

Myoblasts Derived From Normal hESCs and Dystrophic hiPSCs Efficiently Fuse With Existing Muscle Fibers Following Transplantation

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Human embryonic stem cells (hESCs) and human-induced pluripotent stem cells (hiPSCs) have an endless self-renewal capacity and can theoretically differentiate into all types of lineages. They thus represent an unlimited source of cells for therapies of regenerative diseases, such as Duchenne muscular dystrophy (DMD), and for tissue repair in specific medical fields. However, at the moment, the low number of efficient specific lineage differentiation protocols compromises their use in regenerative medicine. We developed a two-step procedure to differentiate hESCs and dystrophic hiPSCs in myogenic cells. The first step was a culture in a myogenic medium and the second step an infection with an adenovirus expressing the myogenic master gene MyoD. Following infection, the cells expressed several myogenic markers and formed abundant multinucleated myotubes *in vitro*. When transplanted in the muscle of Rag/*mdx* mice, these cells participated in muscle regeneration by fusing very well with existing muscle fibers. Our findings provide an effective method that will permit to use hESCs or hiPSCs for preclinical studies in muscle repair.

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INTRODUCTION

Human embryonic stem cells (hESCs) have for all practical purposes, an unlimited capacity for self-renewal and maintain in culture their pluripotent capacity to differentiate in any type of cells found in the human body.¹ For these reasons, they have a tremendous potential to treat injuries or degenerative diseases.² A recent development, with a profound importance for the development of therapies, has been the generation of induced pluripotent stem cells (iPSCs) from somatic cells. Human iPSCs (hiPSCs) provide a similar breadth of opportunities without some of the confounding ethical issues surrounding hESCs. Although these cells may

permit to avoid the requirement for a sustained immunosuppression, their karyotype instability and their teratogenic potential will have to be further investigated.

Cell transplantation is a potential treatment for Duchenne muscular dystrophy (DMD), a lethal X-linked disease due to the absence of dystrophin in myofibers and in several other tissues (brain, heart, smooth muscles, and blood vessels).³ Due to the considerable amount of myogenic cells required for a cell-based therapy of DMD,⁴ hESCs and hiPSCs represent promising avenues for the elaboration of such treatments.⁵ However, at the moment the use of these PSCs in regenerative medicine is compromised by the low number of efficient specific lineage differentiation protocols published.⁶ Many other studies showed that skeletal muscle cells can be derived from mouse ESCs (mESCs) and promising results have been obtained using a gene inducible system in mouse cells.⁷ Myogenic progenitors have also been induced with Pax3 from mESCs and engrafted after intramuscular or systemic transplantation into Frg1 mice.⁸ Mouse iPSCs were also induced to differentiate in myogenic cells with Pax7 and extensive engraftment was obtained in dystrophic mice, which was accompanied by improved contractility of treated muscles.⁹ Other research groups have also obtained myogenic cells from mESCs or mouse iPSCs without transfection, using instead selection with an antibody specific for myogenic cells.¹⁰ On the other hand, for human ESCs, only one protocol has been published. Indeed, Barberi *et al.* have developed a stroma-free induction system to derive engraftable skeletal myoblasts from hESCs.¹¹ However, their technique had a low differentiation rate and was time consuming. Since their publication in 2007, no major new advance has emerged.

The myogenic regulator factor MyoD controls skeletal myogenesis during the embryo development. This basic helix-loop-helix protein binds to thousands of DNA E boxes and induces the expression of muscle-specific genes.¹² Previous studies have shown that MyoD overexpression in different cell types, such as human fibroblasts,¹³ human adult stem cells,¹⁴ and mESCs,¹⁵ is sufficient to induce skeletal muscle differentiation. However, no

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study has yet shown that the expression of MyoD in undifferentiated hESCs and hiPSCs can promote their differentiation in the skeletal myogenic lineage. Since MyoD regulates its own expression, its transitory expression is sufficient to induce the myogenic differentiation of hESCs.¹⁶ Our initial experiments indicated that the direct myogenic differentiation of hESC colonies following an infection with an adenoviral MyoD vector was not very efficient. We thus developed a rapid and effective two-step procedure. The first step was to transfer the hESCs in a myogenic medium, called MB1. This induced a mesenchymal-like differentiation (*i.e.*, formation of CD73⁺ cells as previously done by Barberi *et al.*).¹¹ The second step was an infection of the MB1-hESCs with an adenovirus expressing MyoD under the ubiquitous promoter CAG (Ad.CAG-MyoD). Our results indicate that with this protocol, the hESCs differentiated in cells expressing genes and proteins specific of muscle precursor cells. The conversion was so effective that a selection of the myogenic cells was not necessary to obtain very good fusion not only *in vitro* but also with the host muscle fibers following intramuscular injections and to avoid the formation of teratomas by the nonmyogenic cells.

Our procedure also permitted to convert hiPSCs obtained from a DMD patient in myogenic cells, which formed abundant muscle fibers expressing human spectrin following their transplantation in immunodeficient Rag/*mdx* mice.

RESULTS

Characterization of the hESC culture

After a period of mechanical and enzymatic passages, the differentiated cells of the hESCs H9 cell line were eliminated and the culture showed characteristics of undifferentiated hESCs. The colonies showed typical characteristics of hESCs grown on matrigel, *i.e.*, well-definite boundary, a high nucleus/cytoplasm ratio and single layer growth (Figure 1a). A fluorescence-activated cell sorting (FACS) analysis of the SSEA4, which is specific to the embryonic stage, confirmed the undifferentiated state of our H9 culture with almost 95% of the cells staining positive for this specific antigen (Figure 1c). This result was confirmed by immunohistochemistry (Figure 1b,d). At this stage, in contrast to myoblasts (Figure 1f), the myogenic marker, myosin heavy chain (MHC) was not expressed in the hESCs (Figure 1e). It is with H9 cells expressing these characteristics that the subsequent experiments were performed.

The myogenic differentiation of hESC colonies is low

We initially infected hESC colonies with the adenovirus Ad.CAG-MyoD containing a mouse MyoD gene under a cytomegalovirus early enhancer/chicken β -actin promoter. The exact multiplicity of infection (MOI) could not be established, thus 1×10^5 – 1×10^8 viral particles were used per well of a 24 wells plate. An adenovirus (Ad.CAG-GFP) coding for green fluorescent protein (GFP) under the same promoter was used as a negative control. As soon as 24 hours after the infection, evidences of differentiation were observed regardless of the viral concentration and the type of virus. This could be due to the change of culture medium at the time of viral infection. Colonies started to lose their definite boundaries and the cytoplasm of the cells expanded (Figure 2a). However, an immunostaining performed 5 days after the infection revealed

that only a few cells of the colonies were infected by the Ad.CAG-MyoD and expressed the MyoD transgene (Figure 2b). Brookman *et al.* have already demonstrated that the H9 hESC line that we have used can be infected by an adenovirus coding for GFP.¹⁷ This poor infection rate resulted in an inefficient myogenic conversion of the hESCs based on the expression of desmin, another myogenic marker. In fact, the best results were obtained with the highest viral concentration and the conversion rate to myogenic cells was below 1% based on desmin expression (Figure 2c). However, the desmin-positive cells were only observed when the cells were infected with the Ad.CAG-MyoD and no positive cell was observed in the negative control.

Since our results demonstrated that MyoD expression induced the myogenic differentiation of some hESCs but that colonies limited the infection rate, we infected hESCs grown as single cells with different MOI. For this, we modified the culture conditions before infection with the Ad.CAG-MyoD by adding a Rho kinase (ROCK)-inhibitor to prevent the cell death by anoikis (Supplementary Figure S1 and Supplementary Figure S2a,b). This cell death was due to the fact that the hESCs had less cell/cell contacts, however, despite the presence of that drug, the cell death was still elevated and very few myogenic cells were obtained with this protocol.

Differentiation of the hESCs to mesenchymal-like lineage in a myogenic culture medium (MB1)

We developed a simple culture system to induce mesenchymal-like differentiation of the hESCs. This second protocol was to grow hESCs in a myogenic culture medium (MB1), currently used to proliferate human myoblasts for our clinical transplantations.¹⁸ Undifferentiated hESC colonies were thus transferred from their mTeSR medium to the MB1 medium containing 15% fetal bovine serum (FBS) serum. Following two passages in culture, changes in the cell morphology were observed. These MB1-hESCs cells (Figure 3c) exhibited a flat spindle-like morphology more similar to that of myogenic cells (Figure 3d) than to hESCs (Figure 3a,b). Immunocytochemistry confirmed the loss of the SSEA4 embryonic marker in MB1-hESCs (Supplementary Figure S3a,b). However, the myogenic marker, MHC, was not observed even after 2 weeks in this differentiation medium (Supplementary Figure S3c,d). For a therapeutic application, it is essential to have an unlimited source of cells, thus to verify whether these MB1-hESCs were growing as well as real human myoblasts, their proliferation was studied in MB1 (Figure 3e). We observed that the MB1-hESCs were proliferating even better than the myoblasts. Indeed, the total number of passage three myoblasts derived from an adult donor doubled in 27 hours in MB1 whereas the number of MB1-hESCs doubled in only 16.1 hours.

Expression of genes characteristic of hESCs and of myogenic cells

To induce their myogenic differentiation, the MB1-hESCs were infected with an adenovirus coding for MyoD (Ad.CAG-MyoD). Reverse transcription-PCR (RT-PCR) was used to analyze the changes in gene expression at different steps of our protocol: (i) following the transfer of the hESCs from the mTeSR medium to the MB1 medium with 15% FBS serum (*i.e.*, MB1-hESCs), (ii) following their subsequent infection with the Ad.CAG-MyoD

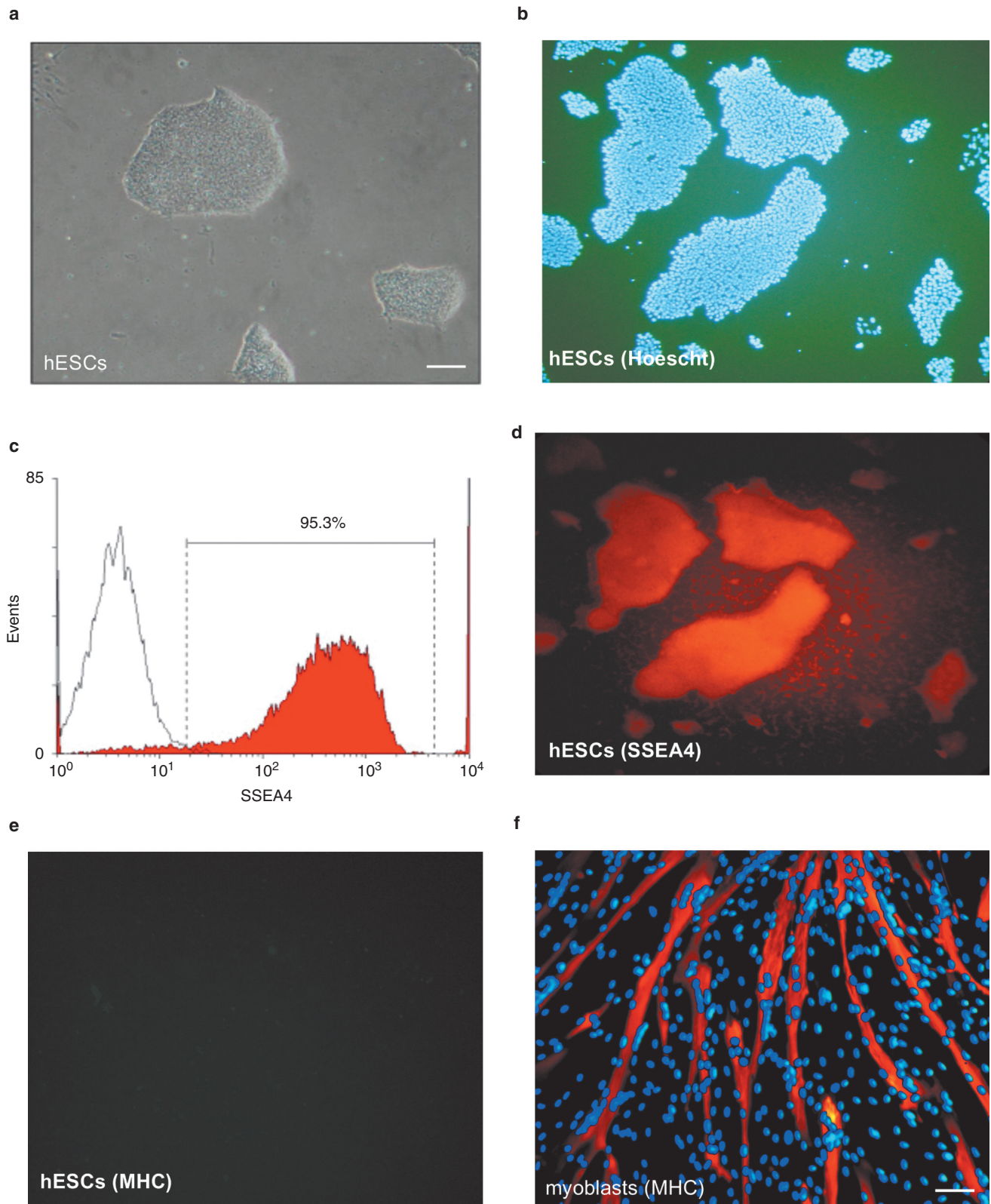


Figure 1 Characteristics of the H9 hESCs. **(a)** The H9 cell colonies had the morphological characteristics of undifferentiated hESCs with defined borders. **(b)** Hoescht staining of hESC colonies. **(c)** The results of the SSEA4 FACS analysis indicated that 95% of the H9 cells were in an undifferentiated state confirming the morphological observations. **(d)** The expression of SSEA4 in the colonies was confirmed by immunohistochemistry. **(e)** No myosin heavy chain (MHC) expression was detected by immunocytochemistry. **(f)** MHC immunocytochemistry of positive control (myoblasts after 4 days in differentiation medium). Bars are respectively 400 μ m in **a** and 120 μ m in **b,d,e**, and **f**. FACS, fluorescence-activated cell sorting; hESC, human embryonic stem cell.

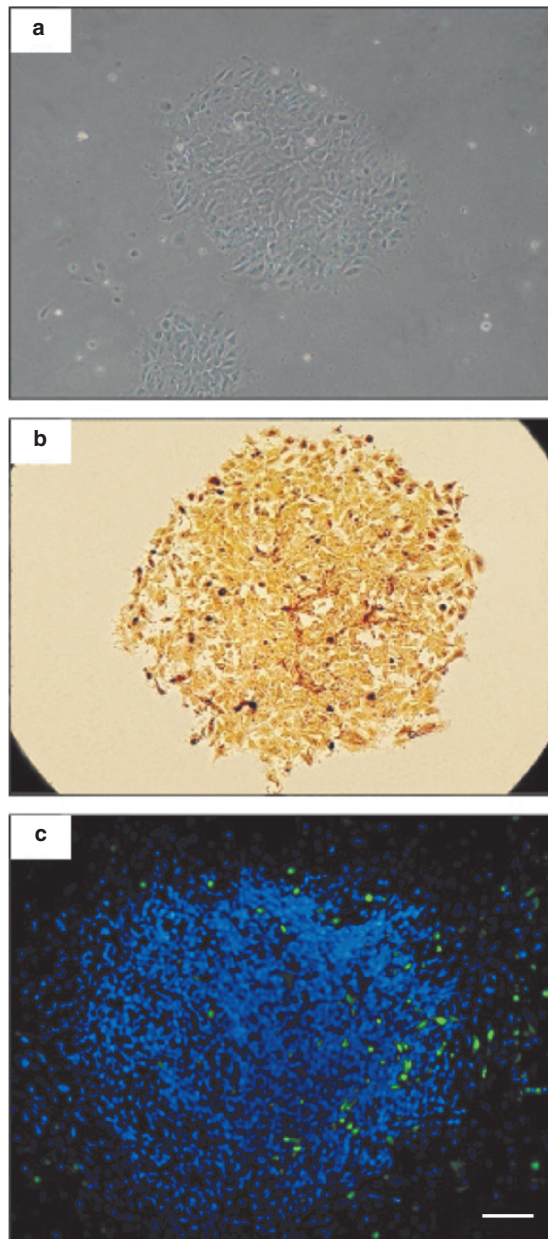


Figure 2 Colony infection. **(a)** Morphological changes of the hESCs were observed 24 hours after the infection regardless of the transgene present in the adenovirus. This change may be attributed to the use of a medium, which did not contain the growth factors necessary for the maintenance of the undifferentiated state. **(b)** Five days following an Ad.CAG-MyoD infection, only a few cells of the colony stained positive for MyoD. **(c)** The number of cells entering the myogenic pathway, based on the desmin staining in green, was even lower than the amount of MyoD positive cells at the same viral concentration as in **b**. However, these desmin-positive cells were only observed when the hESCs were infected with the Ad.CAG-MyoD and none were detected in the Ad.CAG-GFP control. The 200 μ m bar applies to **a**, **b**, and **c**. GFP, green fluorescent protein; hESC, human embryonic stem cell.

(*i.e.*, MB1-MyoD-hESCs), and (iii) following their transfer from the proliferating conditions to a differentiation medium (Dulbecco's modified Eagle's medium (DMEM) with 2% FBS) (Figure 4). RNA was thus collected before and after the MB1 transfer, 3 days after their infections and at days 1, 3, and 5 after

their transfer in the differentiation medium. The undifferentiated specific gene *Rex-1* considerably decreased from hESCs to MB1-hESCs but was still expressed at a very low level following the MyoD infection. As soon as hESCs were transferred in MB1, we also observed the expression of paraxial mesoderm, *TBX4* and *TBX1*, which regulates *Myf5* and *MyoD*. The expression of these markers was further increased after MyoD infection. *TBX4* is important for the development of the limb buds.¹⁹ The premyogenic specification marker *TBX1* is expressed in the premyogenic mesoderm of the first and second branchial arch before the onset of myogenic regulator factor expression.²⁰ The RT-PCR analysis also indicated that the infection with Ad.CAG-MyoD coding for a mouse MyoD gene was sufficient to induce the expression of several human myogenic genes. The MB1-MyoD-hESCs expressed the endogenous, *i.e.*, human, transcription factor MyoD as confirmed by RT-PCR using primers specific for the human mRNA. In addition, we observed the presence of human myogenin and of MHC, which are late myoblast markers that are expressed during the terminal differentiation into myotubes.²¹ Expression of these myogenic genes in MB1-MyoD-hESCs can be directly linked to the MyoD expression since these genes were not expressed in the hESCs control and in the MB1-hESCs. However, compared with the real human myoblasts, the MB1-MyoD-hESCs did not express the transcription factor *Myf5*. These data illustrate the developmental progression of hESCs toward myogenic lineage through a transient mesodermal stage.

Myogenic conversion of MB1-hESCs

We have shown by RT-PCR that MB1-hESCs expressed the mesodermal genes *TBX4* and *TBX1*. To verify a potential mesenchymal-like differentiation, we first verified the expression of the surface antigen CD73 by FACS at around day 3 of culture in MB1 medium (Figure 5a). CD73⁺ is expressed by mesenchymal multipotent precursors, which can be induced to differentiate in bone, cartilage, fat, and skeletal muscle cells.¹¹ Before the infection with Ad.CAG-MyoD, <6% of the CD73-positive MB1-hESCs expressed a high level of CD56, a myoblast marker (Figure 5b). However, the infection of the MB1-hESCs with Ad.CAG-MyoD induced their conversion in cells expressing CD56 (31% of the MB1-MyoD-hESCs were CD56^{high} and an additional 44% were CD56^{low}). Thereafter, the fusion potential of the MB1-MyoD-hESCs was verified by transferring them in the differentiation medium. Most of these cells expressed MHC and formed large multinucleated myotubes containing up to 20 nuclei (Figure 6a–d); 60% of cells were MHC positive after 7 days in differentiation medium (Figure 6f). Moreover, the percentage of MHC-positive cells was not significantly different than that observed with real human myoblast primary cultures. Thus our two-step procedure induced a high myogenic conversion of hESCs. The karyotypes of MB1-hESCs and MB1-MyoD-hESCs did not show abnormalities (Supplementary Figure S2c,d).

Myogenic conversion of hiPSCs

Having established a two-step procedure for the myogenic conversion of hESCs, we applied exactly the same differentiation protocol to hiPSCs derived from a DMD patient skin fibroblast. These hiPSCs were transferred to MB1 medium, infected with Ad.CAG-MyoD and

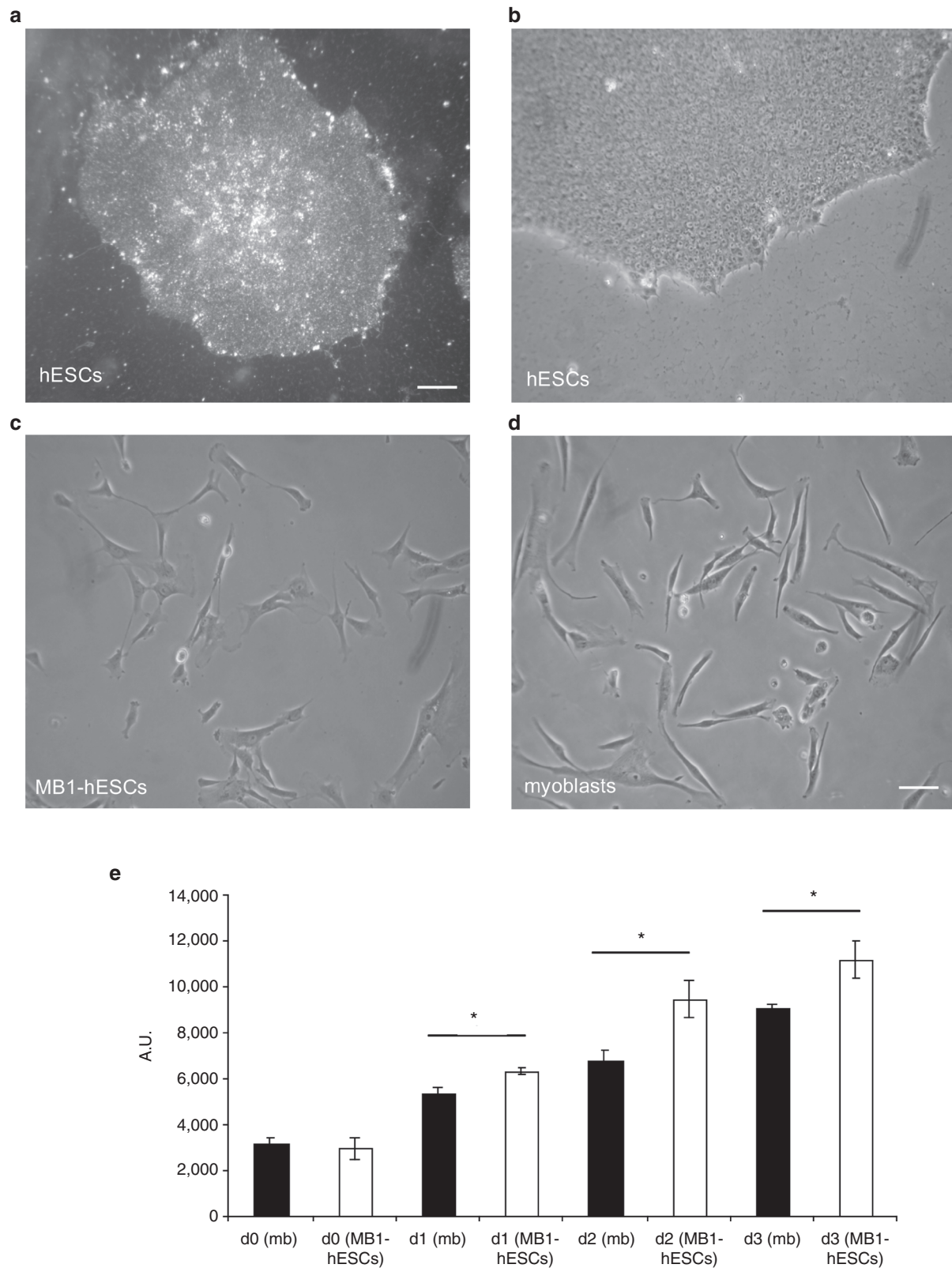
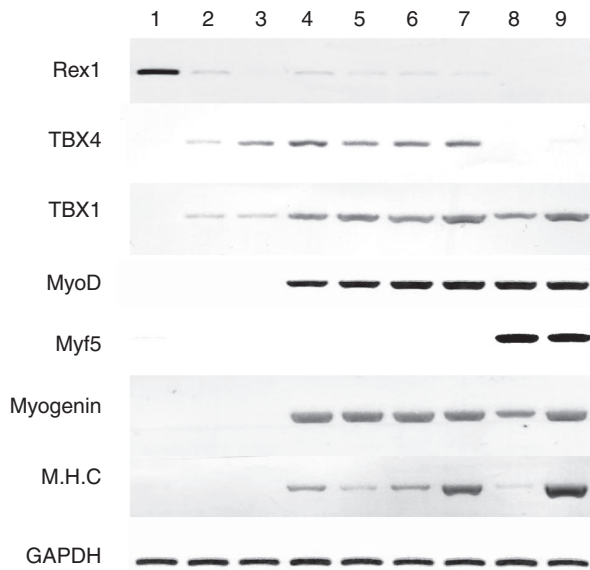


Figure 3 Changes in the human embryonic stem cell (hESC) morphology after culture in MB1 medium. **(a,b)** Illustration of hESCs. **(c)** The hESC morphology changed following their culture in a myogenic medium (MB1) for one passage. **(d)** Illustration of myoblasts. Note that the MB1-hESCs have a flat spindle morphology more similar to that of myoblasts. **(e)** The fluorescence intensity of the CyQUANT GR dye was measured at different times (0, 1, 2, 3 days) to evaluate the proliferation in MB1 medium. An increased proliferation of MB1-hESCs compared with myoblasts was observed at all time intervals. *Indicate statistically different results, $n = 3$, $P < 0.05$. Bars are respectively 300 μm in **a**, 60 μm in **b,c**, and **d**. A.U. arbitrary unit.



1. hESCs in mTesR
2. MB1-hESCs
3. MB1-hESCs, first passage without matrigel
4. MB1-MyoD-hESCs, day 3 in proliferation
5. MB1-MyoD-hESCs, day 1 in differentiation
6. MB1-MyoD-hESCs, day 3 in differentiation
7. MB1-MyoD-hESCs, day 5 in differentiation
8. Myoblasts in proliferation
9. Myoblasts in differentiation

Figure 4 RT-PCR after culture of hESC in MB1 medium and infection with MyoD. The infection of MB1-hESCs with the Ad.CAG-MyoD induced the expression of diverse myogenic genes. Three days following the infection, the MB1-MyoD-hESCs expressed some early and late myogenic genes (MyoD, myogenin, and MHC) but not the transcription factor Myf5. A diminution of embryonic gene expression (Rex1) was observed in the MB1 medium compared with the undifferentiated hESCs. RT-PCR shows a time-dependant, sequential differentiation of hESCs into myogenic cells with a mesenchymal step (Tbx1 and Tbx4). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; hESC, human embryonic stem cell; MHC, myosin heavy chain; RT-PCR, reverse transcription-PCR.

transferred to the DMEM medium with 2% FBS. We investigated the *in vitro* differentiation by MHC expression. As expected, 60% of the MB1-MyoD-hiPSCs became MHC positive as observed with MB1-MyoD-hESCs and no significant difference was observed with real human myoblasts (Figure 6f). However, despite the fact that percentage of MHC was the same, larger myotubes were observed with dystrophic MB1-MyoD-hiPSCs than with MB1-MyoD-hESCs (Figure 6e). Moreover, the karyotypes of MB1-hiPSCs and MB1-MyoD-hiPSCs were normal (Supplementary Figure S2e,f).

MB1-MyoD-hESCs and dystrophic MB1-MyoD-hiPSCs transplanted in Rag/mdx mice formed hybrid muscle fibers

First transplantation experiment without cardiotoxin. As MyoD expression confers to hESCs and to dystrophic hiPSCs the interesting myogenic capacity observed *in vitro*, we speculated that these infected cells might participate in muscle regeneration more efficiently than noninfected cells. We initially tested this hypothesis

by transplanting 500,000 MB1-MyoD-hESCs, 500,000 dystrophic MB1-MyoD-hiPSCs, and 500,000 human myoblasts in the muscles each in two rag/mdx mice. The muscles injected with cells were collected 4 weeks later. The presence of hybrid fibers resulting from the fusion of the human cells with the mouse fibers was investigated by the expression of human spectrin, a gene specifically expressed in the muscle fibers. Human spectrin was clearly detected at the membrane of many fibers but only following the transplantation of human myoblasts, MB1-MyoD-hESCs (Figure 7a) or MB1-MyoD-hiPSCs (Figure 7b). The presence of MB1-MyoD-hESCs-derived muscle fibers was further confirmed by the co-labeling of most of the human spectrin-positive fibers with human-specific anti-dystrophin (Figure 7c). The muscle fibers expressing human spectrin or human dystrophin were often disposed along a more or less linear regions probably close to the injection trajectories, because myogenic cells do not migrate more than a few microns away from the injection trajectories.²² These fibers ranged from very small to large diameters. The proportion of large fibers was variable. We have often made the same observation following the transplantation of primary culture myoblasts.

Second transplantation experiment with cardiotoxin. The transplantation success, however, was restricted by the fact that the implanted cells fused essentially with the myofibers near the injection trajectories, and this could be due to the low number of spontaneously regenerating myofibers present at the time of transplantation in mdx mice. Despite its genetic and biochemical homology to DMD,²³ the mdx mouse has indeed limitations as a model of this disease. We thus further tested the *in vivo* myogenic capacity of the cells derived from hESCs and hiPSCs by transplanting different types of cells (*i.e.*, hESCs, MB1-hESCs, MB1-MyoD-hESCs, dystrophic hiPSCs, MB1-hiPSCs, and MB1-MyoD-hiPSCs) in the tibialis anterior (TA) muscles of immunodeficient Rag/mdx mice. For this second experiment, each type of cells was co-injected with cardiotoxin in seven muscles. The cardiotoxin was used to damage the muscle fibers of the host mice and thus permit the fusion of the transplanted cells with more host muscle fibers. Human myoblasts were again used as a positive control. The muscles injected with cells were also collected 4 weeks later. As for the previous experiment, the success of these transplantations was determined by immunolabeling the muscle cross-section for human spectrin. As much as 500 spectrin-positive fibers were observed in TA grafted either with MB1-MyoD-hESCs or with MB1-MyoD-hiPSCs (Figure 7d).

Human spectrin labeling was used to quantify the success of the transplantation (Figure 7e). The total number of human spectrin-positive fibers was definitively higher following the transplantation of MB1-MyoD-hESCs than with wild-type hESCs or MB1-hESCs. Surprisingly, the total number of human spectrin-positive fibers was higher with MB1-MyoD-hiPSCs than with MB1-MyoD-hESCs. Moreover, the total number of human spectrin-positive myofibers was respectively 37 and 74% higher with MB1-MyoD-hESCs ($P = 0.086$) and MB1-MyoD-hiPSCs ($P < 0.01$) than in muscles injected with the same number of control human myoblasts.

Immunolabeling of the muscle section for human lamin A/C. Human nuclei were identified in the sections of muscles

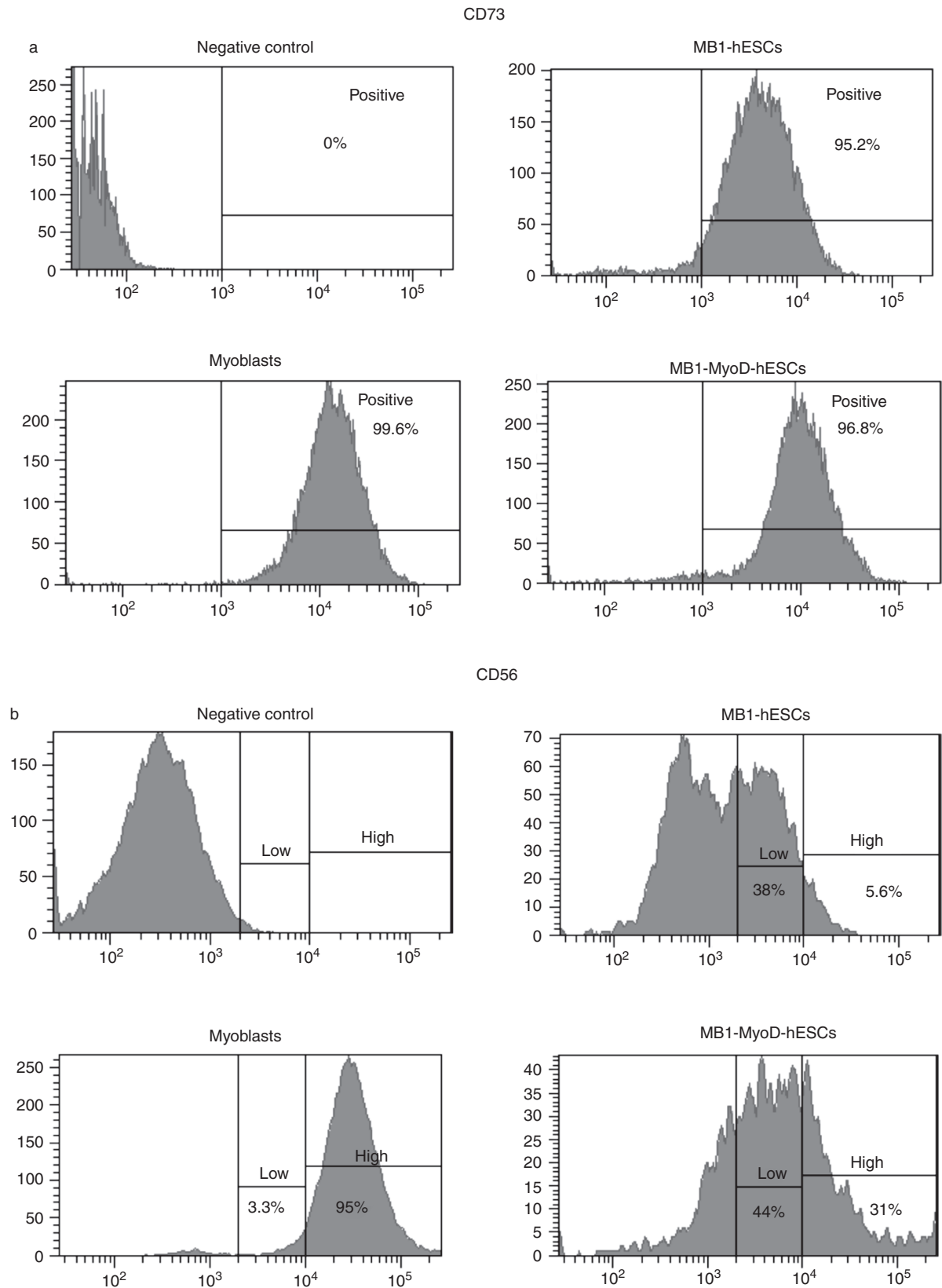


Figure 5 FACS analysis of hESC-derived mesenchymal-like precursors generated by culture in MB1. **(a)** The culture of hESCs in the MB1 culture medium (MB1-hESCs) induced their differentiation in mesenchymal-like stem cells expressing CD73. **(b)** However, in the MB-1 medium, <6% of these CD73 cells also expressed CD56. The infection of these CD73 cells with Ad.CAG-MyoD induced their conversion in cells called MB1-MyoD-hESCs expressing high CD56 level (31%). FACS, fluorescence-activated cell sorting; hESC, human embryonic stem cell.

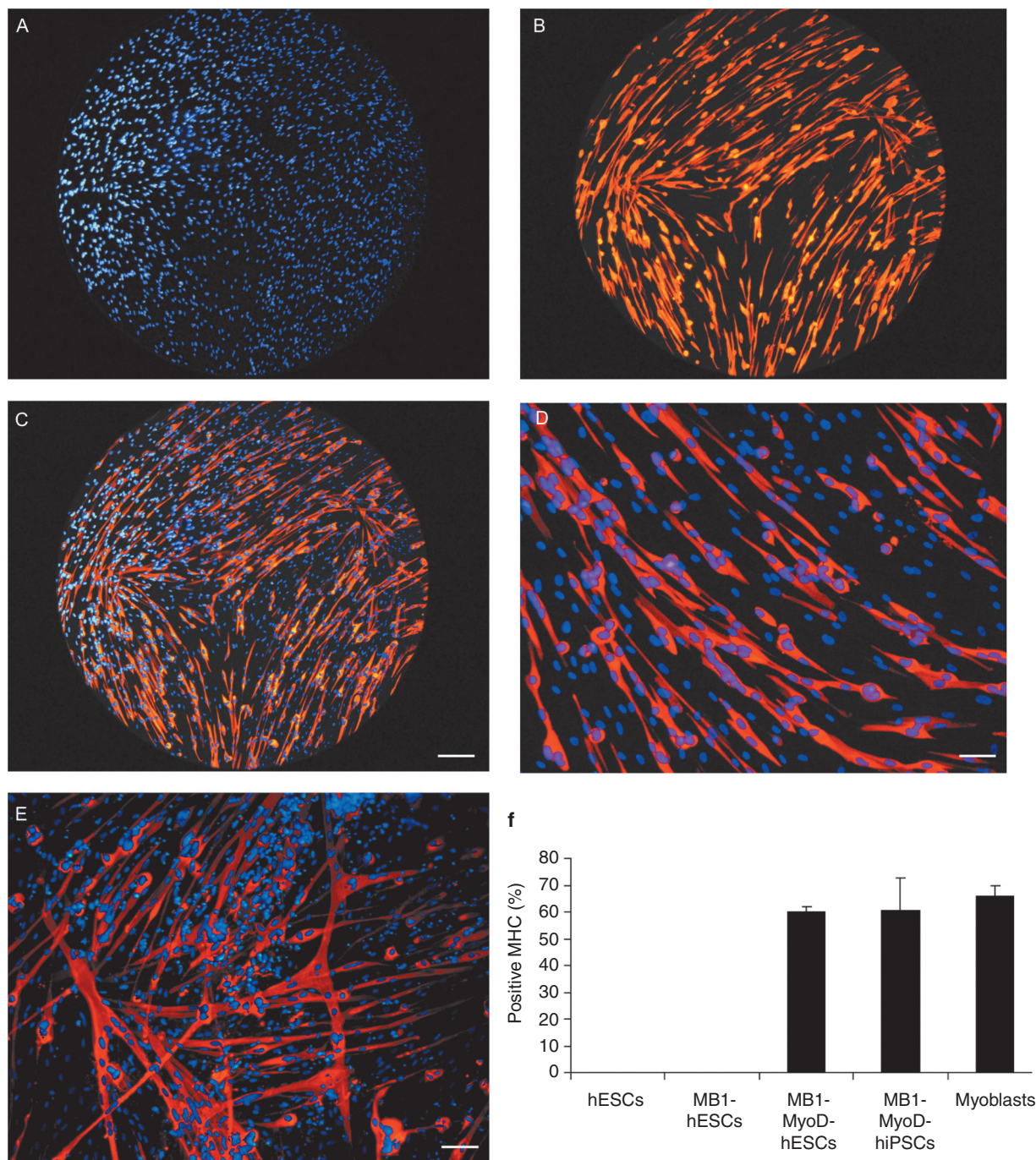


Figure 6 *In vitro* terminal differentiation of MB1-MyoD-hESCs and dystrophic MB1-MyoD-hiPSCs. The MHC immunochemistry in red showed that when cultured in MB1 medium and infected with Ad.CAG-MyoD, (**a-d**) the MB1-MyoD-hESCs and (**e**) the dystrophic MB1-MyoD-hiPSCs acquired skeletal muscle cell properties and fused to form multinucleated myotubes in low-serum condition. The fusion was equal to that of myoblast primary culture. Roughly 60% of the cells expressed MHCs and most of the cells were differentiated in myotubes, some of them containing up to 20 nuclei. (**f**) There was no significant difference between MB1-MyoD-hESCs, MB1-MyoD-hiPSCs, and myoblasts. The bar of 400 μ m in **c** also applies to **a** and **b**. The bar is 30 μ m in **d** and 150 μ m in **e**. hESC, human embryonic stem cell; hiPSC, human-induced pluripotent stem cell; MHC, myosin heavy chain.

transplanted with cells in the two experiments above by immunostaining for human lamin A/C (**Figure 8**). Following the transplantation of MB1-MyoD-hESCs, abundant human nuclei were located inside the muscle fibers expressing human dystrophin. Some human nuclei were outside the muscle fibers in a position similar to that of satellite cells. In fact, we have previously demonstrated that human myoblasts transplanted in mouse

muscles formed satellite cells.²⁴ This observation suggests that myogenic cells derived from hESCs may also form satellite cells (this remains to be proven in subsequent work). A few MB1-MyoD-hESCs cells were also located away from the muscle fibers (as observed when we transplant human myoblasts derived from primary muscle culture). We have no indication as to the nature of these cells.

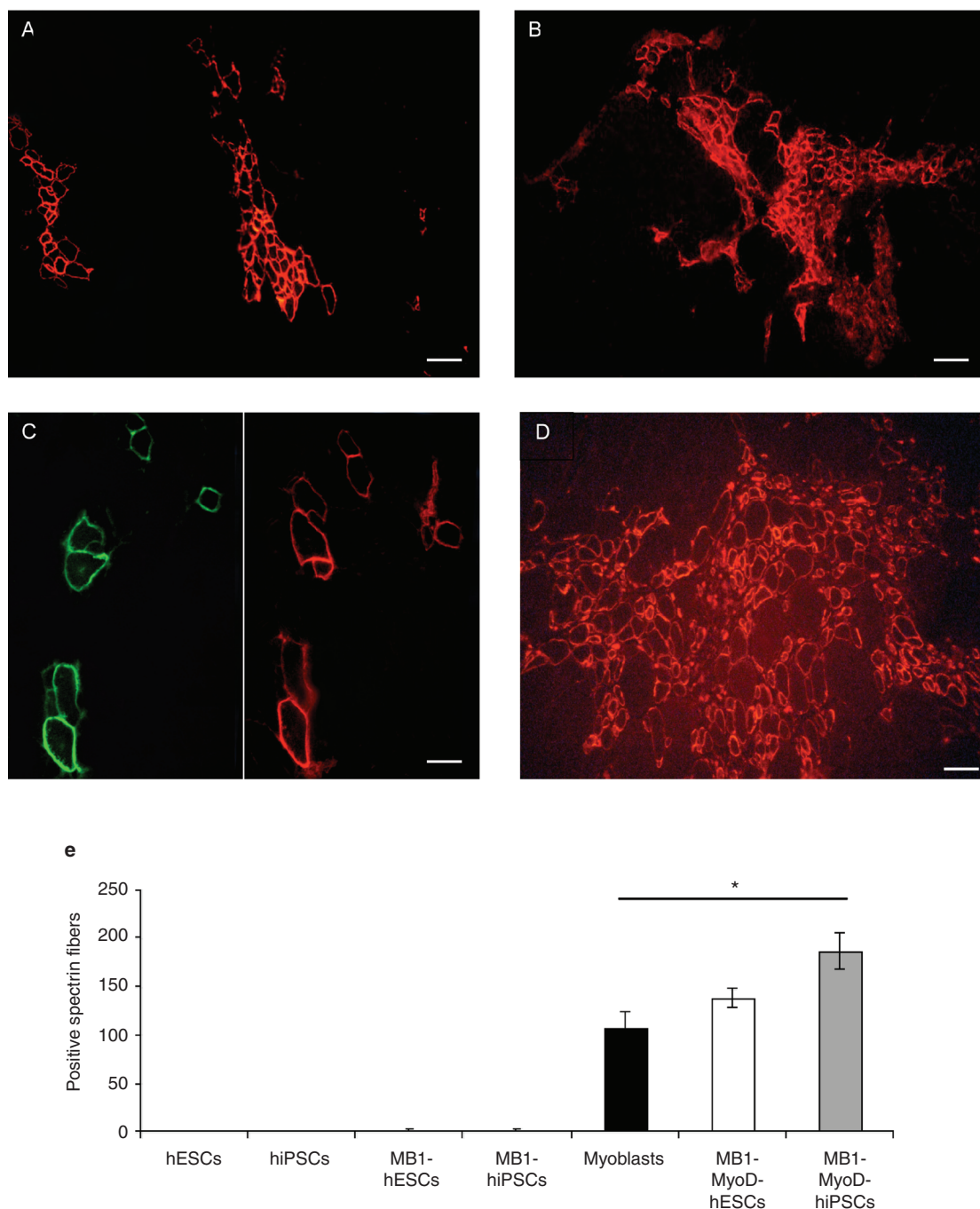


Figure 7 Immunofluorescence showing human spectrin (or dystrophin) positive myofibers after transplantation in *Rag/mdx* mice. Representative cross-sections of *Rag/mdx* TA muscles injected intramuscularly with (a) MB1-MyoD-hESCs or (b) dystrophic MB1-MyoD-hiPSCs and immunostained for human spectrin (in red). (c) The presence of MB1-MyoD-hESCs–derived muscle fibers was further confirmed by the co-labeling of most of the human spectrin-positive fibers in red with human-specific anti-dystrophin in green. (d) Representative cross-sections of *Rag/mdx* TA muscles injected intramuscularly with 0.5 million MB1-MyoD-hESCs and cardiotoxin at several sites (10–15) throughout each muscle. (e) The number of human spectrin-positive muscle fibers per muscle section was counted for the three best sections of each grafted muscle. *Indicates statistically different results between the muscles grafted with myoblasts and those grafted with MB1-MyoD-hiPSCs ($n = 7$, $P < 0.05$). Bars are respectively 120 μm in a, b, and d and 40 μm in c. hESC, human embryonic stem cell; hiPSC, human-induced pluripotent stem cell; TA, tibialis anterior.

Absence of teratoma in the muscles transplanted with hESC and hiPSC or with hESC- and hiPSC-derived cells

It is important to note that no teratoma and no abnormal structure were detected in any of the muscles both in the first and in the second series of transplantation. In addition, following immunolabeling

for human lamin A/C, we did not observe any human nuclei in the sections of muscles transplanted with any type of cells. This suggests that PSCs did not survive either to the transplantation procedure. Indeed, the hESCs and hiPSCs may be sensitive to the pressure used for the intramuscular injection, to cardiotoxin or to the highly

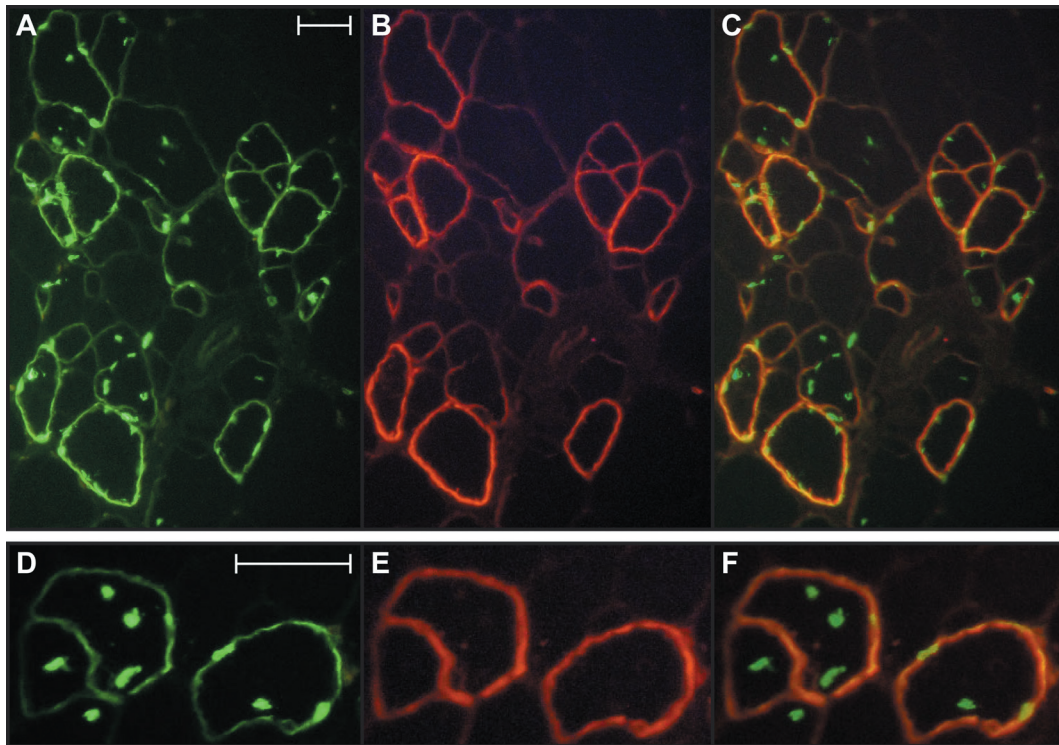


Figure 8 Lamin A/C staining of muscles. Sections of rag/mdx muscles transplanted with MB1-MyoD-hESCs were immunostained with an antibody to (a,d) human lamin A/C (fluorescence green in a and d) and (b,e) for human dystrophin (red fluorescence in b and e). (c,f) The green and red fluorescence were superposed. The figure clearly illustrates that abundant human nuclei were present inside the dystrophin-positive muscle fibers and in close apposition to the muscle fibers. The bars are 50 μm , the bar in a also applies to b and c while the bar in d also applies to e and f. hESC, human embryonic stem cell.

inflammatory environment produced by the damage to the muscle fibers induced by cardiotoxin. An additional hypothesis to account for the absence of teratoma following the transplantation of cells grown in MB1 is that the PSCs differentiated in the MB1 medium, but were not able to survive as pluripotent cells in this medium.

DISCUSSION

In the last 20 years, several different types of cells have been tested for potential clinical applications in muscle pathologies. Rationally in the first study, the cells originating from the skeletal muscle itself, *i.e.*, myoblasts obtained by the proliferation of satellite cells, were transplanted in several clinical trials.²⁵ Mesenchymal stem cells due to their multipotency were also candidates for cell therapy of muscle diseases but their participation to muscle regeneration was rather low.²⁶ hESCs, unlike mesenchymal stem cells, have, for all practical purposes, an unlimited capacity for self-renewal and maintain in culture their pluripotent capacity to differentiate into cell types from all three germ layers. The ability of ESCs to produce a theoretically unlimited supply of cells has focused attention on their use in cellular therapy and *ex vivo* gene therapy.

Advantages and problems of using a MyoD adenovirus

The direct induction of differentiation *via* gene transfer has been shown to be an efficient technique which allowed almost 100% of hESC differentiation under certain conditions.²⁷ Although adenovirus do not integrate the MyoD transgene in the cell genome,

our two-step protocol combining a myogenic culture medium (MB1) and the temporary expression of the myogenic master gene MyoD is sufficient to induce the skeletal myogenesis of hESCs. The initial low infection level of the hESC colonies was possibly due to their compact agglomeration and/or to the presence of an extracellular matrix limiting viral access. To verify the hypothesis that the accessibility of the cells was the factor limiting the viral infection was tested on hESCs grown as single cells. This modification permitted us to use a reproducible MOI. Cell death was the main problem when the hESCs were grown as single cells even when a ROCK inhibitor was used. For this reason, we developed a simple culture system to induce mesenchymal-like differentiation of the hESCs based on selective myogenic culture conditions. Undifferentiated hESCs were thus transferred from the mTeSR1 medium to the MB1 medium before infection with the MyoD adenovirus. Myogenic cell conversion by MyoD was initially reported almost 20 years ago in several differentiated cells.¹³ The excellent myotube formation of MB1-MyoD-hESCs (similar to that of primary culture myoblasts) but not of MB1-hESCs demonstrated that MyoD can also orchestrate a myogenic programming of cells derived from hESCs. Compared with other published techniques to differentiate hESCs in muscle cells,^{5,11,28} it is the first time that such a level of efficiency is obtained.

Gene expression by the hESC-derived cells

Based on the gene expression analysis, it is possible that the MB1-MyoD-hESCs are a mix population of early and late

skeletal myogenic cells. Indeed like myoblasts, MB1-MyoD-hESCs expressed endogenous MyoD, myogenin, which is present in the early stage of differentiation of myoblasts into myocytes,²¹ and the myotube marker, MHC. However, they did not express Myf5. Although both MyoD and Myf5 can activate quiescent satellite cells,²⁹ they play different roles during embryogenesis and are responsible for the formation of two different muscle lineages.³⁰ Therefore, only one of these transcription factors is required for skeletal myogenesis in mutant mice.³¹ Furthermore, MyoD is expressed upstream of Myf5³² and its overexpression has been shown to inhibit the expression of Myf5.³³ Similar result has been also observed with human adipose-derived stem cells infected with a MyoD lentivirus.¹⁴ However, unlike myoblasts, MB1-MyoD-hESCs expressed the paraxial mesoderm gene, *TBX4*, in MB1. This may be related to the presence of two populations of CD56⁺ cells (high and low). These two populations may behave differently *in vitro* and/or *in vivo*. The CD56^{high} population may resemble more closely to original myoblasts, which are also CD56^{high}. Another interesting result is that the mouse MyoD transgene present in the adenovirus upregulated the expression of the human MyoD gene. This result indicated that the murine transcription factor can regulate the endogenous gene as occurs during skeletal muscle development,³⁴ a phenomenon also observed in another study.¹⁴

Myogenic differentiation of hESC-derived cells *in vitro*

The differentiation potential of the MB1-MyoD-hESCs was tested *in vitro* in a low-serum medium. The medium change induced a morphological modification of the infected cells, which became more elongated. Immunocytochemistry revealed that 60% of the infected cells expressed MHC, a protein specific for myotubes. Staining also allowed the observation of myotubes containing up to 20 nuclei. These results indicated that the MB1-MyoD-hESCs do not only undergo myogenesis but that they are functional and can participate in the formation of myotubes. The fusion potential of the MB1-MyoD-hESCs was as high as that of primary culture of myoblasts.

hESC-derived cells participated very effectively to muscle regeneration *in vivo*

On the basis of the very positive results of the *in vitro* study, MB1-MyoD-hESCs cells were transplanted in regenerating muscles of immunodeficient mice. These cells were much more efficient than MB1-hESCs in participating to muscle repair. This result contrasts with those previously obtained by our research team several years ago with human dermal fibroblasts, which were converted to myogenic cells by the forced expression of MyoD.³⁵ Indeed these modified human fibroblasts integrated in mouse regenerating muscles but gave rise to only a few hybrid muscle fibers. MB1-MyoD-hESCs were clearly more efficient. Although, several parameters are different between the two experiments, MB1-hESCs may have a better plasticity than fibroblasts, indeed as undifferentiated cells they can enter different lineages, and consequently, they may develop a more complete myogenic differentiation than differentiated fibroblasts. This highlights the advantage of using genetically modified human multipotent cells over differentiated cells for muscle repair.

Advantages of using cells derived from hiPSCs

A development, with potentially a profound significance for clinical therapy has been the reprogramming of somatic cells in iPSCs.³⁶ The generation and use of iPSCs particularly for autologous stem cell therapy poses fewer ethical problems than the derivation and use of hESCs. Moreover, the main problem of myoblast or mesoangioblast transplantation is the immune response against the donor cells. This problem is currently controlled in clinical trials by a sustained immunosuppression with tacrolimus.³⁷ However, all immunosuppressive drugs have adverse effects and are associated with risks of cancer, infection, and nephrotoxicity. This emphasizes the need to correct the patient own cells to permit autologous transplantations. Unfortunately myoblasts obtained from DMD patients are already close to senescence. The reprogramming of the patient fibroblasts in iPSCs followed by their myogenic differentiation is thus an exciting new approach to obtain an unlimited amount of autologous muscle precursor cells. We are thus working to produce and to genetically correct iPSCs derived from DMD patients to avoid in clinical trials the requirement for immunosuppression. The genetic correction of iPSCs *in vitro* has the great advantage that the patients will not be directly exposed to mutagenic agents or to billions of viral vectors. The additional advantage of correcting iPSCs is that these cells have an indefinite proliferation capacity and thus the corrected cells can be cloned to verify that the genetic correction is adequate before their proliferation in large enough numbers for the cell therapy.

In our *in vitro* studies, the fusion potential of the MB1-MyoD-iPSCs was similar or even superior to that of primary culture of myoblasts. Surprisingly, *in vivo*, the total number of human-positive fibers was even higher with MB1-MyoD-hiPSCs than with MB1-MyoD-hESCs or control myoblasts. A recent article³⁸ reported that while pluripotent genes (*OCT4*, *SOX2*, *REX1*, and *NANOG*) were silenced immediately upon differentiation of hiPSCs, some other genes normally unique to early embryos were not fully silenced in hiPSC derivatives. It is thus possible that MB1-MyoD-hiPSCs are more immature than primary culture myoblasts and thus more able to proliferate and fuse with the host muscle fibers.

The potential immunogenicity problem of iPSCs

A recent article by Zhao *et al.*³⁹ reported that some tumor cells derived from iPSCs can be immunogenic even in syngeneic recipients. This article has since been used to question the possibility of using cells derived from autologous iPSCs for autologous transplantation without immunosuppression. However, this interpretation of Zhao's article goes beyond the author own conclusions. Indeed, Zhao *et al.* concluded that the immune rejection of the tumors derived from iPSCs was due to the overexpression of some genes (Hormad1 or Zg16) not expressed during normal development or differentiation of ESCs, leading to the break of peripheral tolerance. Although, the expression of these minor antigens could be due to the subtle yet apparent epigenetic difference between iPSCs and ESCs,⁴⁰ the genes inducing the rejection may not be overexpressed in nontumorigenic tissues, especially in muscle fibers. In addition, recently discovered mutations in iPSC genes could also contribute to the immunogenicity of iPSC derivatives.⁴¹ Therefore, for an eventual clinical application of cells derived from

iPSCs, current reprogramming technology needs to be optimized to minimize the epigenetic difference between iPSCs and ESCs. Moreover, the hiPSCs used in our experiments were derived from primary fibroblast cultures of adult tissue rather than from an embryonic fibroblast cell line as in the article of Zhao *et al.*³⁹ Thus the problem of the potential immune response of cells derived from an iPSC has to be further investigated in nontumorigenic tissues and from cells not derived from a cell line or cells derived from an embryo.

The potential tumorigenicity problem of hESC- and hiPSC-derived cells

Another important issue is the long-term tumorigenicity risks. The embryonic marker SSEA4 was absent in MB1-hESCs, this is a critical observation since this gene expressed at embryological stage is known to be tumorigenic.¹ However, a very low level of Rex-1 mRNA, another embryonic marker, was still observed in MB1-MyoD-hESCs, it is thus possible that a longer period of post-infection culture is required for the complete repression of that gene. It is also important to note that we did not observe any karyotype abnormality in the cells derived from hESCs or hiPSCs. We have nevertheless examined the mouse muscles transplanted with our various types of cells not only for the presence of muscle fibers expressing human genes, but also for the presence of tumors. No tumor was observed 1 month after transplantation with MB1-MyoD-hESCs or MB1-MyoD-hiPSCs. The chance of developing a tumor may be related to the total number of cells transplanted and to the injection site. Moreover, a recent article reported that mesenchymal stem cells derived from ESCs did not also produce teratoma during a 6 months follow-up.⁴² However, for an eventual clinical application, longer follow-up studies should be done not only in mice but also in monkeys.

Avoiding the use of viral vector

From a therapeutic point of view, another potential problem is the use of viral vectors to transduce the cells. The adenoviral vector has the advantage that it cannot produce mutations due to integration. Moreover, because MyoD is an anti-oncogene,⁴³ its expression should bring an additional security. However, the resulting myogenic cells were grafted to immunodeficient mice. In these mice, there is no immunogenic response raised not only against the human cells, but also against the viral antigens. Indeed adenoviral vectors are generally considered as highly immunogenic, as some antigens are still processed and expressed by host cells. For future experiments, nonviral vectors, MyoD mRNA or the MyoD protein coupled with a cell-penetrating peptide, such as Tat or Pep-1,⁴⁴ may be more suitable on the immunological point of view.

Previous procedure to induce the myogenic differentiation of hESCs

Barberi *et al.*¹¹ have previously reported a procedure to induce the differentiation of hESCs in myogenic cells. However, their procedure was very tedious compared with our procedure. Indeed, Barberi *et al.*¹¹ first cultivated the hESCs for 20 days in an insulin, transferrin, and selenium medium and then in α -minimum essential medium containing serum, for two additional weeks.

The cells then had to be sorted by FACS for expression of CD73. Only 2–10% of these cells expressed skeletal myoblast markers and thus they had to be further separated by FACS for NCAM. The resulting myoblasts formed only small myotubes *in vitro*. In contrast, in our procedure the hESCs or hiPSCs were grown for only 4–6 days in MB1 medium and infected with a MyoD adenovirus and transferred to the DMEM medium. Our procedure did not require any purification of the myogenic cells by FACS since 60% of the cells were MHC positive. Moreover, large abundant myotubes were obtained with the myogenic cells derived with our procedure.

Barberi *et al.*¹¹ studied the survival of the hESC-derived skeletal myoblasts by labeling them with luciferase. However, that article did not provide good evidence that the hESCs-derived cells really fused with the muscle fibers of the host mice. Indeed **Figure 6g** of Barberi *et al.* only illustrated that human nuclei (identified by human nuclear antigen) were present outside the muscle fibers. **Figure 6h** of Barberi *et al.* illustrated that an extracellular human protein (*i.e.*, laminin) was present. On the contrary, our **Figures 7** and **8** clearly illustrate the presence of many muscle fibers expressing human spectrin and human dystrophin. These proteins are expressed only in muscle fibers and therefore these figures clearly prove that the myogenic cells derived from both hESCs and hiPSCs by our procedure did fuse with the host muscle fibers, whereas there is no such demonstration was provided by Barberi *et al.*¹¹ Since the aim of using myogenic cells derived from both hESCs and hiPSCs is to develop a therapeutic application for muscular dystrophies, our demonstration of significant participation to muscle repair is very important.

Conclusions

There are many additional experiments that remain to be done before such a cell therapy approach can be considered for a clinical application. A non-immunogenic method for inducing MyoD expression has to be developed, early mesoderm markers such as Brachiury and Meox should be investigated, upstream genes such as Pax3 and Pax7 have to be tested, detection of human cells with anti-lamin A/C may indicate if the cells are surviving better, longer term evaluation of the absence of tumors have to be done. However, our results clearly demonstrated that hESCs and hiPSCs have been efficiently committed to myogenic lineage by the use of myogenic medium combined with the forced expression of MyoD. An important finding is that the resulting myogenic cells efficiently formed hybrid muscle fibers following intramuscular transplantation. Such an effective muscle transplantation of MPCs derived from human iPSCs has never been reported before.

MATERIALS AND METHODS

Reagents. The reagents were purchased from the following companies: FBS from Biomedica (Drummondville, Québec, Canada); penicillin/streptomycin, trypsin from Gibco (Burlington, Ontario, Canada); matrigel from BD Biosciences (Mississauga, Ontario, Canada); mTeSR1 media and dispase from Stem Cell Technologies (Vancouver, British Columbia, Canada); MB1 medium from Hyclone (Logan, UT), random primers, GoTaq, Oligo(dT), and RNasin from Promega (Madison, WI); mouse monoclonal anti- β chain of spectrin (NCLSPEC1) antibody from Novocastra (Newcastle upon Tyne, UK); mouse anti-MyoD (CS-304) from Santa

Cruz Biotechnology (Santa Cruz, CA); anti-human MHC (Mab 4470) from R&D System (Minneapolis, MN); mouse anti-human desmin (clone D33, cat. no. M0760) from DAKO (Burlington, Ontario, Canada); mouse anti-SSEA4 (clone MC813, cat. no. ab16287) from Abcam (Cambridge, MA); mouse anti-human CD73 conjugated with APC (clone AD2, cat. no. 17-0739-42) from eBioscience (San Diego, CA); mouse anti-CD56 conjugated with PE (cat. no. 340685) from BD Biosciences; goat anti-mouse IgG conjugated with Alexa 546, goat anti-mouse IgG conjugated with Alexa 488 from Molecular probes (Eugene, OR); mouse mAb for human and dog dystrophin MANDYS104 from CIND (Oswestry, UK); goat anti-mouse biotinylated antibody from DAKO diagnostics (Mississauga, Ontario, Canada); DAB Substrate Kit for detection of horseradish peroxidase activity from Vector laboratories (Burlington, Ontario, Canada); CyQUANT cell proliferation assay kits from Molecular Probes; the ROCK inhibitor Y-27632 from VWR (Mississauga, Ontario, Canada); the Cardiotoxin and all the other products from Sigma-Aldrich (St Louis, MO).

Ethical approval. All the experiences were approved by the animal care committee of the CHUL (Centre Hospitalier de l'Université Laval). *Mdx* mice (dystrophic mouse model with dystrophinopathy on a C57BL10J genetic background) were purchased from Jackson laboratory (Bar Harbor, ME) and reproduced in our animal facility. The *Rag/mdx* mice were produced in our laboratory by crossing *mdx* mice with *Rag*^{-/-} mice. The experiments with the hESCs and hiPSCs were authorized by the Stem Cell Oversight Committee of Canada.

hESCs culture. The H9 cells line was bought from WiCell Research Institute (Madison, WI). The undifferentiated cells were grown on matrigel-coated 6-well plate in the mTeSR1 medium as described by Ludwig *et al.*⁴⁵ The cells were enzymatically passaged every 5–7 days using 1 mg/ml of dispase. For mesenchymal differentiation, the culture medium of hESCs still on matrigel was changed for MB1, the culture medium that we normally used for the proliferation of human myoblasts. After 4–6 days in MB1 medium, hESCs were trypsinized to a single-cell suspension and plated on three matrigel-free 6 wells plate. Before confluence, cells were trypsinized and plated in one T25 or T75 flask (MB1-hESCs). Only when a large number of cells were required for intramuscular transplantation, the MB1-hESCs were proliferated for and additional three to four passages, this required about 2 weeks. The cells were then infected or not with Ad.CAG-MyoD (MOI 30) at 60% confluence.

Viral production. The Ad.CAG-MyoD was produced by our collaborator Dr Isao Fujii (Sojo University). This nonreplicative type 5 adenoviruses contained the murine MyoD under the cytomegalovirus early enhancer/chicken β -actin (CAG) promoter.⁴⁶ An adenovirus containing the enhanced GFP gene was used as control. The adenoviruses were produced in 293 cells line. The cells were plated at ~70% confluence on attachment petri dishes in DMEM 10% FBS + 1% penicillin/streptavidin. The following day, the cells were infected with the Ad.CAG-MyoD at a MOI of 3 and the cells were maintained in culture for an additional period of 48 hours. The cells were then gently detached by doing up and down with the culture media. The medium was recuperated and three freeze and thaw cycles were performed to lyse the cells. The virus was then purified from the medium using the CaCl₂ technique.

Infection of mononucleated cell suspension. The hESC colonies that reached confluence were first treated with 10 μ mol/l of ROCK inhibitor for a period of 1 hour. The colonies were then dissociated using a traditional solution of 0.05% Trypsin. Cells were seeded on a petri dish coated with matrigel and returned to culture in the mTeSR1 medium containing 10 μ mol/l ROCK inhibitor for a period of 24 hours. The cells were then infected for 6 hours at different MOI in α -minimum essential medium media containing 10 μ mol/l of ROCK inhibitor. Finally, the cells were washed twice with phosphate-buffered saline (PBS) and returned to culture in mTeSR1 medium supplemented with 10 μ mol/l of ROCK inhibitor.

hESC colony infection. hESC colonies that had not reached confluence, ~3–4 days after the cell passage, were infected with 1×10^5 – 1×10^8 viral particles, for a period of 6 hours in serum-free α -minimum Eagle's medium. After this period, the cells were washed twice with PBS and placed back in a culture medium, composed of α -minimum Eagle's medium, 20% FBS, 11% L-Glu, 1% penicillin/streptomycin, and 10 μ mol/l monothioglycerol. The cells were grown for a period of five more days and fixed with 95% ethanol for immunocytochemistry.

Dystrophic hiPSC culture. The dystrophic hiPSC cell line was bought from Georges Daley laboratory (Harvard University, Boston, Massachusetts). The derivation of these cells from skin fibroblasts has been previously described.⁴⁷ The cells were grown on matrigel-coated petri dishes using the mTeSR1 media. The undifferentiated cells were enzymatically passaged every 5–7 days using 1 mg/ml of dispase. For mesenchymal-like differentiation, hiPSCs were grown in the MB1 medium. After 5 days in MB1 medium, the hiPSCs were trypsinized to a single-cell suspension and plated on three matrigel-free 6 wells plate. Before confluence, cells were trypsinized and plated in one T75 flask (MB1-hiPSCs). The cells were then infected or not with Ad.CAG-MyoD (MOI 30) at 60% confluence.

Myotube formation. Myogenic differentiation was induced by growing the cells to 70% confluence and changing the medium to DMEM containing a low concentration of serum (2% FBS) and 1% penicillin/streptomycin. The cells were then cultured for 7 days before fixation with 95% ethanol.

RNA isolation and RT-PCR. RNA was isolated using trizol and its purity was determined by spectrophotometry. A DNaseI treatment was then made for a period of 1 hour at 37°C. This enzyme was then inactivated with 25 μ mol/l EDTA and by heating at 42°C for 15 minutes. The RNA was then transcribed in cDNA using the Omniscript RT kit (Qiagen, Valencia, CA). The cDNA was then amplified using Taq polymerase. The primer sequences, the temperature, the number of cycles, and the size of the amplicons were indicated in **Supplementary Table S1**.

FACS analysis. The cells were detached from the dish using 0.05% trypsin, pelleted and washed with PBS. The cells were then incubated with an appropriate antibody against SSEA4, CD73-APC or CD56-PE at a dilution of 1:65 in FACS buffer (PBS, 5% FBS) for 1 hour. For double labeling, cells were incubated with CD73-APC and CD56-PE antibodies at the same time using the same protocol. For SSEA4 labeling, the cells were washed then incubated with an anti-mouse immunoglobulin antibody coupled with fluorescein isothiocyanate for 45 minutes at a dilution of 1:300 in FACS buffer. The cells were then analyzed by FACS.

Immunocytochemistry. The cells were first fixed with 95% EtOH for 15 minutes. After a wash, nonspecific binding of antibodies was blocked by a 1 hour incubation with 10% FBS in PBS. The first antibody was then incubated in PBS containing 1% FBS for 1 hour and at concentration corresponding to the manufacturer recommendation, which was 1:75 for the desmin (d33; DAKO) and 1:50 for the MHC (mouse anti-MyHC mAb MF20, DSHB, Iowa City, IA). The second antibody coupled either with Alexa 488 or Alexa 546 was incubated at a dilution of 1:250 in PBS for 45 minutes. The cells were finally stained with DAPI (4',6-diamidino-2-phenylindole) diluted at 1:10,000 for 3 minutes. For the analysis ($n = 3$), the cells of three random fields were manually counted under a microscope. All values were expressed as means \pm SEM.

Immunoperoxidase. Cells were fixed in 100% methanol for MyoD staining. Nonspecific reactions were blocked with 1% bovine serum albumin. Cells were then incubated overnight at 4°C with the primary antibodies at the dilutions recommended by the manufacturer (1/200). After three washes, the cells were incubated with the secondary antibodies for 45 minutes at room temperature. Biotin-conjugated secondary antibodies (1:150) were used for immunoperoxidase staining. These antibodies were revealed with a streptavidin-coupled horseradish peroxidase signal amplification kit (Life Technologies, Burlington, Ontario, Canada) followed by DAB detection.

Immunohistochemistry. Tibialis anterior (TA) muscles of Rag/mdx mice were removed 1 month after myoblast transplantation. Frozen muscle cross-sections were blocked in PBS with 10% FBS and 2% bovine serum albumin for 1 hour and then incubated overnight at 4°C with the mouse mAb for human dystrophin (MANDYS104, a generous gift from Dr Glen Morris, MRIC Biochemistry Group, Wrexham, UK) diluted 1:10. Finally, muscle sections were incubated 1 hour with a goat anti-mouse Alexa 488 (diluted 1:250). Cross-sections were washed with PBS before and after incubation with both antibodies. A mouse monoclonal anti- β chain of spectrin (NCLSPEC1) antibody was used diluted 1:100 to detect spectrin. Muscle cross-sections were blocked in PBS with 10% FBS, 2% bovine serum albumin for 1 hour, and then incubated overnight at 4°C with the primary antibody. Muscle sections were incubated 1 hour with a goat anti-mouse Alexa 546.

Cell proliferation assay. The cell proliferation assay was performed using a CyQUANT cell proliferation assay kit (Life Technologies), which measures the nucleic acid content in the test samples. The cells were harvested after various treatment times and stored at -80°C until the analysis. The frozen micro-plates were then thawed at room temperature and the CyQUANT GR dye/cell lysis buffer was added. After incubating for 5 minutes, the fluorescence was measured (excitation/emission: 495/520 nm) using a micro-plate reader.

Cell transplantation. Primary normal human myoblasts were obtained and proliferated as described previously.^{48,49} The day of transplantation, cells were trypsinized and washed first in DMEM containing 10% FBS and then in Hank's balanced salt solution, before being resuspended in 20 μ l of Hank's balanced salt solution. The left and right TAs were surgically exposed and 0.5 million cells were co-injected with cardiotoxin (100 μ g/ml) at 10–15 sites throughout each TA muscle as previously described by our group.⁵⁰ Rag/mdx mice were transplanted either with myoblasts, hESCs, MB1-hESCs, MB1-MyoD-hESCs, hiPSCs, MB1-hiPSCs or MB1-MyoD-hiPSCs. Another negative control mouse group was injected in one TA only with Hank's balanced salt solution. The fusion of human cells with the mouse muscle fibers was assessed 1 month later by detecting the presence of human spectrin or human dystrophin.

Karyotyping. Dividing cells were arrested in metaphase with colcemid overnight, hypotonically shocked with KCl, and fixed with methanol/acetic acid (3/1; vol/vol). Chromosomes were identified using RHG-banding technique. At least 30 mitoses were examined for each karyotype.

Statistical analysis. All data are expressed as means \pm SEM and are representative of at least three separate experiments. The statistical significance of the difference between groups was determined by a Student's *t*-test. A value of *P* < 0.05 was considered significant.

SUPPLEMENTARY MATERIAL

Figure S1. Infection of isolated hESCs with Ad.CAG-MyoD.

Figure S2. Cytogenetic analysis.

Figure S3. Immunocytochemistry for embryonic marker SSEA4 and myogenic marker MHC on MB1-hESCs.

Table S1. Primer sequences, PCR conditions, and amplicon sizes for the RT-PCR analysis.

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