Table 1 Flow cytometric analysis of primary cultures without inductive stimuli. Percentages of TTCVs and FTCVs immunostained for CD29, CD44, CD73, CD105 and CD34 (mean±SD, n=3; *FITC* fluorescein isothiocyanate, *PE* phycoerythrin, *PC5* phycoerythrin-cyanine 5)

Cells	CD29 FITC	CD44 FITC	CD73 PE	CD105 PE	CD34 PC5
TTCVs	96.9±2.2	94.7±4.86	87.3±10.8	91.5±9.25	0.27
FTCVs	98.8±1.0	97.2±1.89	93.6±4.23	98.1±2.03	0.27

were depleted of hematopoietic cells. Furthermore, we found that the average level of MSC marker expression on the FTCVs was higher than that on the TTCVs but this difference was not statistically significant.

Expression of pluripotent stem cell markers by FTCVs

We also analyzed the gene expression of pluripotent stem cell markers in uninduced FTCVs and TTCVs obtained between the 3rd and 6th passages of culture by qPCR analysis. FTCVs collected during the 9th week of gestation (FTCV-9th) expressed both *Nanog* and *Sox2* mRNA but not *Oct4* mRNA (Fig. 1c). In contrast, FTCVs collected during later weeks of gestation (FTCV-10th, FTCV-11th) and TTCVs exhibited no detectable expression of these pluripotent stem cell markers. Moreover, immunocytochemical analysis revealed the presence of the Nanog protein in uninduced FTCV-9th cells (Fig. 2a).

Myogenic differentiation

To evaluate their myogenic potential, the differentiation of the FTCVs into a myogenic lineage was induced by incubation with 5-azacytidine in DMEM with 20% FBS for 24 h, after which they were cultured in DMEM supplemented with 2% horse serum for 21 days. We found that FTCV expression of both *Nanog* and *Sox2* mRNA decreased markedly after 1 day of myogenic induction (Fig. 2g); indeed, the Nanog protein was also down-regulated after myogenic induction (Fig. 2d). During myogenic induction, the morphology of the FTCV population changed to multinucleated myotubes (Fig. 3). We evaluated the expression of the myogenic markers *MyoD*, *myogenin*, *desmin* and *dystrophin* in the FTCVs by qPCR analysis. These experiments revealed that myogenic differentiation of the FTCVs induced both *MyoD* and *myogenin* mRNA expression by day 3, although this expression decreased again by day 7. Moreover, FTCV expression of *desmin* mRNA

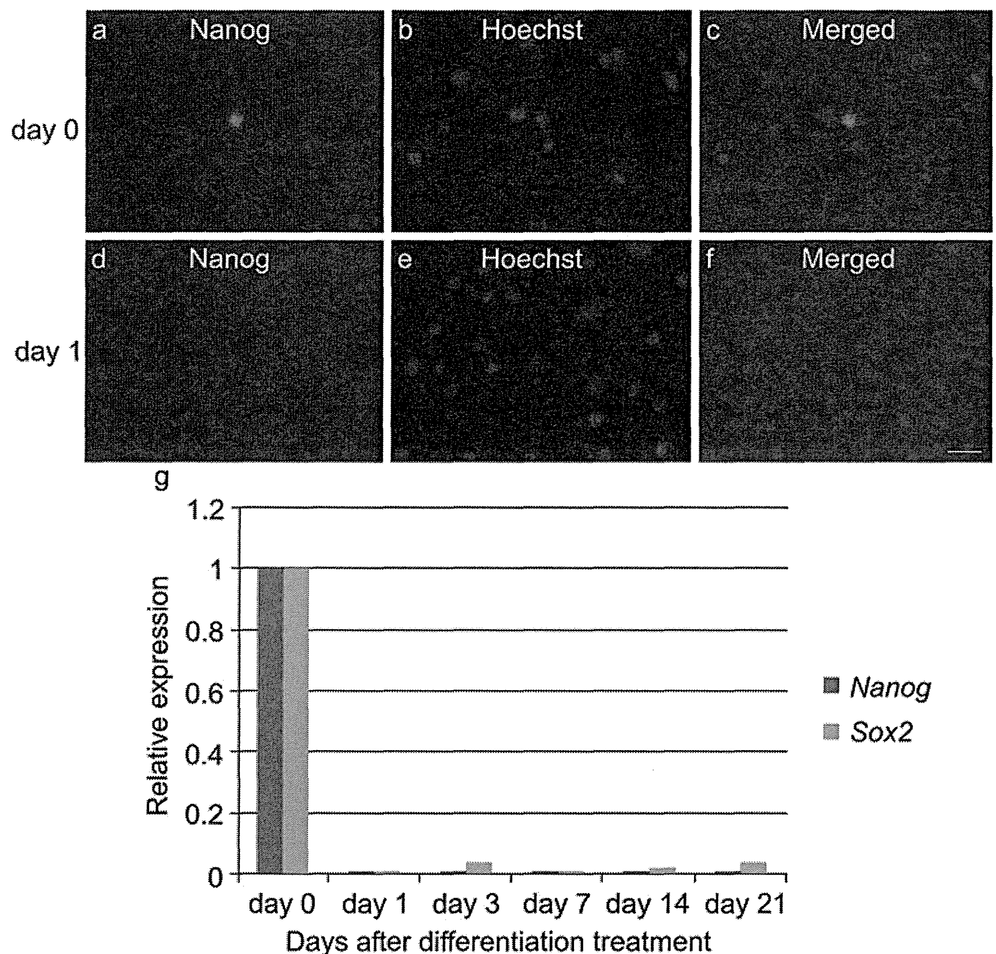

Fig. 2 Immunocytochemistry of the Nanog protein. **a** Nanog (day 0). **b** Hoechst dye (day 0). **c** Merged image of **a**, **b** (day 0). **d** Nanog (day 1). **e** Hoechst dye (day 1). **f** Merged image of **d**, **e** (day 1). *Bar* 50 μm. **g** qPCR analysis of *Nanog* and *Sox2* expression in FTCV-9th cells. Time course of *Nanog* and *Sox2* mRNA expression at the indicated day after treatment with 5-azacytidine. The value obtained before treatment with 5-azacytidine was set to 1 in each experiment

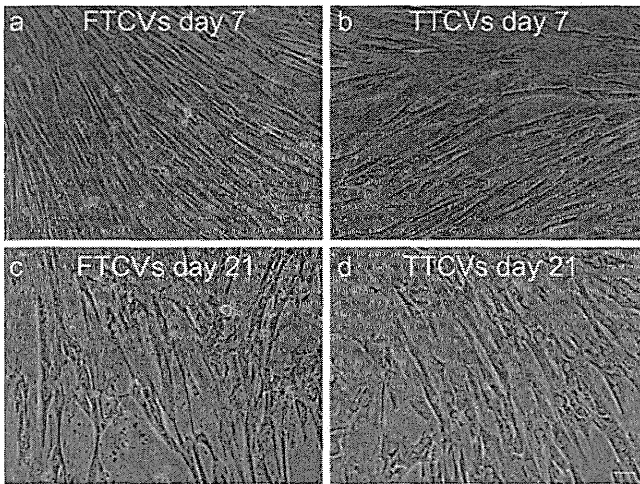

Fig. 3 Phase contrast microscopic images of the FTCVs and TTCVs after treatment with 5-azacytidine. **a** FTCVs (day 7). **b** TTCVs (day 7), **c** FTCVs (day 21), **d** TTCVs (day 21). *Bar* 50 μm

was relatively high on day 1 of myogenic differentiation and subsequently decreased to a moderate level (Fig. 4). In addition, RT-PCR analysis clearly showed that *dystrophin* mRNA expression was increased from day 14 (Fig. 5a), as confirmed by qPCR analysis (Fig. 5b). Immunocytochemistry and Western blot analysis also confirmed that the dystrophin protein was detected in the FTCVs at day 21 (Fig. 6a, d). To evaluate the efficiency of myotube formation, FTCVs and TTCVs were fixed at day 0 and day 21 after plating and stained as described in Materials and methods with an antibody to the myosin heavy chain protein (MF20) in order to determine the fusion index. The results revealed that FTCV-9th cells with a fusion index of 57.3±11.1% had significantly higher myogenicity than FTCV-11th cells (20.9±6.4%) and TTCVs (9.9±1.4%) at day 21 (Fig. 7).

In vitro differentiation of multiple lineages

qPCR analysis revealed that FTCVs treated with 5-azacytidine and maintained in myogenic culture conditions also expressed *RUNX2*, *Sox9*, *PPARγ*, *nestin*, *ACTA2* and *GATA4*, which are markers of the osteoblast, chondrocyte, adipocyte, neural, smooth muscle and cardiac lineages, respectively (Fig. 8). Furthermore, the relative levels of expression of these non-myogenic markers were lower than the expression levels of myogenic markers such as *desmin* and *dystrophin*.

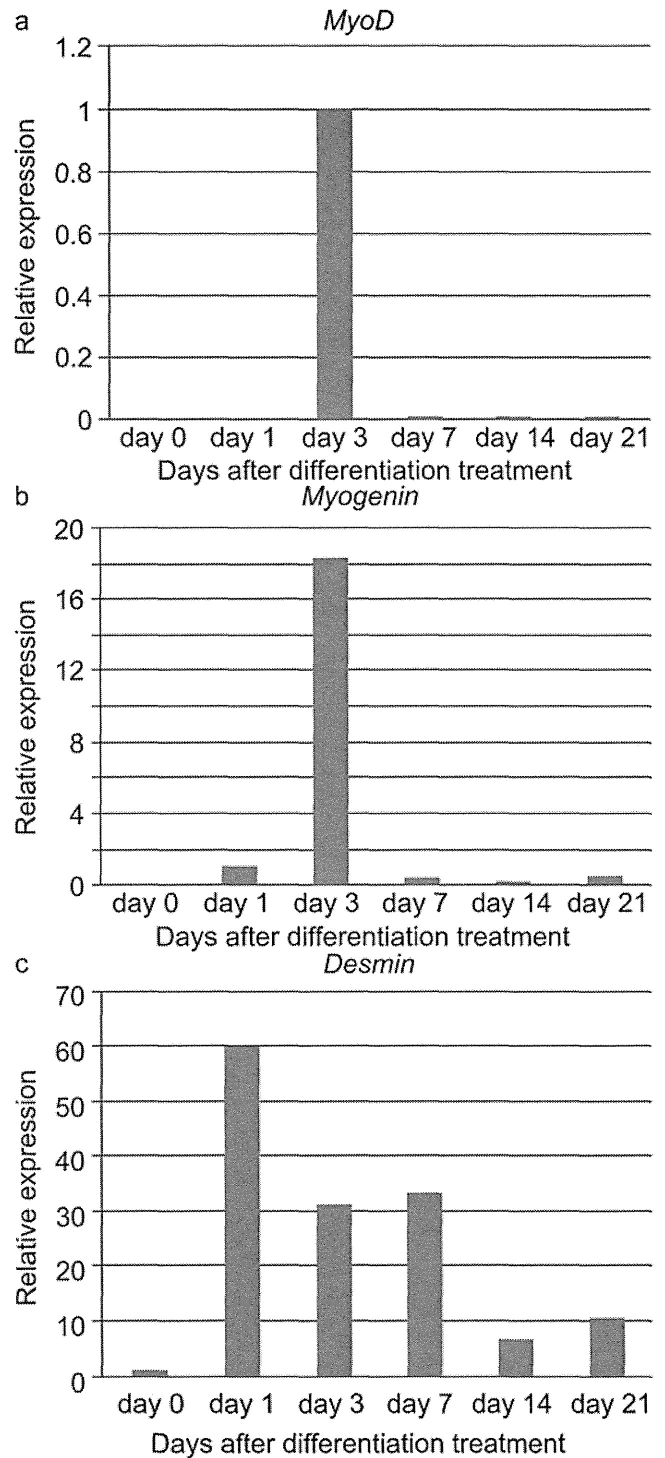

Fig. 4 qPCR analysis of skeletal muscle markers in the FTCVs. Time course of *MyoD* (**a**), *myogenin* (**b**) and *desmin* (**c**) mRNA expression at the indicated day after treatment with 5-azacytidine. *MyoD* mRNA was detectable after day 3. *Myogenin* mRNA was undetectable at day 0. The earliest detectable value was set to 1 in each experiment

Discussion

In this study, we have investigated the myogenic potential of FTCVs as a cell source for muscular dystrophy cell therapy. To our knowledge, no previous studies have examined the effect of combining 5-azacytidine treatment with culture in myogenic differentiation media on the expression of dystrophin in FTCVs. Recently, stem cells have been investigated intensively for their potential use in various therapeutic regimens, including tissue engineering and cell therapy. Stem cells

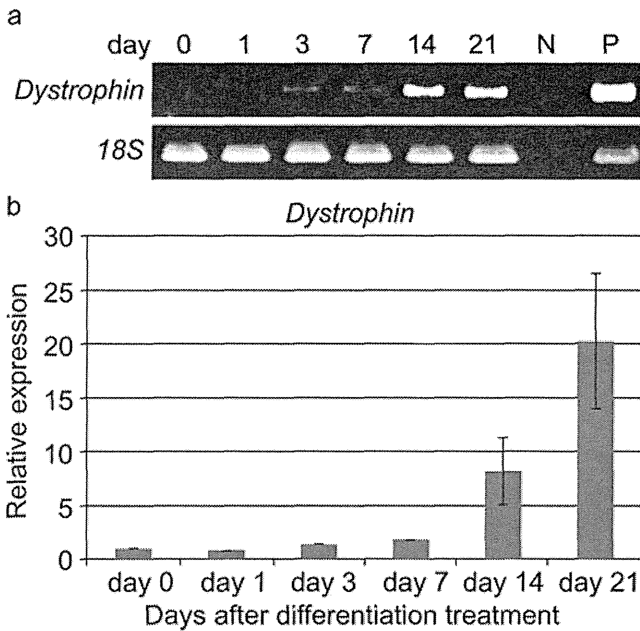

Fig. 5 Expression of *dystrophin* mRNA in the FTCVs during myogenic induction. **a** RT-PCR analysis. Time course of *dystrophin* and ribosomal RNA *18S* mRNA expression at the indicated day after treatment with 5-azacytidine (*N* negative control lanes without reverse transcriptase, *P* RNAs from human skeletal muscle as positive control). **b** qPCR analysis. Time course of *dystrophin* mRNA expression at the indicated day after treatment with 5-azacytidine. The value obtained before treatment with 5-azacytidine was set to 1. Each value (n=3) represents the mean±SD

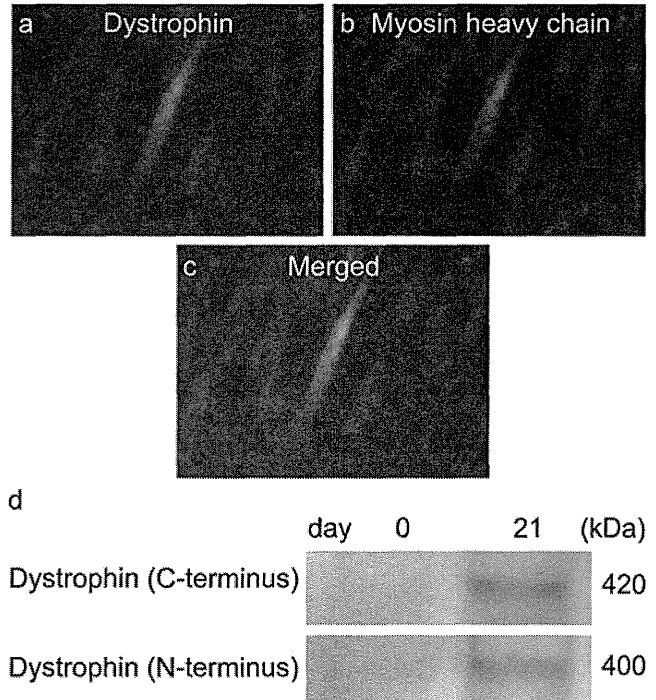

Fig. 6 Immunocytochemistry of dystrophin (*green*) and myosin heavy chain (*red*) proteins in FTCVs at 21 days after 5-azacytidine treatment. The nuclei were counterstained with Hoechst dye (*blue*). **a** Dystrophin. **b** Myosin heavy chain (MF20). **c** Merged image of **a**, **b**. *Bar* 50 μm. **d** Western blot of dystrophin (using C-terminus [NCL-DYS2] and N-terminus [NCL-DYS3] antibodies) in the FTCV lysate before 5-azacytidine treatment (day 0) and at day 21 after 5-azacytidine treatment

can be loosely classified into three groups: embryonic stem (ES) cells, fetal stem cells (i.e., first-trimester chorionic villi and placenta) and adult stem cells. In particular, FTCVs possess a number of advantages that contribute to their potential therapeutic use. First, FTCVs might have a higher myogenic potential for differentiation than cells taken from other tissues because they are derived from the earliest fetal cell population. Second, the ability to isolate pluripotent autogenic progenitor cells such as FTCVs during gestation might be advantageous for the timely treatment of genetic diseases in newborns. Third, fetal cells do not form teratomas when injected into adults and are less immunogenic than other cell population, making them particularly suitable for transplantation (In't Anker et al. 2003; De Coppi et al. 2007). Fourth, the use of FTCVs for treatment avoids the ethical issues associated with ES cells (Weiss and Troyer 2006). Finally, FTCVs are obtained within the process of prenatal diagnosis and can be taken without an additional invasive procedure.

Our present results suggest that FTCVs express *Nanog* and *Sox2* mRNA, the proteins of which have been previously identified as crucial factors for maintaining stem cell characteristics in human ES cells (Hoffman and Carpenter 2005; Kazuki et al. 2010). The expression level of *Nanog* mRNA in the FTCVs collected during the 9th week of gestation is not significantly different from that of NTERA-2 cells, which express pluripotent stem cell markers (Pal and Ravindran 2006; Fig. 1c). Our investigation of pluripotent stem cell marker expression in the FTCVs collected between the 9th and 11th weeks of gestation has revealed that both *Nanog* and

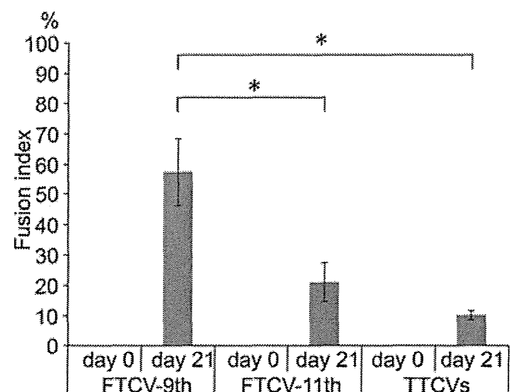

Fig. 7 Quantification of MF20-positive (MF20+) cells in FTCV-9th cells, FTCV-11th cells and TTCVs during in vitro differentiation. Fusion index was determined by calculating the percentage of nuclei among MF20+ cells, as described. Each data point (n=3) is presented as the mean±SD. Quantification of MF20+ cells in each population was compared by using the Student's t-test (*, P<0.05)

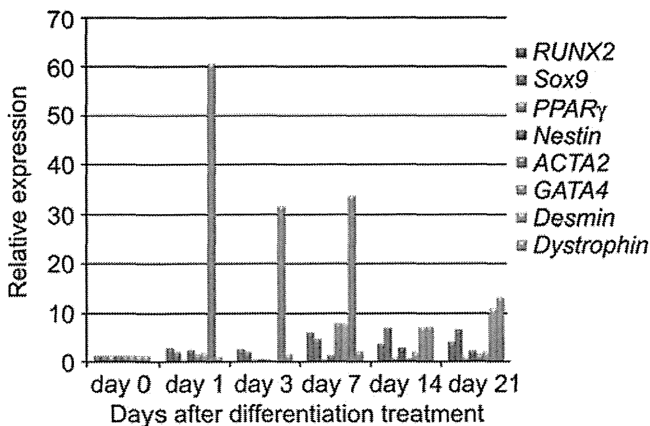

Fig. 8 qPCR analysis of multiple tissue markers in FTCVs. Time course of *RUNX2*, *Sox9*, *PPARγ*, *nestin*, *ACTA2* and *GATA4* and skeletal muscle marker (*desmin* and *dystrophin*) mRNA expression at the indicated day after treatment with 5-azacytidine. The value obtained before treatment with 5-azacytidine was set to 1 in each experiment

Sox2 mRNA are expressed in the cells collected during the 9th week. Immunocytochemistry has revealed that Nanog-protein-positive cells constitute only a small population of the FTCV-9th cells (Fig. 2a). Because FTCVs exhibit heterogeneity, which suggests the presence of other stem-cell-like cells, additional studies are required to assess further the expression pattern of stem cell markers. However, these data confirm that the FTCV-9th cells contain a subpopulation of uncommitted progenitors that express *Nanog* and *Sox2* mRNA and that they are likely to be distinct from the committed MSC population. We have also confirmed that FTCVs expressed MSC markers at a high level (Table 1). Although the FTCVs from the 10th and 11th weeks do not express *Nanog* and *Sox2* mRNA, they do express myogenic markers, including *dystrophin*, after myogenic induction. These data indicate that FTCVs possibly down-regulate the expression of *Nanog* and *Sox2* mRNA between the 9th and 10th weeks of gestation (Fig. 1c). On the basis of these findings, we conclude that the characteristics of our FTCV-9th population require further detailed analysis. Furthermore, our results indicate that FTCVs can be efficiently directed to differentiate in vitro into skeletal muscle cells that express dystrophin (Fig. 6). Dystrophin is a mature skeletal muscle protein produced during cell fusion (Edmondson and Olson 1993).

Under the myogenic differentiation conditions used in this study, FTCVs exhibit a greater increase in the expression of myogenic markers than any other lineage markers (Fig. 8). Indeed, MSCs derived from TTCVs can be induced to differentiate into skeletal muscle by treatment with 5-azacytidine in DMEM with 20% FBS and subsequent culture in 2% horse serum. Initially, we optimized the cell concentration used in the present study from this previously reported culture method (Kawamichi et al. 2010) but our modified method led to the further formation of myotubes in the FTCVs. Although such myogenic induction was probably caused by transient and random gene modifications attributable to treatment with the DNA demethylation agent 5-azacytidine, our myogenic culture conditions might have increased the effectiveness of myogenesis in the FTCVs.

Since DMD-derived FTCVs are regularly obtained during early pregnancy for prenatal diagnosis purposes, they are a stable source of cells with pluripotent and myogenic differentiation potential. Furthermore, the DMD-derived FTCVs can be easily cultured in vitro. These cells can be manipulated by using gene-targeting protocols that involve a mini-dystrophin gene (Tang et al. 2010), other genes using viral vectors (Odom et al. 2008), or antisense morpholinos (Saito et al. 2010). Alternatively, they can be treated with pharmacological agents, such as aminoglycoside (Barton-Davis et al. 1999; Malik et al. 2010) or negamycin (Arakawa et al. 2003; Allamand et al. 2008), which target the nonsense mutation in the *dystrophin* gene of about 15% of DMD patients. These particular properties of the FTCVs might enable effective DMD treatment by allowing the use of a patient's own FTCVs for self-transplantation with one of the above-mentioned gene therapies. Myogenic differentiation of reimplanted FTCVs *in situ* could theoretically repopulate and repair the dysfunctional skeletal muscles in DMD. Furthermore, a combination of targeted gene manipulation and pharmacological treatments that enable stem cells to grow and differentiate indefinitely could further extend the possible therapeutic potential of these cells. Moreover, such manipulations of stem cells offer the potential of developing protocols to cure genetic defects and to assess in great detail the functionality and safety of therapeutic protocols in vitro. Indeed, patient-derived FTCVs might be suitable for self-transplantation with nonimmunogenicity or low immunogenicity and non-tumorigenicity. Even FTCVs with a different genetic background might represent a novel cell source for transplantation because of their low immunogenicity and lack of need for immunosuppressive agents.

The expression of markers for MSCs, pluripotent stem cells and myogenic cells in the FTCV population opens up new possibilities in cell therapy for DMD patients and individuals with other neuromuscular diseases. Further in vivo studies, which are currently under way, will be essential for identifying the factors that determine the conditions for definitive myogenic differentiation, survival and dissemination and the potential clinical effects of these cells in animal models.

In summary, FTCVs that have been previously obtained for prenatal diagnosis express markers of MSCs and pluripotent stem cells and undergo myogenesis efficiently in vitro. The accessibility of FTCVs and the minimal ethical issues involved with obtaining these cells make them promising therapeutic candidates for patients suffering from DMD.

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＝症例報告＝

脊髄性筋萎縮症0型の1例

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石垣 景子[1] 舟塚 真[1] 大澤真木子[1]

要旨 脊髄性筋萎縮症は，発症年齢と重症度に基づきⅠ型，Ⅱ型，Ⅲ型に分類される．今回我々は0型といえる最重度の症例を経験した．症例は2カ月，女児．在胎41週2日に出生，出生直後より呼吸不全，筋緊張低下，哺乳不良を認めた．当科入院時は著明な筋緊張低下，筋力低下，胸郭の変形，四肢の関節拘縮，嚥下不能，誤嚥性肺炎を認めた．舌の線維束性収縮を認め，深部腱反射は消失し，運動神経伝導速度検査ではM波の出現なく有意な筋収縮を認めなかった．遺伝子検査より*SMN*遺伝子，*NAIP*遺伝子の欠失を認めた．最重度の経過であり，胎児期発症と考えられ，脊髄性筋萎縮症0型と診断した．0型の報告は少なく，若干の考察も含めて報告する．

見出し語 脊髄性筋萎縮症，0型，最重症型，新生児，関節拘縮

はじめに

脊髄性筋萎縮症（spinal muscular atrophy，以下SMAと略す）は，脊髄の前角細胞の変性による筋萎縮と進行性筋力低下を特徴とする下位運動ニューロン病である．体幹，四肢の近位部優位の筋の脱力，筋萎縮を示す．国際SMA協会より診断基準が示されている（表1）[1]．発症年齢，臨床経過に基づき，Ⅰ型，Ⅱ型，Ⅲ型に分類される．Ⅰ型は発症が6カ月までで重症型である．

今回我々は診断基準の除外項目のうち，関節拘縮と運動神経伝導速度測定不能に該当したが，SMAと確定診断し，最重度である0型と考えられた症例を経験したので報告する．

Ⅰ 症 例

症 例 日齢54，女児．

家族歴 神経筋疾患，代謝性疾患なし．

妊娠経過 胎動微弱の自覚はなかった．妊娠12～13週に前期破水のため，10日間入院した．

現病歴 他院にて，在胎41週2日に，回旋不良，用手補助，頭位経腟分娩にて出生した．出生体重は2,558 g，Apgar scoreは2点/7点（1分/5分）であった．出生時に啼泣は認められず，気管挿管を施行され，人工呼吸管理が1日間行われた．筋緊張低下，哺乳不良を認めていたが，徐々に改善し，日齢20頃には哺乳は65～70 ml/回程度まで可能となった．

しかし，その後，徐々に嚥下困難が認められた．日齢23に誤嚥性肺炎を認め，嚥下困難が進行するようになった．日齢26に経管栄養を開始された．日齢30に嚥下造影検査を施行された．異常所見は指摘されなかったが，口腔内より唾液が噴出するようになった．日齢40にシーソー様呼吸が認められた．日齢46に施行された胸部X線にて右上中肺野の無気肺を認めた．日齢50にnasal CPAPを開始され，頻回の吸引を必要とした．日齢54に当科に転院した．

入院時現症 身長54.0 cm（＋0.36 SD），体重3,426 g（－1.32 SD），頭囲37 cm（＋0.69 SD），胸囲30.5 cm（－3.31 SD）であった．体温36.2度，心拍数166回／分，呼吸数66回／

表1 国際SMA協会の脊髄性筋萎縮症の診断基準（1992）

・包含項目
Ⅰ．筋力低下
　対称性
　近位筋＞遠位筋
　下肢＞上肢
　体幹および四肢
Ⅱ．脱神経
　舌の線維束性攣縮，手の振戦
　筋生検─萎縮筋線維の群
　筋電図─神経原性変化
・除外項目
1．中枢神経機能障害
2．関節拘縮症
3．外眼筋，横隔膜，心筋の障害，聴覚障害，著しい顔面筋罹患
4．知覚障害
5．血清CK値＞正常上限の10倍
6．運動神経伝導速度＜正常下限の70%
7．知覚神経活動電位の異常

第52回日本小児神経学会総会推薦論文

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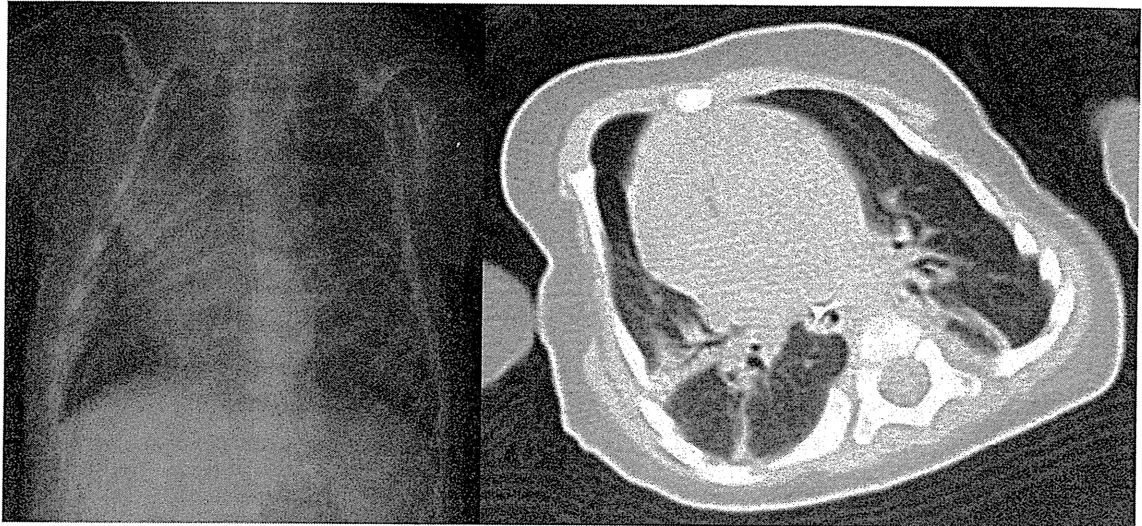

図1 胸部X線(正面像)と胸部CT(肺野条件)
両肺背側に気管支透亮像を伴う浸潤影を認めた.

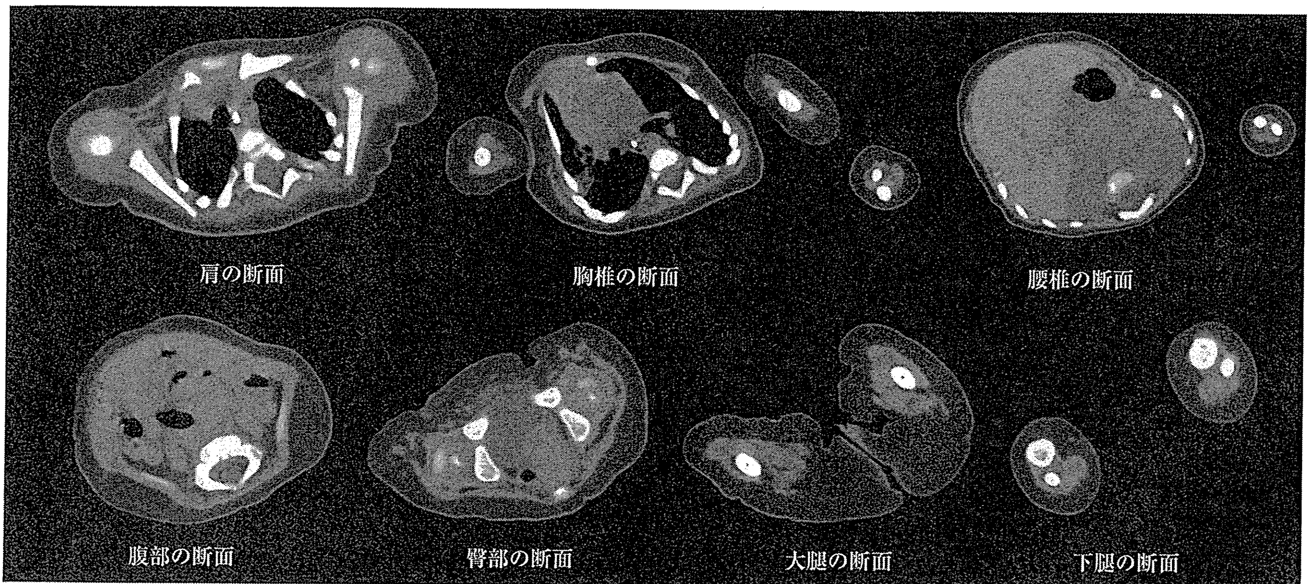

図2 筋CT検査(日齢54, window レベル30)
体幹筋・四肢・大殿筋においてX線低吸収域は認めず,筋量が少なく萎縮が著明であった.

分,SpO_2 90～99%(鼻マスク式非侵襲的陽圧換気,酸素使用下)であった.全身状態は不良で,吸啜は微弱であった.全身の著明な筋緊張低下,筋力低下がみられ,蛙肢姿勢であった.固視,追視がみられた.眼球運動制限はなかった.舌に線維束性収縮を認めた.閉眼可能であるが表情は乏しく,啼泣時の表情も乏しかった.

胸部は胸郭が右方向に突出した変形があり,軽度のベル様変形を認めた.奇異呼吸を認め,呼吸音は両側に著明なラ音を聴取し,右上肺野の空気の入りが低下していた.両手の下垂手はあったが,尺側偏位は明らかでなかった.四肢関節はMP関節,PIP関節,DIP関節の伸展制限があった.足関節の背屈制限があった.肘,股,膝関節の運動制限はなかった.手の把握反射をわずかに認めた.深部腱反射は両上下肢ともに消失し,Babinski反射は陰性であった.

検査所見 前医にて,頭部MRI検査,脊髄MRI検査,聴性脳幹反応(ABR),髄液一般検査,髄液中NSE,染色体検査,有機酸代謝,アミノ酸代謝を検査されたが,明らかな異常は認めなかった.

当科での末梢血検査では,血算,生化学検査,甲状腺機能検査は異常を認めなかった.CKは91 U/lであり,正常範囲であった.

胸部X線と胸部CT検査では,両肺背側に気管支透亮像を伴う浸潤影を認めた(図1).

日齢54の胸椎,腰椎,臀部,大腿中央,下腿中央で撮影された筋CT検査(window レベル30)上,体幹筋・四肢・大殿筋において,X線低吸収域は認めず,筋量が少なく萎縮が

著明であった（図 2）.

運動神経伝導速度（左正中神経刺激）では，有意な筋収縮やＭ波の出現を認めず（図 3），重度の末梢神経障害パターンと考えられた.

診断と臨床経過 鑑別診断として，SMA の他，末梢神経障害（Dèjèrine-Sottas 症候群，congenital hypomyelinating neuropathy），新生児仮死の影響，先天性ミオパチーなどを考えた．末梢神経障害は痛み刺激への反応から感覚神経障害は否定的であった．新生児仮死の影響は病歴と頭部 MRI 検査や脳波検査が正常であることより，先天性ミオパチーは筋 CT 検査で筋そのものの X 線吸収値変化を認めないことより共に否定的であった.

遺伝子検査にて *SMN* 遺伝子，*NAIP* 遺伝子の欠失を認め，SMA と確定診断した.

早期より関節拘縮を認めたことより，胎児期発症と考えられ，Dubowitz[2]の述べる SMA 0 型と診断した.

入院後，非侵襲的陽圧換気，排痰理学療法，陽・陰圧体外式人工呼吸器を施行した．日齢 61 に前医へ転院となった.

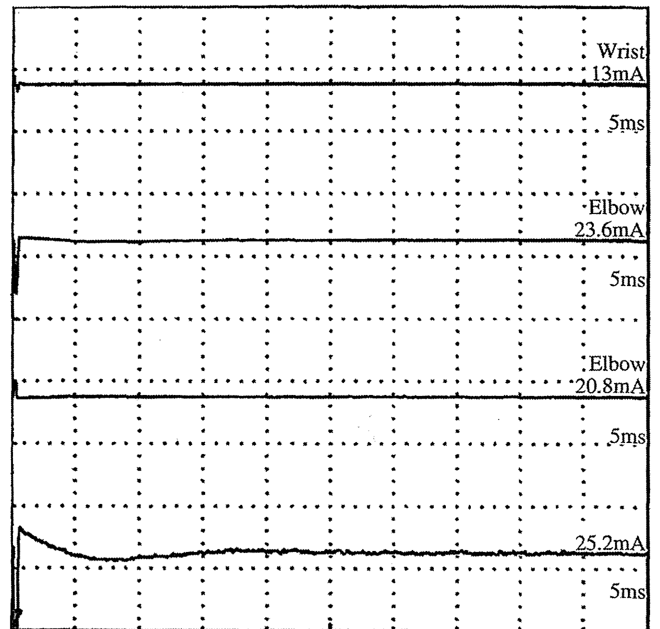

図 3 運動神経伝導速度（左正中神経刺激）
有意な筋収縮やＭ波の出現を認めなかった.

Ⅱ 考 察

SMA は脊髄の前角細胞の変性による筋萎縮と進行性筋力低下を特徴とする常染色体性劣性遺伝病である．SMA の遺伝子同定のためには明確な診断基準と分類を確立することが必要であるという考えのもとに，国際 SMA 協会が組織され，表 1 に示す診断基準が作成された．これを満たすものが典型的 SMA であり，特に小児期発症の SMA においては，遺伝子的に単一の疾患単位である[3]．SMA の分類としては発症年齢，臨床経過に基づき，Ⅰ型（OMIM#253300），Ⅱ型（OMIM#253550），Ⅲ型（OMIM#253400）に分類される.

Ⅰ型は重症型，急性乳児型，Werdnig-Hoffmann 病とも呼ばれ，生後 6 カ月までの発症で，筋力低下が重症で全身性である．発症後に運動発達は停止し，体幹を動かすこともできず，筋力低下，筋緊張低下のために体が柔らかいフロッピーインファントの状態を呈する．支えなしに座ることができず，哺乳困難，嚥下困難，誤嚥，呼吸不全を伴う．舌の線維束性収縮がみられる．人工呼吸管理を行わない場合，死亡年齢は平均 6 ～ 9 カ月であり，18 カ月までにほぼ全例が死亡する[3].

Ⅰ型（重症型）の中に最重症型である例がみられる．Dubowitz は 1999 年の総説で新生児期発症，顔面筋の筋力低下，進行性で致死的な臨床経過をたどる SMA の最重症型を 0 型と提唱している[2]．これらの乳児では，上肢より下肢に，遠位筋より近位筋に強い全身の筋力低下と筋緊張低下がみられると述べている．その総説の中で新生児仮死，重度の筋力低下，顔面の筋力低下，関節拘縮などの症状がみられた症例が示されている．Dubowitz はこれらの最重症型について，prenatal または congenital という言い方は，SMA Ⅰ型の 1/3 程度が出生前の発症で出生時に症状が存在するため適切ではないと述べており，0 型と呼ぶことを提唱している．しかし，現時点で 0 型の診断基準は存在しない.

ドイツの後方視的な検討では，*SMN1* 遺伝子の欠失により診断された SMA Ⅰ型の 66 例のうち，*SMN2* コピー数が 1 つであった 4 例（6.1%）が，出生時に関節拘縮と呼吸不全が認められ，0 型と診断されている[4]．その 4 例全てで人工呼吸管理を必要とし，生存期間は数カ月以内であった.

その他，在胎 20 週の胎児で，左室低形成がみられ，剖検と遺伝子検査の結果により SMA と診断された例[5]や出生時に関節拘縮を認めたが，6 歳まで生存し，出生時を除いて人工呼吸管理を要しなかった例[6]が報告されており，SMA の臨床的多様性が示唆される.

国内からの SMA 0 型の報告を検討するため，検索しうる範囲で国内からの 30 例以上の症例報告（学会抄録を含む）を検討した．遺伝子検査で *SMN* 遺伝子の欠失を認めない症例や奇形を伴うなど非典型的と思われた症例は除外した．学会抄録が多数であったが，我々が新生児期発症であり 0 型の可能性があると考えた症例の報告を表 2 に示す[7)~11)]．2005 年の報告[11]によると，SMA Ⅰ型 17 例を検討し，*SMN2* コピー数が 1 つであった 3 例では，いずれも出生直後より症状が出て，生後 3 カ月までには呼吸障害を認め，最も重症度が高い症例であり，0 型である可能性が示唆された.

SMA において，*SMN2* コピー数が少ないほど，重症化することが知られている．本症例で *SMN2* コピー数は測定していないが，前述の少数例の報告において，*SMN* コピー数が 1 個であった 7 例は，新生児期発症であり，SMA 0 型が示唆された[4)11)]．SMA Ⅰ型の検討では *SMN2* コピー数 2 個が多く，*SMN2* コピー数 1 個である割合は，66 例中 4 例（6.1%）[4]，17 例中 3 例（18%）[11]，14 例中 4 例（29%）[12]と報告されて

表2 SMA 0型の可能性のある国内からの報告

報告年度	報告者	出生時の症状	症例	遺伝子検査	筋生検	筋電図
1988	竹島ら（学会抄録）[7]	全身の筋緊張低下，深部腱反射消失，舌の線維束性収縮	ABR結果より第8・11脳神経障害も併発	不明	施行	施行
2000	藤井ら（学会抄録）[8]	先天性多発性関節拘縮，羊水過多	先天性多発性関節拘縮症と診断	不明	神経原性筋萎縮（2カ月時）	不明
2002	武隈ら（学会抄録）[9]	not doing well	症例2 日齢117死亡	不明	施行	不明
2005	横須賀ら（学会抄録）[10]	新生児仮死，一過性多呼吸	症例3 手の尺側偏位，胸郭のベル様変形，生後80日で永眠	不明	不明	不明
2005	斎藤ら[11]	出生直後より症状を認め，生後3カ月までには呼吸障害	SMA I型17例中，*SMN2*コピー数が1個の3例（内，*NAIP*遺伝子の欠失2例）	施行	不明	不明

いる．

斎藤らはI型50/121家系（41%），II型7/99家系（7%），III型2/75家系（3%）で*NAIP*遺伝子の欠失を認めたと報告しており[3]，より重症型で*NAIP*遺伝子の欠失率が高くなることが知られている．そのため，最重症型である0型では*NAIP*遺伝子の欠失率が高いことが示唆され，本症例でも*NAIP*遺伝子の欠失を認めた．しかし，少数例の報告にとどまり，*SMN2*コピー数との関連，表現型との関連など今後の検討が必要であると思われた．

本症例では除外項目である関節拘縮が認められ，運動神経伝達速度が測定不能であった．SMAと関節拘縮については，SMA I型の約10%に関節拘縮が認められたという報告[13]や致死的な関節拘縮を認めた4人の新生児の剖検例で2人に*SMNt*遺伝子の欠損を認めたという報告がみられる[14]．このように最重度の場合，SMAの診断の除外項目に該当することがある．筋力低下，筋緊張低下，呼吸不全がみられ，SMAも疑われた場合，除外項目が該当しても積極的に遺伝子検査などを考慮するべきである．SMA 0型は予後不良であり，早期診断は本人のケアやご家族のサポートのために重要である．

最重症型，0型，先天性など用語は統一されていないが，SMA I型に分類されている症例の中に最重症型が存在する．

フロッピーインファントで，周産期の関節拘縮，呼吸不全，顔面筋罹患がみられた場合，国際SMA協会の診断基準の除外項目が該当してもSMA 0型も鑑別にあげるべきである．SMA 0型は非常に予後が不良であるため，診断の遅れは患者の管理やご家族の診断がつかないことへの不安にも関わりうる．そのため，十分な遺伝カウンセリングの下に積極的に遺伝子検査を実施して早期診断に努めるべきと考えた．

最後に，患児のご冥福を心からお祈りいたします．また，今回の発表をご許可いただいた患児のご両親に深謝申し上げます．

遺伝子検査施行に関して，臨床遺伝専門医・指導医による説明を行い，ご家族の自由意志により同意を得た．

本発表の要旨は第52回日本小児神経学会総会（2010年8月，福岡）にて発表した．

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A Case of Spinal Muscular Atrophy Type 0 in Japan

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The patient was a 2-month-old female infant born at 41 weeks and 2 days of gestation presenting multiple arthrogryposis, severe muscle hypotonia and respiratory distress with difficulty in feeding. She suffered from repeated complications with aspiration pneumonia. On admission to our hospital, she exhibited fasciculation and absence of deep tendon reflexes. Examination of the motor nerve conduction velocity (MCV) revealed no muscle contraction. Deletions of the *SMN* and *NAIP* genes were noted. Based on severe clinical course and disease development *in utero*, she was given a diagnosis of spinal muscular atrophy (SMA) type 0 (very severe type).

Arthrogryposis and disappearance of MCV are exclusion criteria for SMA. However, the clinical course of the infant was very severe and included such exclusion items. Consequently, when an infant presents muscle hypotonia and respiratory distress, SMA must be considered as one of the differential diagnoses, even though arthrogryposis is an exclusion criterion for SMA.

We discuss this case in relation to the few extant reports on SMA type 0 in Japanese infants in the literature.

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脊髄性筋萎縮症の診断とケア

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〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜

小児期発症の遺伝性神経筋疾患においては、日常診療、治療、リハビリテーションから、遺伝学的検査による診断、疾患の自然歴や最新の治療に関する情報提供、次子の再発率や出生前診断に関する遺伝カウンセリングなど、疾患と患者さんの状況に合わせたオーダーメイド医療が必要となります。当センターでは、臨床遺伝専門医、小児神経専門医、認定遺伝カウンセラー、臨床心理士、看護師が、診療、情報提供、遺伝カウンセリングの全てにおいてチームで対応しています。また、遺伝性神経筋疾患の病態解明、治療法開発を目指して、末梢血、胎盤由来の細胞、絨毛細胞などを用いた研究を患者さんとご家族の同意のもとで推進しています。今回は、当センターで遺伝子診断、遺伝カウンセリングを多く行っている脊髄性筋萎縮症（spinal muscular atrophy: SMA）の診断とケアについてまとめます。

〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜〜

1．SMAについて

SMAは、脊髄の前角細胞の病変によって起こる筋萎縮症であり、運動ニューロン病のひとつです（図１）。体幹、四肢の近位筋優位に筋力低下と筋萎縮を示します。発症年齢と重症度によってⅠ型からⅣ型に分類されます。生後すぐの新生児から高齢者まで、幅広い年齢層の患者さんがいます。最近の調査では、日本におけるSMAの患者さんは1,000人〜2,000人と推定されています。本稿では、小児期に発症するⅠ型、Ⅱ型、Ⅲ型を中心に解説します。

2．症　状

脊髄前角細胞の消失により、骨格筋萎縮を伴う筋力低下と脱神経の症状を示します。症状は左右対称性で、遠位筋よりも近位筋でより明らかに、上肢より下肢でより明らかに認められます。また体幹および四肢のいずれでも症状が認められるという特徴があります。脱神経の症状としては、舌の線維束性収縮や手指の細かい震えが特徴的です。知的発達は年齢相当もしくは高い方が多く、Ⅱ型のお子さんの中には、３歳頃から電動車椅子をハツラツと乗りこなす方もいます。

Ⅰ型（Werdnig-Hoffmann病：重症型）：

生後６か月ごろまでに発症し、全身性の筋力低下がみられ、支えなしに座位を保持することが困難で

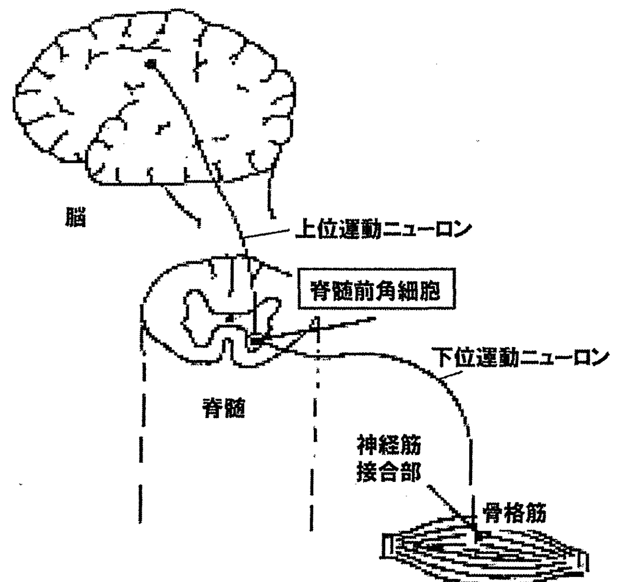

図１

す。発症後には運動発達がとまり、体幹を動かすことができません。哺乳困難、嚥下困難、誤嚥、呼吸不全を伴います。人工呼吸器を用いない場合の寿命は生後6～9か月とされ、95%は18か月までに亡くなるといわれています。したがって多くの場合では、生命を救うために気管内挿管または気管切開と、人工呼吸器管理が必要となります。

Ⅱ型（Dubowitz病：中間型）：

生後1歳6か月までに発症し、支えなしに立ったり、歩いたりすることができません。成長とともに、関節拘縮と脊柱側弯が著明になります。上気道感染から肺炎や無気肺へと進展し、呼吸不全に陥ることがあります。

Ⅲ型（Kugelberg-Welander病：軽症型）：

生後1歳6か月以降に発症し、立ったり歩いたりできていたのに転びやすくなる、歩けない、立てないという症状が見られます。次第に、上肢の挙上が困難になります。思春期以前に歩行不可能になった方では脊柱側弯が生じます。

Ⅳ型（成人型）：

成人期に発症し、筋力低下が認められます。発症年齢が遅いほど、症状進行のスピードは緩やかとなります。下位運動ニューロンのみが障害されます。筋萎縮性側索硬化症（ALS）では上位運動ニューロンも障害され、この点が異なります。脊柱側弯は見られません。

3．診　断

筋萎縮を伴う筋力低下と脱神経の所見により、臨床的に本症と診断が可能です。また末梢神経伝導速度検査、針筋電図、骨格筋画像検査が、診断の一助となります。しかしこれらの検査は鎮静が必要となったり、苦痛を伴うものが含まれるため、近年ではこのような検査より優先して、適切な遺伝カウンセリング後の遺伝子検査で診断を確定する例が増えています。小児期発症のSMAは、ほとんどの場合*SMN1*（survival motor neuron 1）遺伝子の変異により発症します。*SMN1*遺伝子は、5番染色体長腕q13領域に存在し、SMNタンパク質をコードします[1]。多くのSMA患者さんでは、二つある*SMN1*遺伝子の両方に欠失が認められます。また一部の方では、一方の*SMN1*遺伝子に欠失があり、他方の*SMN1*遺伝子に遺伝子内の微小な変異が認められます。

遺伝子解析では5～10mlの末梢血を採取し、血液に含まれるリンパ球からDNAを抽出して検査を実施します。*SMN*遺伝子のエクソン7とエクソン8をそれぞれ独立にポリメラーゼ連鎖反応（PCR）で増幅し、*SMN1*遺伝子欠失の有無を判定します。この方法で欠失が明らかにならない場合でも、臨床的にSMAが疑われる場合には、MLPA（multiplex ligation-dependent probe amplification）法により、*SMN*遺伝子の各エクソンを調べることも可能となってきています。しかしSMA患者さんの全ての方で、*SMN1*遺伝子変異が確認されるわけではなく、*SMN1*遺伝子以外の原因遺伝子が存在すると推測されます。現行の方法で*SMN1*遺伝子変異が検出されない患者さんにおいては、次世代シークエンサーを用いた解析により*SMN1*遺伝子のみならず、さらに広い範囲での詳細な研究が開始されています。

4．遺伝カウンセリング

SMAの原因として、乳児期発症のⅠ、Ⅱ型については95%以上、幼児期・小児期発症のⅢ型の40～50%に*SMN1*遺伝子欠失が認めらます。*SMN1*遺伝子変異により発症するSMAは、常染色体劣性遺伝形式に従います。すなわち、父由来の*SMN1*遺伝子と母由来の*SMN1*遺伝子の両方に変異がある場合に、SMAを発症します。父由来または母由来の遺伝子のどちらか1つだけに変異がある場合には、この方は全く無症状であり「保因者」と呼びます。保因者は、生涯症状がありません。

両親がいずれも保因者の場合、お子さんがSMAになる確率は1/4（25%）です。Ⅰ型の保因者の頻度は欧米では60～80人に1人、Ⅱ型、Ⅲ型は76～111人に1人ですが、日本では欧米よりその頻度が少ないといわれています。SMAの自然歴など疾患概要や遺伝のことをより詳しく知りたい場合には、遺伝カウンセリングを受けるという方法もあります。「病気について、治療を含めた今後のことを知りたい」、「次の子を希望しているので、検査が可能かどうか知りたい」など、来院された方の状況に応じて、遺伝カウンセリングが提供されます。日本では、2012年1月現在76の大学病院を含む91施設に、遺伝カウンセリングを受けられる外来があります。遺伝

カウンセリングを担当するのは、臨床遺伝専門医や認定遺伝カウンセラー、臨床心理士などで、遺伝性疾患について詳しい知識を持っています。

5．治　療

SMAでは根本治療はいまだ確立していませんが、小児神経科医、神経内科医、整形外科医、理学療法士などが連携をとり、チーム医療による継続的な医療管理を受けられる体制が望まれます。乳児期に発症するI型やII型の方では、哺乳や嚥下が困難なため経管栄養や胃瘻が必要となる場合があります。また、呼吸器感染症、無気肺を繰り返す方が多く、これらが予後を大きく左右します。鼻マスク人工換気法（非侵襲的陽圧換気法; non-invasive positive pressure ventilation: NIPPV）は有効ですが、乳児期には使用困難な場合が少なくありません。

また、筋力にあわせた運動訓練、関節拘縮の予防などのリハビリテーションが必要です。III型の方では歩行可能な状態をなるべく長期に維持し関節拘縮を予防する目的で、リハビリテーションを行います。装具の使用が日常生活動作の向上や機能維持に有用な場合もあります。

SMAの治療法開発については、世界中で精力的に研究が行われています。5番染色体長腕q13には、*SMN1*遺伝子と*SMN2*遺伝子が存在します（図2）。*SMN2*遺伝子は、*SMN1*遺伝子とその配列がとてもよく似ていて、SMNタンパク質の遺伝情報をもっています。しかし*SMN1*遺伝子と5塩基のみ異なる部分があることにより、エクソン7が組み込まれない短縮型*SMN2* mRNAが多く産生され、エクソン7が組み込まれている機能的な完全長*SMN2* mRNAは少ししか作られません。エクソンがmRNAに組み込まれることを、スプライシングといいます。*SMN2*遺伝子の場合、エクソン7がうまくスプライシングされない状態にあると考えられます。

エクソン7が組み込まれない短縮型*SMN2* mRNAからは、機能的なSMNタンパク質はできません。これまでの分子遺伝学的研究から、*SMN1*遺伝子の欠失があるSMA患者さんには必ず*SMN2*遺伝子が存在していることがわかっています。SMAモデルマウスの研究からは*SMN2*遺伝子のコピー数が多いほど、SMAの症状は軽くなるということが示唆されています。これらの知見から、「*SMN2*遺伝子から機能的なSMNタンパク質を十分産生させる」ための研究が進められています。この機序に基づいて、バルプロ酸ナトリウムなどヒストン脱アセチル化酵素阻害剤を用いた研究が行われています[2]。

この他にもSMNタンパク質の産生増加を目指して、ウイルスベクターを用いて*SMN1*遺伝子を導入する研究が進められています[3]。また運動ニューロン保護を目的とした治療として、神経保護作用が期待されるグルタミン酸遊離抑制剤のガバペンチン、リルゾール、神経栄養因子としてのTRH（甲状腺刺激ホルモン放出ホルモン）などの検討がなされています[4][5][6]。

再生治療の分野では、2009年に米国でSMA患者由来皮膚細胞からヒトiPS細胞が作製されました[7]。SMA患者由来iPS細胞においては、細胞移植治療へ

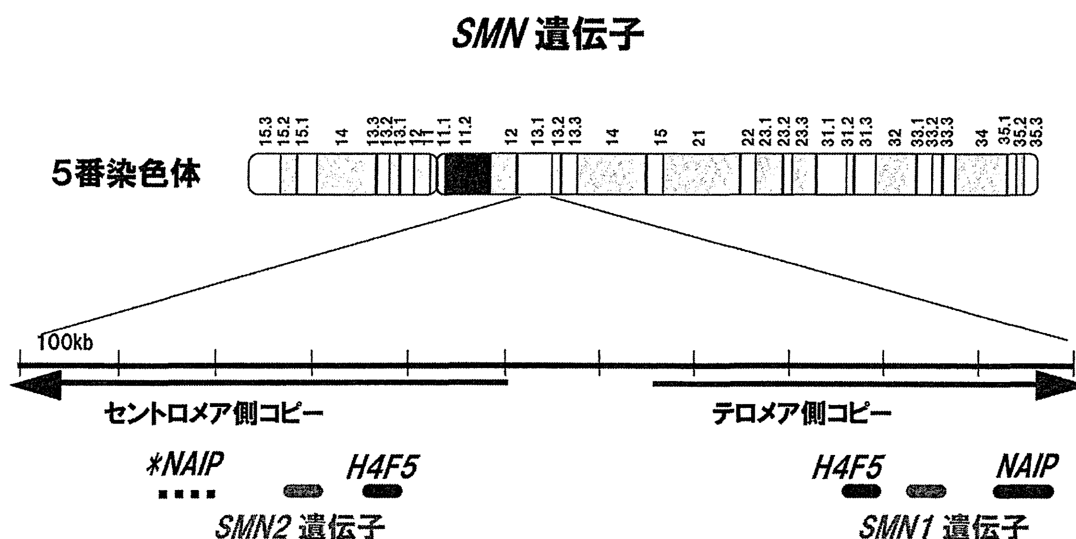

図2

の応用が期待されることに加え、SMAの病態を再現できるモデル作製につながる可能性が広がりました。疾患モデルの作製により、病態を解明する研究や薬物スクリーニングへの寄与が期待されています。

SMAにおけるロボットスーツHAL (hybrid assistive limb)の使用についての検討も進行中です。HALは装着者の運動意図を基にしてリアルタイムに四肢の運動機能を増強する機能を持つ装着型ロボットです。神経・筋難病患者さんが希少性神経・筋難病疾患に対して開発された下肢装着型ロボットであるHAL神経・筋難病下肢用モデルを定期的、間欠的に治療的に装着し、適切に筋収縮を助けられることで、筋萎縮と筋力低下の疾患の進行が抑制されると考えられています[8]。現在、医師主導治験の準備が進められています。

欧米では、神経保護剤のOlesoxime、RNAプロセシング酵素阻害剤のRG3039、アンチセンス・オリゴヌクレオチド製剤のISIS-SMNRx等の治験が公表されています。更に詳しい国際的な治験の情報については、アメリカ合衆国のThe U.S. National Institutes of healthが提供しているClinical Trials.gov (http://clinicaltrials.gov/)に掲載されています。

6．SMA（脊髄性筋萎縮症）家族の会

SMAの患者さんをとりまく環境を快いものにして、共に支えあう場をもつために、1999年に、「SMA（脊髄性筋萎縮症）家族の会」(http://www.sma.gr.jp/) が発足されました。現在、会員数は約300世帯にのぼり、全国レベルでの活動をしています。ホームページや機関誌の発行を通じて関連情報を発信し、定例会の開催やメーリングリストを通じて知識の共有などが行われています。

おわりに

臨床現場では患者さんの治療への熱意が高く、患者さんやご家族に説明の上、それぞれの医師の裁量でバルプロ酸ナトリウムやTRHなどの薬剤が投与されることがあります。しかし、これらの薬剤の健康保険の対象疾患としてSMAが認められているわけではありません。そして、薬剤の投与方法や評価基準が統一されていないために、薬剤の効果を正確に評価することは困難です。できるだけ早く、承認を受けたSMA治療薬を届けるためには、日本においても治験を進めていく必要があります。

有効な治験を行うためにも、患者さんの臨床情報、遺伝子情報を合わせた調査研究を行うことは重要な課題となっています。これらの課題を解決する目的で、現在、「脊髄性筋萎縮症患者登録システム」の構築および、多施設間で統一した運動機能評価法の導入について、厚生労働科学研究費補助金（難治性疾患克服研究事業）「脊髄性筋萎縮症の臨床実態の分析、遺伝子解析、治療法開発の研究」の研究者を中心に、検討を進めています(http://plaza.umin.ac.jp/~SMART/)。今後、研究や治験への参加など、治療法開発の発展のために患者さんにご協力いただく機会が多くなってくることと思います。

本年3月に、SMAの患者さんを診察する医療施設が、高い水準で診療できること、また根本治療を目指す研究の発展について、患者さん、ご家族、医療関係者が情報を共有することを目的として、「脊髄性筋萎縮症診療マニュアル」を発刊いたしました[9]。疾患の基本知識から、ケア、治療研究などが網羅されたものとなっておりますので、是非、参考にして下さい。

謝辞　本原稿の一部の内容は、厚生労働科学研究費補助金難治性疾患等克服研究事業（難治性疾患克服研究事業）「脊髄性筋萎縮症の臨床実態の分析、遺伝子解析、治療法開発の研究」（研究代表者　斎藤加代子）および、文部科学省科グローバルCOEプログラム「再生医療本格化のための集学的教育研究拠点」の一環として実施したものです。

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Original article

Enhanced expression of myogenic differentiation factors and skeletal muscle proteins in human amnion-derived cells via the forced expression of *MYOD1*

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Abstract

Objectives: Mesenchymal stem cells are expected to be an ideal cell source for cellular and gene therapy. We previously showed that cells derived from the human placenta can be induced to differentiate into myotubes *in vitro* and to express dystrophin in mdx/scid mice *in vivo*. In this study, we examined whether amnion-derived cells can be efficiently transduced and differentiated using lentiviral vectors carrying human *MYOD1*. *Methods:* The amnion-derived cells were isolated from human preterm placentas. They were transduced with the *MYOD1* vector, and mRNA levels for *MYOD1*, *MYF5*, *MYOG*, *MYH2* and *DMD* were determined by quantitative-reverse transcriptase-polymerase chain reaction, and also examined immunocytochemically. *Results:* Approximately 70% of amnion-derived cells were efficiently transduced by the lentiviral vectors. *MYOD1* activates *MYF5* and *MYOG*, *MYH2* and *DMD* after a 7-day culture. The concerted upregulations of these myogenic regulatory factors enhanced *MYH2* and *DMD* expressions. *PAX7* was below the detectable level. Both myosin heavy chain and dystrophin were demonstrated by immunocytochemistry. *Conclusions: MYOD1* activates *MYF5* and *MYOG*, the transcription factor genes essential for myogenic differentiation, and the concerted upregulation of these myogenic regulatory factors enhanced *MYH2* and *DMD* expressions. The amniotic membrane is an immune-privileged tissue, making *MYOD1*-transduced amnion-derived cells an ideal cell source for cellular and gene therapy for muscle disorders. This is the first report showing that amnion-derived cells can be modified by exogenous genes using lentiviral vectors. Furthermore, *MYOD1*-transduced amnion-derived cells are capable of the dystrophin expression necessary for myogenic differentiation.
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Keywords: Duchenne muscular dystrophy; Mesenchymal stem cell; Cellular therapy; Gene therapy; Dystrophin; Placenta

1. Introduction

Duchenne muscular dystrophy (OMIM# 310200) is an X-linked recessive inherited disorder that affects 1 in 3500 males. The onset of Duchenne muscular dystrophyis usually before the age of 3 years, and patients die of respiratory failure around the age of 20 [1]. Duchenne muscular dystrophyis caused by structural mutations in

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the dystrophin gene (*DMD*), which encodes dystrophin, a large membrane-associated protein that plays an important role in linking intracellular cytoskeletal actin filaments to the sarcolemmal membrane [2]. Approximately 60% of *DMD* mutations are large deletions or insertions, whereas 40% are nonsense, missense, or small insertion-deletion mutations.

No curative therapeutic approaches for Duchenne muscular dystrophy currently exist. However, cell-based treatments in addition to gene therapy [3], exon skipping therapy [4], and read-through therapy with aminoglycosides [5] remain promising options.

Mesenchymal stem cells (MSC) are expected to be an ideal cell source for cellular and gene therapy because they can easily be obtained from bone marrow, adipose tissues, and the placenta, they are abundant and non-tumorigenic, and they have the useful characteristics of homing and chemokine secretion. MSC are already utilized for the treatment of graft versus host disease [6] and inflammatory bowel disease [7]. Several laboratories have shown that MSC can be obtained from amnion-derived cells and induced to differentiate into myocytes [8].

Although the myogenic differentiation of MSC can be induced by treating them with demethylating agents such as 5-azacythidine (5AZA), there is no marked enhancement of either *MYOD1,* the human myogenic differentiation factor 1 gene, or *MYH2* expression, nor does5AZA treatment substantially increase the myogenic differentiation of MSC [9]. In addition, there have been several attempts to enhance the myogenesis by introducing *MYOD1* into cells [10]. It was recently shown that human adipose-derived cells displayed enhanced myogenic differentiation after being forced to express *MYOD1* [11], and another group showed that forced expression of *MYOD1* led to the trans-differentiation of human fibroblasts into myotubes [12].

In this study, we introduced human *MYOD1* into amnion-derived cells using a lentiviral vector and examined the precise gene expression levels of *MYF5*, *MYOG*, *MYH2* and *DMD*. We demonstrated significant upregulations of the genes for essential transcription factors involved in myogenesis. The potential applications of*MYOD1*-transduced amnion-derived cells are also discussed.

2. Materials and methods

2.1. Isolation of human amnion-derived cells

Ethics approval for the tissue collection was granted by the Institutional Review Board of Tokyo Women's Medical University, Japan. Written informed consent was obtained prior to sample collection. Amnion tissue samples were obtained from normal full-term pregnancies at the time of caesarean section before the onset of labor. None of these pregnancies were complicated by premature membrane rupture or chorioamnionitis. The placentas were processed within 24 h of collection; i.e., they were thoroughly washed with phosphate-buffered saline (a solution containing sodium chloride, sodium phosphate, potassium chloride and potassium phosphate), and, after separation from the placentas, the amnions were minced into 5 mm sections using knives on a clean bench. The amnion tissue was placed in collagen I coated dishes (Iwaki, Japan), and after 20 min, Mesenchymal Stem Cell Basal Medium (MSCBM, Lonza, USA) was carefully poured onto the attached cells, which were then maintained at 37 °C in 5% CO_2. After 48 h, the non-adherent cells were removed, and the medium was changed twice a week. After about one week, a few colonies were found in the dishes. At 70–80% confluence, the amnion-derived cells were harvested with 0.5% Trypsin–EDTA (Life Sciences, USA) and plated onto new dishes. Cells were processed from 24 placentas, and primary cultures from 8 placentas were used for this study.

2.2. Flowcytometric analysis

The amnion-derived cells were used for fluorescent activated cell sorting (FACS) analysis employing the EPICS ALTRA XL-MCL analyzer (Beckman Coulter, USA), and the data were analyzed with EXPO™32 ADC software (Beckman Coulter). Antibodies against human CD14, CD29, CD34, CD44, CD45, CD73, CD105, CD166, HLA-ABC, and HLA-DR were obtained from Beckman Coulter and BD Biosciences Pharmingen (USA), AbD Serotec (UK) and Cytognos (Spain).

2.3. Production of lentiviral vectors and MYOD1 transduction of human amnion-derived cells

A full-length human *MYOD1* cDNA clone (Genome Network Project Clone, WW01A62C23) was provided by the RIKEN Bioresource Center (Ibaraki, Japan) through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan [13–16]. A lentiviral vector carrying the *MYOD1* cDNA, pLenti6/human*MYOD1,* was constructed using the pLenti6/UbC/V5-DEST Gateway Vector kit and the ViraPower Lentiviral Expression System (Life Technologies, USA). A GFP expression vector, pRRL.PPT.SF.IRES-GFP, was kindly provided by Taiju Utsugisawa.

Three micrograms of the purified pLenti6/UbC/V5-DEST – human*MYOD1* cDNA and pRRL.PPT.SF. IRES-GFP were used for the transfection of 4×10^6 293FT cells together with Lipofectamine 2000 (provided with the kit) reagent and ViraPower packaging Mix (provided with the kit). After 48 h, the supernatant

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was collected. Eight milliliters of viral supernatant were added to 6–8 × 10^5 amnion-derived cells. To examine the transfection efficiency of our procedure, the GFP expression of the cells was analyzed by FACS.

2.4. In vitro myogenesis

The amnion-derived cells were transduced with the pLenti6/UbC/V5-DEST – human *MYOD1* supernatant and seeded onto collagen I coated cell culture dishes (IWAKI) at a density of 1 × 10^4 per ml in growth medium (Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum) for 24 h. The cell cultures were then washed twice with phosphate-buffered saline (−), and maintained in differentiation medium (DMEM supplemented with 2% horse serum (Iwaki, Japan)). The medium was changed twice a week until completion of the experiment.

2.5. Quantitative-reverse transcriptase-polymerase chain reaction using MYOD1-transduced amnion-derived cells

Total RNA was purified from the *MYOD1*-transduced amnion-derived cells using the RNeasy mini kit (QIAGEN, Germany) at 7 and 14 days after transduction. Two micrograms of total RNA were subjected to reverse-transcription using Expand Reverse Transcriptase (Roche, USA). The gene expression levels of *MYOD1, MYF5, PAX7, MYOG, MYH2*, and *DMD* were analyzed using the quantitative-reverse transcriptase-polymerase chain reaction (Q-RT-PCR), the primers listed in Supplementary Table 1, and Mx3000™ (Stratagene, USA). The non-transduced amnion-derived cells were designated the day 0 cells. Q-RT-PCR was performed at 95 °C for 10 min for 45 cycles, with each cycle consisting of 95 °C for 15 s, followed by treatment at 60 °C for 60seconds after completion of the last cycle. Relative gene expression levels were calculated using RNA extracted from normal human skeletal muscle myoblast cells (Lonza), which had been cultured for 4–5 days using the SkGM-2 BulletKit (Lonza) or for 7 days in total DMEM supplemented with 2% horse serum as a myoblast control [17].

2.6. Immunocytochemical analysis

The cultures were fixed in 4% paraformaldehyde and stained with either a mouse anti-human dystrophin IgG1 monoclonal antibody (NCL-DYS2, Novocastra, UK) or a mouse anti-myosin heavy chain IgG2 monoclonal antibody (MF-20, Developmental Studies Hybridoma Bank, USA). The cells were visualized with appropriate AlexaFluor488 goat anti-mouse IgG secondary antibodies (Invitrogen, USA). Total cell nuclei were stained with Hoechst solution (Sigma Aldrich, UK).

3. Results

3.1. Morphology of the amnion-derived cells

We isolated amnion-derived cells from placentas, and a large number of primary cells were successfully obtained. These cells consisted of small fibroblast-like and cobblestone-like cells (Fig. 1a), and after 3 passages, they had formed a homogeneous population of the fibroblast-like cells (Fig. 1b). The yield was approximately 2 × 10^7 cells per gram of amnion tissue after three weeks.

3.2. Surface markers of amnion-derived cells

The surface marker expressions of amnion-derived cells were evaluated by flowcytometric analysis (Fig. 2). After 3 passages, the amnion-derived cells were positive for CD29, CD44, CD73, CD105, CD166, and HLA-ABC. However, they did not express any hematopoietic lineage markers such as CD34, CD14, CD45 or HLA-DR.

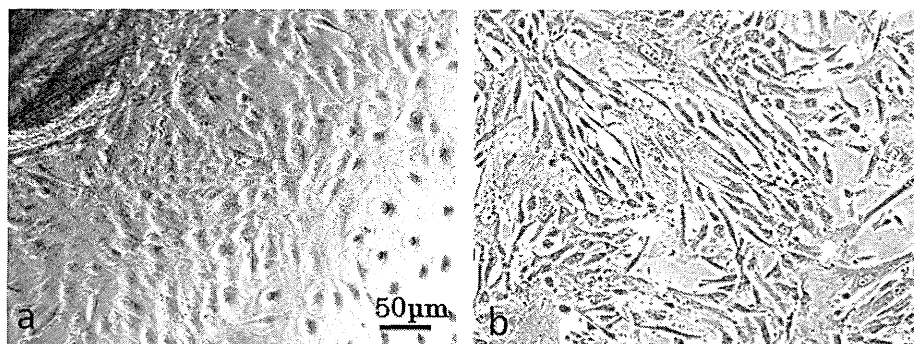

Fig. 1. Photomicrographs of amnion-derived cells in primary cultures in which two cell types, cobblestone- and fibroblast-like cells, can be seen (a). After three passages, the cells had formed a homogeneous population of fibroblast-like cells (b). Scale bar: 50 μm.

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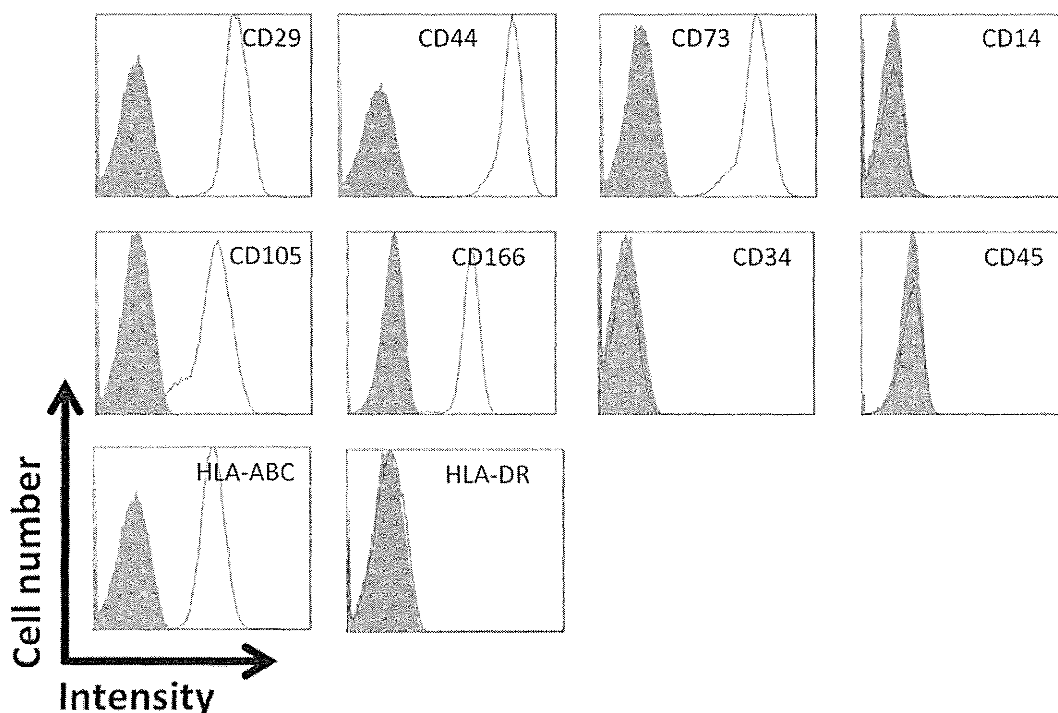

Fig. 2. Surface marker expressions of amnion-derived cells were evaluated by flowcytometric analysis after 3 passages. They expressed the following MSC markers: CD29, CD44, CD73, CD105 CD166, and HLA-ABC. However, they did not express any hematopoietic lineage markers such as CD34, CD14, CD45, or HLA-DR.

3.3. Transduction of amnion-derived cells with a GFP vector

The amnion-derived cells were transduced with a GFP vector, and the transduction efficiency of our procedure was evaluated by flowcytometric analysis after 72 h. In each experiment, approximately 70–80% of amnion-derived cells were positive for GFP (data not shown).

3.4. Q-RT-PCR of MYOD1 transduced amnion-derived cells

The mRNA levels of *MYOD1, MYF5, MYOG, PAX7, MYH2, and DMD* in the *MYOD1*-transduced amnion-derived cells were determined by Q-RT-PCR. Forced expression of human *MYOD1* in amnion-derived cells markedly enhanced their *MYF5* and *MYOG* gene expression levels after 7 days of culture (Fig. 3). On day 7, the *MYOD1*mRNA level of these cells was increased 50-fold as compared to that observed on day 0. The mRNA level corresponded to 4.8% of that in the control myoblasts. On day 14, the *MYOD1*mRNA level had decreased to 1.1% of that in the controls, suggesting that the enhanced *MYOD1* mRNA expression was transient.

It should be noted that *MYF5*gene expression was highly upregulated in the *MYOD1*-transduced amnion-derived cells. On day 7, it was increased by over 500-fold, and the relative mRNA level had reached 5.6% of that in control myoblasts. However, on day 14, the *MYF5* expression of these cells had been almost completely abrogated.

The *MYOG* mRNA level in the amnion-derived cells was extremely low (0.02%) on day 0. On day 7, it had reached 0.125% of that in the control myoblasts, but was undetectable by day14.

On days 7 and 14 the mRNA levels of *MYH2* were 0.11% and 0.33% and those of *DMD* were 20% and 28%, respectively, of corresponding levels in the control cells. These results suggest that genes encoding skeletal muscle proteins were activated following the concerted activation of myogenic regulatory factors such as *MYOD1*, *MYF5,* and *MYOG.* On the other hand, the level of *PAX7* was not measurable in either the non-transduced or transduced amnion-derived cells (data not shown).

3.5. Immunocytochemistry of myogenic differentiated cells

The MYOD1-transduced amnion-derived cells were subjected to immunocytochemical analysis after a 28-day culture in differentiation medium. Both myosin heavy chain 2 and dystrophin were immunologically detected in their cytosol and nuclei, suggesting these cells to be capable of differentiating into myotubes (Fig. 4).

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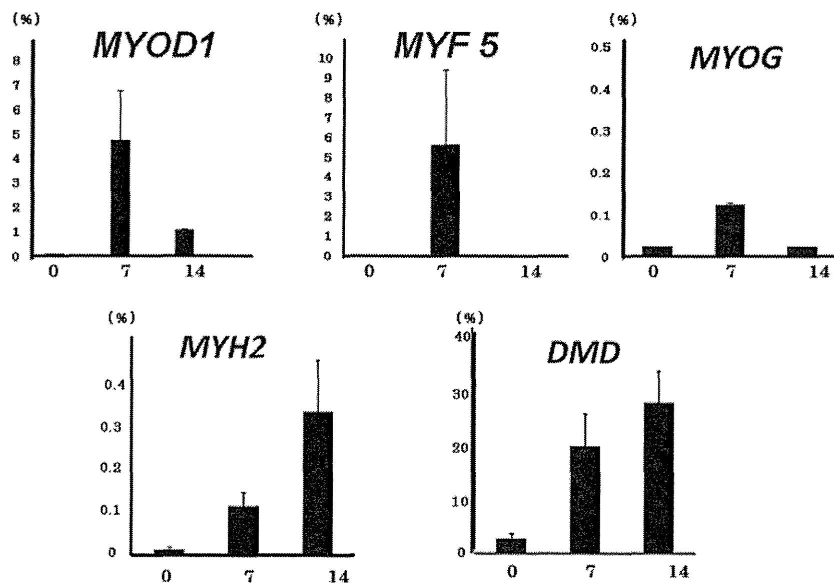

Fig. 3. The mRNA levels of *MYOD1, MYF5, MYOG, PAX7, MYH2*, and *DMD* in the *MYOD1* transduced amnion-derived cells were determined by Q-RT-PCR using the Universal Probe Library system (Roche). Relative gene expression levels were calculated using normal human skeletal muscle myoblast cells (Lonza) as a control. Day 0 represents the mRNA level of the amnion-derived cells on the day of transduction. Days 7 and 14 represent the mRNA levels of the *MYOD1*-transduced amnion-derived cells. The mRNA levels of the cells were as follows: *MYOD1*, 0.10 ± 0.05%, 4.8 ± 2.0%, and 1.09 ± 0.07%; *MYF5*, 0.013 ± 0.001%, 5.6 ± 3.8%, and < 0.0025%; *MYOG*, ≦0.00%, 0.13 ± 0.01%, and <0.0025%; MYH2, 0.01 ± 0.001%, 0.11 ± 0.03%, and 0.33 ± 0.12%; and *DMD*, 2.8 ± 1.0%, 20 ± 6.0%, and 28 ± 6.0%. The mRNA levels of the *MYOD1*-transduced amnion-derived cells were markedly upregulated, as shown in the figure; however, *PAX7* was not detected in either the untreated or the transduced amnion-derived cells.

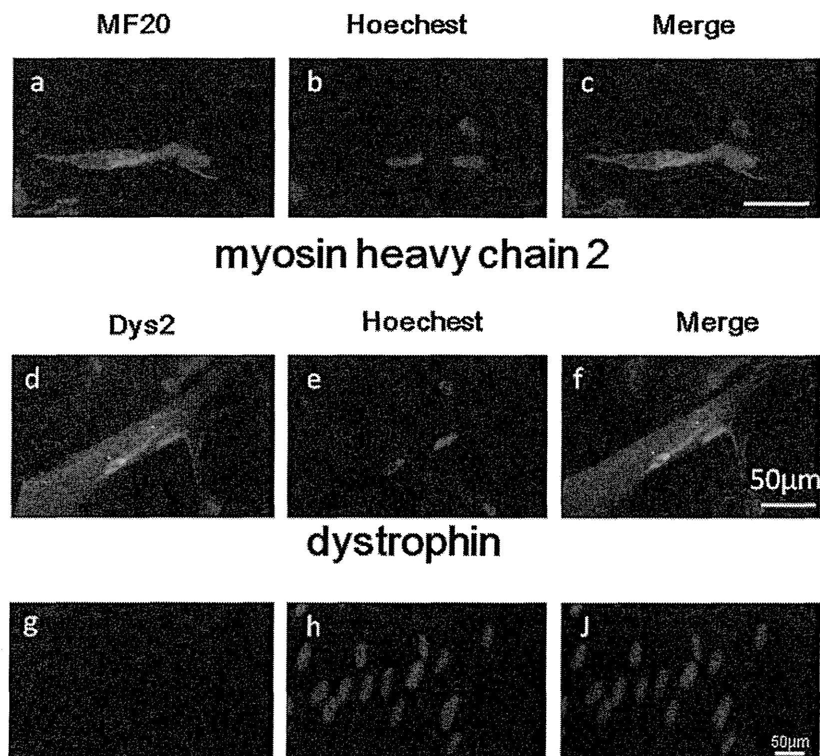

Fig. 4. Expressions of muscle specific genes during the differentiation of amnion-derived cells. *MYOD1*-transduced amnion-derived cells were cultured in differentiation medium for 28 days and stained using immunohistocytochemical methods. (a) Myosin heavy chain molecule (green, MF20); (b) nuclei (blue, Hoechst); (c) merged image of a and b; (d) dystrophin molecule (green, Dys2); (e) nuclei (blue, Hoechst); (f), merged image of d and e;The untreated amnion-derived cells expressed neither myosin heavy chain (g–h) nor dystrophin (data not shown). Scale bars: 50 μm.

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4. Discussion

Myogenesis is classified into two modes, skeletal myogenic differentiation during development and regeneration mediated through satellite cells after injury. Previous studies have revealed that several transcription factors are essential for regulating embryonic and adult muscle formation. Among these factors, MyoD, Myf5, and myogenin are considered to be master transcription factors that are essential for myogenesis and are known as myogenic regulatory factors [18]. During myogenesis, *MYOD1* and *MYF5* are able to functionally substitute for one another [19]. In contrast, MyoD and Myf5 have distinct functions in the processes involved in recovery from muscle injury: MyoD is indispensable for the commitment of satellite cells to become myogenic precursor cells, and Myf5 plays an important role in myoblast proliferation [20]. On the other hand, myogenin is important for terminal muscle differentiation and lineage maintenance [21]. In this study, we clearly showed *MYOD1* transduction to activate both *MYF5* and *MYOG*, leading to myogenic differentiation resembling the developmental stages between myogenic progenitors and myoblasts. It is still unclear whether the concerted activation of *MYF5* and *MYOG* is due to direct transactivation by MyoD. Nonetheless, sequential activation of myogenic differentiation factors subsequently increased the gene expression levels of *MYH2* and *DMD*. As a result, the myosin heavy chain2 and dystrophin were immunocytochemically detected, as shown in Fig. 4. However, we were unable to demonstrate the protein expression of MYOD1, MYF5, MYOG, or PAX7, since examining the expressions of transcription factors by Western blotting or other immunological procedures is quite difficult.

Satellite cells, which are generated around myofibers during fetal development, express another transcription factor, Pax7. They are mitotically quiescent, but are activated in response to the stress induced by muscle injury [22]. Although the *MYOD1*-transduced amnion-derived cells did not display *PAX7* expression, the biological significance of this transcription factor remains obscure.

We previously demonstrated that human MSC transplanted into skeletal muscles of mdx mice successfully differentiated and fused with murine muscles, suggesting cellular therapy to be a promising strategy for Duchenne muscular dystrophy [23]. A previous report showed that dystrophin Dp71 and dystrophin-associated proteins are co-localized to the nuclei of muscle cells. This result implies that allogenic *MYOD1*-transduced amnion-derived cells would be a useful tool for cellular therapy [24].

The amniotic membrane is an immuno-privileged tissue and has been used as a biological membrane for treating burns, injuries, and skin ulcers as well as for corneal transplantation [25–27], and the transplantation of amniotic membrane-derived cells has been experimentally applied to the treatment of lung fibrosis [28]. Recently, a preclinical study reported their use for regenerative therapy targeting central nervous system tissues [29]. It is also known that the amniotic epithelium produces anti-inflammatory and growth factors that are beneficial for the treatment of inflammatory corneal diseases [30]. Taken together, these observations explain the increasing attention that the human amniotic membrane has received due to its anti-scarring, anti-inflammatory, and wound-healing properties, as well as its multipotent differentiation ability and immunomodulatory features [31].

In conclusion, the placenta has several advantages as the cellular source for devising novel cellular and gene therapies, especially for muscle disorders. The placenta can be obtained non-invasively, the amniotic membrane has been shown to be an immune-privileged tissue and*MYOD1*-transduced amnion-derived cells are capable of the dystrophin expression needed for myogenic differentiation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.braindev.2012.05.012.

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