ持つ細胞であり，基礎研究への貢献，再生医療や創薬への応用など，さまざまな面が期待されている．われわれは当初から，遺伝性疾患を中心に患者皮膚などからiPS細胞を作製し，それを用いた疾患の病因，病態解析を中心に研究している[3]．

疾患特異的iPS細胞

疾患特異的iPS細胞を用いることにより，患者に還元されるさまざまな研究の進展が期待される．いくつかの可能性を列記してみたい．

1．診断への応用

iPS細胞から生検が困難な組織の細胞に分化させ，それを用いた診断が期待される．患者皮膚などから樹立したiPS細胞を生検困難な大脳，小脳，脊髄などの中枢神経組織に分化させ，それを使った診断や病態解析が考えられる．心筋，軟骨，肺，膵臓などの組織は生検可能であるが，大量の組織を繰り返し採取することはできないので，そのような場合も対象となりうるだろう．

2．疾患の発症機序の解明

患者から作製したiPS細胞を患部と同じ組織に分化させることができれば，病気の発症機構を詳細に解析できる可能性がある．健常人と患者のiPS細胞を同じように分化させて，各分化段階の細胞を回収して比較することにより，今までとは違った手法で疾患の本態に迫ることが期待される．

Induced Pluripotent Stem Cells and Genetic Diseases
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また，HAX1 遺伝子異常を伴う Kostmann 症候群では好中球減少と中枢神経の異常を，SBDS 遺伝子異常を伴う Shwachman 症候群では膵外分泌の異常と造血障害を合併することが知られているが，関係のなさそうな２つの異常がなぜ合併するのか分かっていない．これらの患者から疾患特異的 iPS 細胞を樹立し，その細胞から血液(好中球)，神経，膵臓に分化させ，その過程を正常と比較することにより，新たな知見を得るための研究が開始されている．

3. 疾患モデルの構築

iPS 細胞技術は，今までモデル動物のなかった疾患に対して新しいモデルを提供すると考えられる．たとえば，脊髄性筋萎縮症（spinal muscular atrophy ; SMA）と筋萎縮性側索硬化症(amyotrophic lateral sclerosis ; ALS)は，筋組織を支配している運動ニューロンが選択的に変性，死滅し，その結果，筋力低下，筋萎縮が生じ，不幸な転帰をとる疾患である．共に良いモデル動物がないことから，病態解明が進んでいないが，iPS 細胞技術を使うことで，SMA，ALS 患者の皮膚細胞から疾患特異的 iPS 細胞を樹立し，この iPS 細胞から SMA，ALS 患者と同じ遺伝子セットをもつ運動ニューロン細胞を大量に作り出すことができるようになった．この運動ニューロン細胞を使うことで，これらの疾患の病態解析や，薬剤探索のスタートラインに立つことができるようになった．

実際，最近，SMA や familial dysautonomia（家族性自律神経失調症）患者から作製された iPS 細胞を用いて，新規薬剤を開発した事例が報告され[4,5]，疾患特異的 iPS 細胞はさまざまな疾患の新規治療法の開発に貢献できることが明確に示された．

4. 時空を超えて疾患の本態に迫る

Duchenne 型筋ジストロフィー患者は一般に 10 歳ころに歩けなくなり，20 歳ぐらいで人工呼吸器が必要となる．たとえば 10 歳の患者から筋生検をして診断する際，皮膚または筋肉の細胞から iPS 細胞を樹立すると，その細胞から分化させた骨格筋組織は，いわば生まれたての筋肉の細胞と同じ状態であることが予想される．つまり 10 年間という時間を過去に遡って，そのときの筋肉の状態と現在の筋組織を比較することが可能になると考えられる．このような手法は今までには考えられなかったことであり，iPS 細胞がもつ強力なインパクトではないかと思っている．

5. 患者自身の細胞を使った薬物試験

新薬の開発に当たっては，一般的に，前臨床試験として動物実験を行った後，健常人である程度安全性を確認してから患者での臨床試験に移ることが多い．しかし，動物実験の結果は必ずしもヒトに当てはまらず，健常人には異常がなくても，患者では重篤な副作用が出現することがしばしば問題となる．実際に薬を投与されるのは患者なのだから，患者の細胞そのものを使って毒性試験や有効試験をするほうがよいはずであり，疾患特異的 iPS 細胞から目的とする細胞に分化させ，その細胞を用いて毒性試験や有効試験を行う時代が始まろうとしている．

6. 新しい遺伝子治療の可能性

従来の遺伝子治療は，限定した自己複製能しかもたない体性幹細胞に遺伝子を導入して，患者に細胞を戻すという方法で実施されてきたが，あまりうまくいかなかった．

iPS 細胞はほぼ無限の自己複製能をもっているので，患者から作製した iPS 細胞の遺伝子を修復して，正常化されたクローンだけを選択して増やすことができる．すでにマウスでは，鎌状赤血球のモデルマウスから作製した iPS 細胞の異常遺伝子を修復し，修復された iPS 細胞から作られた造血前駆細胞を用いた移植実験で，鎌状赤血球マウスの貧血が改善されたモデルが報告されている[6]．将来の遺伝子治療は，iPS 細胞を使って行う治療法となっていくのではないかと筆者は考えている．

7. 個別化医療への応用

最近，米国ではQT延長症候群のiPS細胞樹立が盛んに行われている．QT延長症候群は8つ以上のタイプに分かれ，タイプによって使用する薬が異なるため，タイプを決めるための薬物負荷試験を行う必要がある．ところが，この薬物負荷試験が非常に危険な検査で，致死的な不整脈が出現することがあることが知られている．そこで，QT延長症候群の患者の皮膚から作製したiPS細胞を*in vitro*で心筋に分化させ，この細胞を用いて薬物負荷試験が行われている．今まで患者で行ってきた負荷試験を，iPS細胞で代用できるようになるかもしれない．

おわりに

遺伝性疾患ではiPS化(初期化)した細胞を再び分化させることで，疾患特異的な現象を再現できる可能性が高いと考えられる．京都大学ではすでに複数の診療科が参画して，遺伝性疾患を中心にさまざまな疾患特異的iPS細胞が樹立され，疾患の病態解析や新薬の開発に向けた研究が進んでいる．海外でも米国を中心に疾患特異的iPS細胞を用いた研究成果が報告され[7]，熾烈な競争が繰り広げられている．iPS細胞を用いた研究は遺伝性疾患の研究に新たな光を与え，画期的な治療法を提供する可能性を秘めている．研究の一層の発展を期待したい．

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TABLE 2 Details of response to sequential treatments where applicable ($n = 10$)

No.	Severity of disease	First treatment		Second treatment		Third treatment	
1	Severe	Amlodopine	×	Nifedipine	√	–	–
2	Moderate	Amlodopine	×	GTN	×	–	–
3	Moderate	Amlodopine	×	GTN	×	–	–
4	Severe	Nifedipine	×	Amlodopine	×	–	–
5	Severe	Nifedipine	×	Amlodopine	×	GTN	√
6	Moderate	Nifedipine	×	GTN	×	–	
7	Severe	GTN	×	Amlodopine	×	Nifedipine	√
8	Moderate	Nifedipine	×	GTN	√	–	–
9	Severe	Amlodopine	×	Nifedipine	×	GTN	×
10	Moderate	Amlodopine	√	GTN	√	–	–

×: no response/inadequate response; √: response.

Overall, GTN patches were effective in 55% of the treated patients. Efficacy was better than that of nifedipine and amlodipine (33 *vs* 25% response rate, respectively), but small numbers and retrospective analysis does not allow statistical comparison. Response was similar in primary and secondary RP. Children with severe RP had a better response to nifedipine and amlodopine than children with moderate disease. The sub-group with severe disease was more likely to be using a disease-modifying drug, which may have had an impact. However, numbers are too small for any conclusion to be drawn from this.

Application of GTN patches allows removal if adverse events occur. Together with absence of tablets, this may make treatment with GTN attractive in paediatric practice. All patients received Deponit GTN patches. Alternative brands may not have adequate skin adhesion when cut into quarters for this off-license use.

GTN patches, nifedipine and amlodipine offer symptomatic relief for patients with moderate primary/secondary RP. Further studies, including head-to-head trials, are needed to determine if one agent is superior. Meanwhile, GTN patches offer an alternative to oral calcium channel blockers for symptomatic relief of paediatric RP.

Rheumatology key message

- GTN patches are an efficacious treatment option in paediatric RP.

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A case of early-onset sarcoidosis with a six-base deletion in the *NOD2* gene

SIR, We present the first case of early-onset sarcoidosis (EOS, MIM no. 609464) with a six-base deletion in the *NOD2* gene, resulting in the replacement of one amino acid and the deletion of two additional amino acids. All previous mutations reported for EOS and Blau syndrome (BS, MIM no. 186580) were single-base substitutions that resulted in the replacement of a single amino acid [1–3].

The patient was a Japanese male born after an uncomplicated pregnancy and delivery. His family had no symptoms of skin lesions, arthritis or uveitis. At 5 years of age, he was diagnosed with bilateral severe uveitis. He became blind in both eyes during adolescence. He had swollen ankles without pain during childhood,

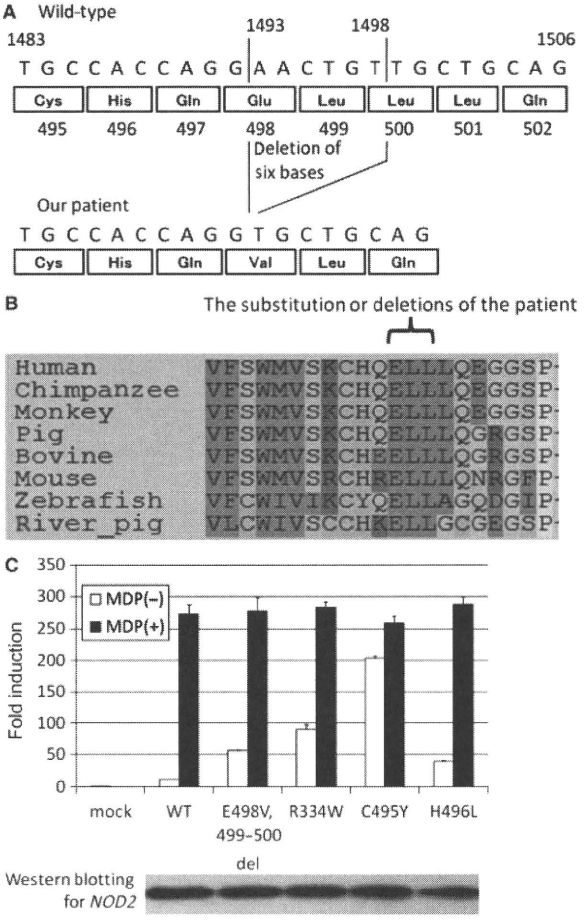
and developed arthritis in his both knees and ankles at 15 years of age. At 30 years, a skin rash had developed on his extremities after his first BCG vaccination. The skin lesions were scaly erythematous plaques with multiple lichenoid papules and some pigmentation. At the same age, camptodactyly without obvious synovial cysts of the hands was observed, and the deformity in all fingers developed by 35 years. At 41 years, he had low-grade fever for 1 year. He had no pulmonary lesions. His laboratory investigations showed normal white blood cell count, mildly elevated CRP (1.0 mg/dl) and ESR (20 mm/h). A skin biopsy from his left forearm revealed non-caseating granulomas without lymphocyte infiltration. There were no indications of infection by *Mycobacterium*.

The clinical symptoms and pathological findings on the biopsied skin indicated that the patient suffered from EOS. It has been reported that EOS and BS have a common genetic aetiology due to mutations in the *NOD2* gene that cause constitutive Nuclear Factor (NF)-κB activation [4, 5]. Thus we analysed the *NOD2* gene from the patient to look for mutations that might correlate with the pathology of EOS. A written informed consent was obtained from the patient and his families, according to the protocol of the institutional review board of Kyoto University Hospital and in accordance with the Declaration of Helsinki. Genomic sequencing analysis of the patient's *NOD2* gene showed the presence of a heterozygous deletion of six bases in exon 4, which resulted in c.1493_1498delAACTGT, p.E498V, 499–500del (Fig. 1A). The mutation was novel and was not identified in 100 normal controls. A genome alignment of *NOD2* among several species showed that E498, L499 and L500 are conserved from zebrafish to human (Fig. 1B). These data strongly suggested that the identified deletion of six bases in the *NOD2* gene is not a single nucleotide polymorphism (SNP), but is probably responsible for EOS in the patient.

Previous studies report that *NOD2* mutations causing EOS/BS show constitutive activation of NF-κB [6–8]. Therefore, we investigated the level of NF-κB activity associated with the new mutation identified here. First, we confirmed the level of mRNA expression of the mutated allele by subcloning analysis of *NOD2*-cDNA, which showed that the mutated allele was expressed as well as the wild type allele (data not shown). We then evaluated the ability of the *NOD2* mutant to constitutively activate NF-κB by using an *in vitro* reporter system in HEK293T cells transfected with both *NOD2* mutants and NF-κB reporter plasmids (Fig. 1C). The deletion mutant demonstrated almost five times more NF-κB activity than wild type without muramyl dipeptide (MDP) stimulation. Western blot analysis confirmed that NOD2 mutant protein expression was similar to that of wild type (Fig. 1C). Thus, like other mutations of *NOD2* identified previously, the deletion mutant identified here also showed constitutive activation of NF-κB.

The mechanism underlying EOS/BS has not been totally understood, although two pathways downstream from *NOD2* have been identified: NF-κB activation through receptor-interacting protein (RIP) like interacting caspase-like apoptosis regulatory protein kinase (RICK) and MAP kinase activation through the caspase recruitment domain 9 (CARD9) [9]. We previously tested 10 *NOD2* missense mutations that have been identified in our cohort of EOS/BS patients in Japan, and all of them demonstrated constitutive activation of NF-κB [3]. By analysing this newly identified deletion mutant, we have further confirmed the importance of constitutive activation of NF-κB by mutated *NOD2* for the pathogenesis of EOS/BS. We would like to emphasize the

FIG. 1 (**A**) Summary of the mutations identified in our patient. (**B**) NOD2 protein alignment among different species on the mutated amino acids. (**C**) NF-κB reporter assay using the *NOD2* deletion mutant. *In vitro* NF-κB reporter assays were performed as previously described [1, 3, 6, 7]. Mock vector, wild type *NOD2* (WT) and three *NOD2* variants (R334W, C495Y, H496L) derived from EOS/BS patients, were used as controls. Values represent the mean of normalized data (mock without MDP = 1) of triplicate cultures, and error bars indicate S.D. Shown is one representative result of three independent experiments. Protein expression levels of *NOD2* mutants analysed by western blotting are shown in the bottom panel.



usefulness of the NF-κB reporter assay with mutant *NOD2* for observing its role in EOS/BS, although the MAP kinase activation pathway and other possible pathways need to be evaluated to more completely understand the pathogenesis of the *NOD2* mutation in EOS/BS.

We have identified the first deletion mutation in the *NOD2* gene responsible for EOS/BS, and the mutant showed constitutive activation of NF-κB, which is one of the key features that lead to the pathogenesis of EOS/BS.

Rheumatology key message

- A six-base deletion in *NOD2* gene causes EOS.

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Comment on: Hepatotoxicity rates do not differ in patients with rheumatoid arthritis and psoriasis treated with methotrexate

Sir, We read with interest the recent article by Amital *et al*. [1] that compared hepatotoxicity rates in PsA and RA patients treated with MTX based on the evaluation of standard liver function tests. The authors conclude that the incidence of hepatotoxicity does not differ between the two disease groups after adjusting for the cumulative dose of MTX.

Several studies in MTX-treated psoriasis patients have reported that isolated abnormalities of liver enzymes (i.e. alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase) were poor predictors of the severity of liver histopathology. The authors state that the combined sensitivity of aspartate aminotransferase, alanine aminotransferase and bilirubin for detecting an abnormal liver biopsy has been rated at 0.86 based on a previous study [2]. This figure implies that 14% of those with normal liver function tests will have undetected hepatic disease. Larger studies have suggested that 30–50% of the psoriasis patients on MTX have normal standard liver function test results despite histology showing fibrosis and cirrhosis [3]. The lack of correlation between liver enzymes and hepatic fibrosis and cirrhosis has been the major factor leading to the recommendation that liver biopsies be done to monitor potential hepatotoxicity. In this study, the liver function tests were performed with varying frequency which could allow abnormal liver function tests to be missed. The authors acknowledge that the rates of other hepatotoxic agents such as alcohol use and the occurrence of other hepatic comorbidities were not known. We believe that these are significant confounding variables, which make the interpretation of the results of this study difficult. The British Association of Dermatologists recommends serial monitoring

The FASEB Journal • Research Communication

Generation of skeletal muscle stem/progenitor cells from murine induced pluripotent stem cells

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ABSTRACT **Induced pluripotent stem (iPS) cells, which are a type of pluripotent stem cell generated from reprogrammed somatic cells, are expected to have potential for patient-oriented disease investigation, drug screening, toxicity tests, and transplantation therapies. Here, we demonstrated that murine iPS cells have the potential to develop *in vitro* into skeletal muscle stem/progenitor cells, which are almost equivalent to murine embryonic stem cells. Cells with strong *in vitro* myogenic potential effectively were enriched by fluorescence-activated cell sorting using the anti-satellite cell antibody SM/C-2.6. Furthermore, on transplantation into *mdx* mice, SM/C-2.6$^+$ cells exerted sustained myogenic lineage differentiation in injured muscles, while providing long-lived muscle stem cell support. Our data suggest that iPS cells have the potential to be used in clinical treatment of muscular dystrophies.—Mizuno, Y., Chang, H., Umeda, K., Niwa, A., Iwasa, T., Awaya, T., Fukada, S., Yamamoto, H., Yamanaka, S., Nakahata, T., Heike, T. Generation of skeletal muscle stem/progenitor cells from murine induced pluripotent stem cells. *FASEB J.* 24, 2245–2253 (2010). www.fasebj.org**

Key Words: Duchenne muscular dystrophy · Pax7 · long-term engraftment · no teratoma formation · high engraftment efficiency

TO MAINTAIN HOMEOSTASIS, SKELETAL muscle fibers are continuously regenerated by activated satellite cells (1), the muscle-specific stem cells that differentiate into myoblasts and form myotubes to replace the myofibers damaged by exercise and daily activities (2). The muscular dystrophies are inherited myogenic disorders of variable distribution and severity that are characterized by progressive muscle wasting and weakness (3). In many forms of muscular dystrophy, the common molecular defect of the encoded proteins, which are involved in muscular structural integrity, is observed in both immature satellite cells and mature myofibers (4). Duchenne muscular dystrophy (DMD), which is the best-described and most serious form of muscular dystrophy, results from mutations in the X-linked dystrophin gene (5). Dystrophin and its associated proteins are commonly known to be indispensable for the functioning of the intracellular actin cytoskeleton, as are laminins in the extracellular matrix of muscle fibers, which protect myofibers from contraction-induced damage (6). Loss of dystrophin causes the rapid and continuous damage of muscles, which leads to the exhaustion of both skeletal muscles and satellite cells, even though muscular regeneration occurs at a higher frequency in DMD patients than in nonaffected individuals (7). Despite extensive efforts to establish pharmacological agents that halt the clinical course of DMD, the disease still results in high mortality in patients during late adolescence.

Skeletal muscle stem/progenitor cell transplantation is considered to be one of the most promising therapies for the muscular dystrophies. In fact, a recent report has shown that the transplanted satellite cells can engraft as myofibers with normal dystrophin expression in the muscles of *mdx* mice, a mouse model of DMD (8, 9). Most of the clinical trials involving allogeneic transplantation of DMD, however, have not obtained satisfactory results due to immune rejection, rapid death, and limited migration of transplanted myoblasts (10).

Embryonic stem (ES) cells have considerable advantages over somatic stem cells as a cell source of transplantation due to their capacity for unlimited proliferation in an undifferentiated state over a prolonged period, and their ability to differentiate into various lineages of cells in the same way as observed *in vivo* (11). Recently, mouse and human induced pluripotent stem (iPS) cells have been established by introducing 3 or 4 pluripotency-associated genes into somatic cells (12–21). Like ES cells, these reprogrammed somatic cells possess properties of self-renewal and pluripotency, and yield germline adult chimeras. Furthermore, the iPS cell technology enables us to generate individualized stem cells, which is expected to contribute to patient-oriented disease studies, drug screenings, toxicity tests, and transplantation therapies (22, 23).

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We demonstrated previously that long-lived muscle stem cells can successfully be induced from murine ES (mES) cells *in vitro* by plating embryoid bodies (EBs) onto Matrigel-coated plates (24). These ES cell-derived $Pax7^+$ cells can be enriched effectively by the SM/C-2.6 antibody, an anti-satellite cell antibody (25), and possess a potential to differentiate into myofibers both *in vitro* and *in vivo*. Here, we demonstrated that by using our induction system, SM/C-2.6^+ myogenic lineages were induced successfully from murine iPS (miPS) cells. This system enabled quantitative assays of mature and immature skeletal muscular lineage cells both *in vitro* and *in vivo* and may serve as a useful experimental tool for the treatment of various muscular dystrophies.

MATERIALS AND METHODS

Cell lines

A 4-factor miPS cell line reprogrammed by the introduction of *Oct3/4, Sox2, Klf4,* and *c-Myc* (clone 38D2), and a 3-factor iPS cell line that lacks *c-Myc* (clone 256H-18), were established from murine embryonic fibroblasts. These fibroblasts carried the Nanog-GFP-IRES-$Puro^r$ reporter and the tail-tip fibroblasts of adult *Discosoma* sp. red fluorescent protein (DsRed)-transgenic mice, respectively, and were maintained as described previously (13, 14). The enhanced green fluorescent protein (GFP)-transfected ES cell line D3, a kind gift from Dr. Masaru Okabe (Osaka University, Osaka, Japan), was maintained as reported elsewhere (24, 26).

In vitro differentiation of ES cells and iPS cells into a muscle cell lineage

Differentiation of mES and miPS cells was based on a previously established protocol (24). Briefly, in order to eliminate feeder cells, undifferentiated mES and miPS cells were treated with 0.5% trypsin/ethylenediaminetetraacetic acid (Life Technologies, Inc., Grand Island, NY, USA; http://www.invitrogen.com) and transferred onto tissue culture dishes (Falcon; BD Biosciences, San Diego, CA, USA; http://www.bdbiosciences.com) coated with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO, USA; http://www.sigmaaldrich.com) in maintenance medium supplemented with 5000 U/ml leukemia inhibitory factor (LIF), at a concentration of 5×10^3 cells/cm^2. For embryoid body (EB) formation, mES and miPS cells were cultured in hanging drops for 3 d at a density of 800 cells/20 μl differentiation medium, consisting of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 5% horse serum (Sigma), 0.1 mM 2-mercaptoethanol, 0.1 mM nonessential amino acid, and 50 μg/ml penicillin/streptomycin. EBs were then transferred to a suspension culture in the differentiation medium for an additional 3 d. Finally, each EB was plated onto 48-well tissue culture plates (Falcon) coated with Matrigel Basement Membrane Matrix (BD Bioscience, Bedford, MA, USA; http://www.bdbiosciences.com). The medium was changed every 5 d.

Immunostaining

Immunofluorescence and immunocytochemical analyses were performed as described previously (24). Primary antibodies (Abs) used in this study included mouse anti-Pax3, mouse anti-Pax7 (R&D Systems, Minneapolis, MN, USA; http://www.rndsystems.com), rabbit anti-Myf5 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; http://www.scbt.com), mouse anti-MyoD1, mouse anti-myogenin (Dako, Carpinteria, CA, USA; http://www.dako.com), mouse anti-myosin heavy chain (MHC) (Zymed Laboratories, San Francisco, CA, USA; http://www.invitrogen.com), rabbit anti-DsRed (Clontech Laboratories Inc., Palo Alto, CA, USA; http://www.clontech.com), rat anti-laminin-α2 (4H8-2; Alexis Biochemicals, San Diego, CA, USA; http://www.axxora.com), and mouse anti-dystrophin (MANDRA1; Sigma). The secondary Abs used in this study were Cy3-conjugated anti-mouse, anti-rabbit, or anti-rat IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA; http://www.jacksonimmuno.com), fluorescein isothiocyanate (FITC)-conjugated anti-mouse or anti-rat IgG (Jackson ImmunoResearch), and Alexa 633-conjugated anti-rat IgG (Molecular Probes, Eugene, OR, USA; http//probes.invitrogen.com). Vectastain ABC Kit and DAB substrate Kit (Vector Laboratories, Burlingame, CA, USA; http://www.vectorlabs.com) was used for diaminobenzidine (DAB) staining. Hoechst 33342 (Molecular Probes) was used for nuclear staining. For the *in vivo* myogenic differentiation assay, the engrafted muscles were isolated and frozen in liquid nitrogen-cooled isopentane (25). The Vector® M.O.M.™ Immunodetection Kit (Vector Laboratories) was used to prevent nonspecific secondary antibody from binding to Fc receptors in the frozen sections. The samples were then examined using a fluorescent microscope (FluoView System; Olympus, Tokyo, Japan; http://www.olympus-global.com) or an AS-MDW system (Leica Microsystems GmbH, Wetzlar, Germany; http://www.leica.com). Photographs were acquired with an AxioCam (Carl Zeiss Vision GmbH, Hallbergmoos, Germany; http://www.zeiss.com) or an AS-MDW system (Leica Microsystems GmbH). DAB and HE staining were performed as previously reported (27).

RT–PCR analysis

RNA isolation and RT–PCR were performed according to previously established protocols (24). The oligonucleotide primers for *Pax3, Pax7, Myf5, MyoD, Myogenin,* and *GAPDH* were described elsewhere (24).

Flow-cytometric (FCM) analysis and cell sorting

Staining procedures, FCM analysis, and cell sorting were performed as described previously (24). On d 20, EBs were collected and treated with enzyme-free Hank's-based Cell Dissociation Buffer (Invitrogen, Carlsbad, CA, USA; http://invitrogen.com) for 30 min at 37°C, and gently dissociated into single cells. The resultant cells were stained with biotin-conjugated or rat SM/C-2.6 antibody (25) and then with allophycocyanin (APC)-conjugated streptavidin or anti-rat IgG (Becton Dickinson Labware, San Jose, CA, USA; http://www.bd.com). The primary Abs used for FCM analysis included mouse anti-CD34 (Becton Dickinson), mouse anti-CD56 (Biolegend, San Diego, CA, USA; http://www.biolegend.com), mouse anti-M-cadherin (Calbiochem, San Diego, CA, USA; http://www.calbiochem.com), mouse anti-c-Met, and mouse anti-integrin α7 (R&D Systems). The secondary Abs used in this study were PE- or FITC-conjugated anti-mouse IgG (Becton Dickinson). Dead cells were excluded by propidium iodide (PI) (Sigma) or 4′,6-diamidino-2-phenylindole (DAPI; Sigma) staining. Samples were analyzed using a FACSCalibur apparatus and the Cell Quest software (Becton Dickinson). Cell sorting with the SM/C-2.6 Ab was performed using a FACSVantage flow cytometer (Becton Dickinson).

Intramuscular cell transplantation

Male *mdx* mice (Central Laboratories for Experimental Animals, Kanagawa, Japan; http://www.clea-Japan.com), aged 6–8 wk, which originated from the C57BL/10 strain, were used as

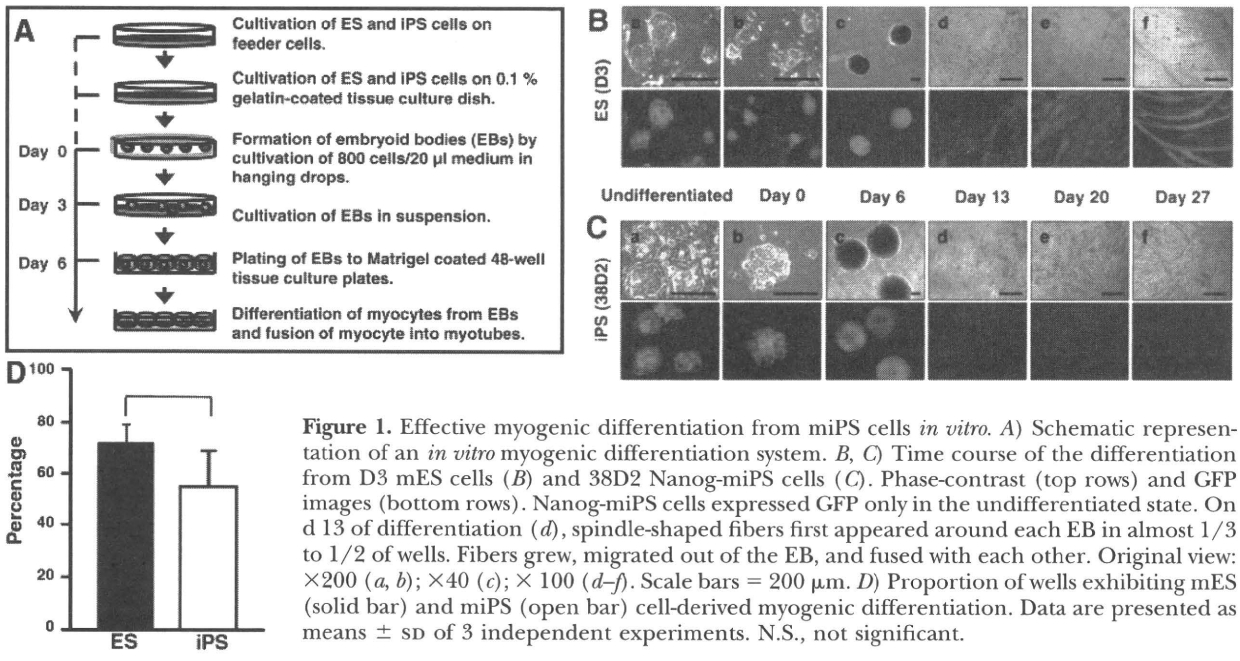

Figure 1. Effective myogenic differentiation from miPS cells *in vitro*. *A*) Schematic representation of an *in vitro* myogenic differentiation system. *B, C*) Time course of the differentiation from D3 mES cells (*B*) and 38D2 Nanog-miPS cells (*C*). Phase-contrast (top rows) and GFP images (bottom rows). Nanog-miPS cells expressed GFP only in the undifferentiated state. On d 13 of differentiation (*d*), spindle-shaped fibers first appeared around each EB in almost 1/3 to 1/2 of wells. Fibers grew, migrated out of the EB, and fused with each other. Original view: ×200 (*a, b*); ×40 (*c*); × 100 (*d–f*). Scale bars = 200 µm. *D*) Proportion of wells exhibiting mES (solid bar) and miPS (open bar) cell-derived myogenic differentiation. Data are presented as means ± SD of 3 independent experiments. N.S., not significant.

recipient mice throughout the experiment. The miPS cell line (clone 256H-18), which arose from B6, was used as the source of donor cells. In the major histocompatibility complex, the H2 haplotypes of *mdx* mice and iPS cells are both of type *b*.

Intramuscular cell transplantation was performed according to an earlier experiment (24). Briefly, the recipient *mdx* mice (28) were injured with 50 µl of 10 µM cardiotoxin (CTX; Latoxan, Valence, France; http://www.latoxan.com) in the left

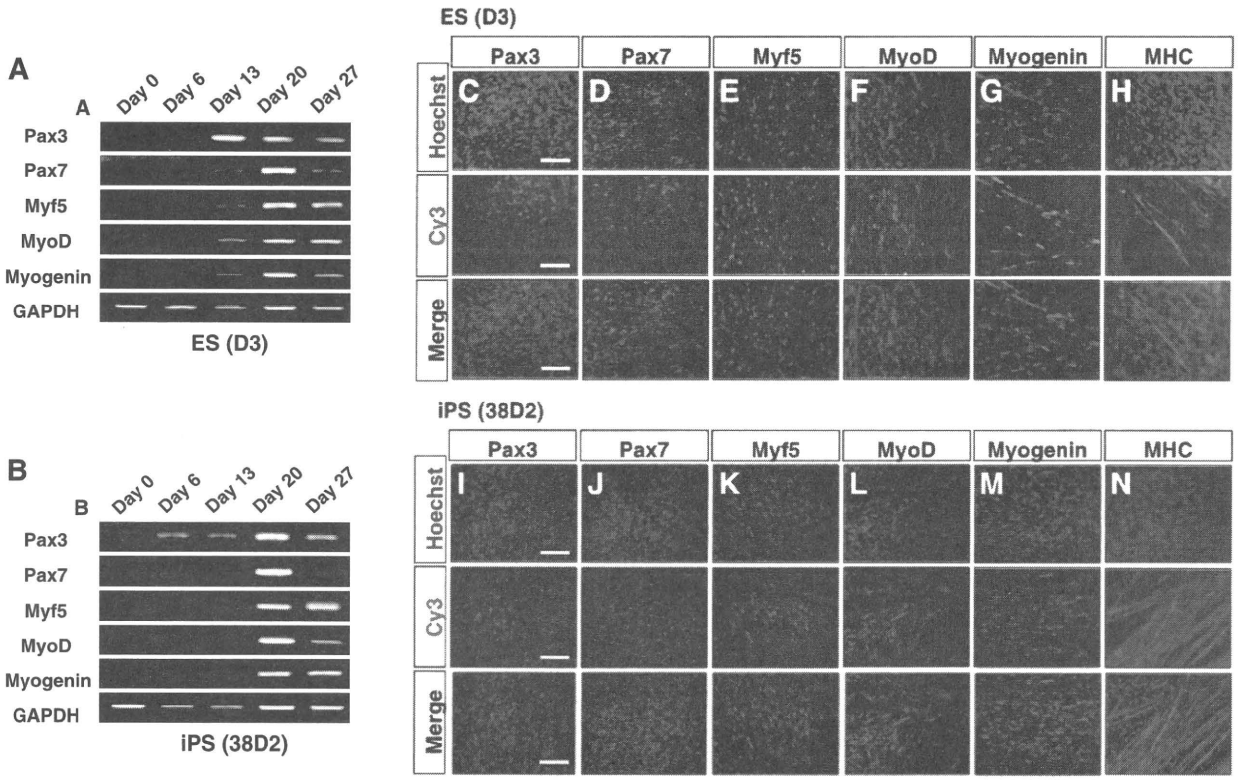

Figure 2. Myogenic marker analysis in mES and miPS cell-derived cultures. *A, B*) Sequential RT–PCR analysis of skeletal myogenesis-related genes. Data for D3 mES cells (*A*) and 38D2 miPS cells (*B*). For semiquantitative comparison, samples were normalized by dilution to produce equivalent signals for *GAPDH*. *C–N*) Skeletal myofibers were observed in cultures, which were positive for anti-Pax3 (*C, I*), anti-Pax7 (*D, J*), anti-Myf5 (*E, K*), anti-MyoD (*F, L*), anti-myogenin (*G, M*), and anti-MHC (*H, N*) Abs on d 20 of differentiation. Data for D3 mES cells (*C–H*) and 38D2 miPS cells (*I–M*). mES and miPS cell-derived cultures were analyzed using Cy3. Nuclei were costained with Hoechst 33342 (blue). Original view: ×200. Scale bars = 100 µm.