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Differentiation of Murine Embryonic Stem Cells in Skeletal Muscles of Mice

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Possible myogenic differentiation of SSEA-1- and OCT-4-positive murine embryonic stem cells (ESCs) and embryoid bodies (EBs) was studied in vitro and in vivo. In vitro, ESC- or EB-derived ESCs (EBs/ESCs) showed only traces of Pax 3 and 7 expression by immunocytochemistry and Pax 3 expression by immunoblot. By RT-PCR, myogenic determinant molecules (myf5, myoD, and myogenin) were expressed by EBs/ESCs but not by ESCs. However, in such cultures, very rare contracting myotubes were still present. Suspensions of LacZ-labeled ESCs or EBs were injected into anterior tibialis muscles (ATM) of different cohorts of mice for the study of their survival and possible myogenic differentiation. The different cohorts of mice included isogenic adult 129/Sv, nonisogenic CD1 and mdx, as well as mdx immunosuppressed with 2.5 mg/kg daily injections of tacrolimus. Ten to 90 days postinjections, the injected ATM of nonisogenic mice did not contain cells positive for LacZ, SSEA-1, OCT-4, or embryonic myosin heavy chain. The ATM of intact mdx mice contained very rare examples of muscle fibers positive for dystrophin and/or embryonic myosin heavy chain. In the ATM of the isogenic normal and the immunosuppressed mdx mice, as expected, large teratomas developed containing the usual diverse cell types. In some teratomas of immunosuppressed mdx mice, small pockets of muscle fibers expressed dystrophin and myosin heavy chain. Our studies indicated that in muscles of animals nonisogenic with the used ESCs, only very rare ESCs survived with myogenic differentiation. These studies also indicated that ESCs will not undergo significant, selective, and preferential myogenic differentiation in vitro or in vivo in any of the models studied. It is probable that this strain of murine ESC requires some experimentally induced alteration of its gene expression profile to secure significant myogenicity and suppress tumorogenicity.

Key words: Murine embryonic stem cells; Embryoid bodies; Myogenic differentiation; mdx mice; Immunosuppression; Tacrolimus; Dystrophin expression; Teratoma formation

INTRODUCTION

There are several types of myogenic progenitor cells (2,29,37). During normal embryonic development, a group of mesodermal cells becomes earmarked for subsequent myogenic differentiation (3). These cells can be considered presumptive myogenic stem cells. These cells express Bcl2, MNF, c-MET, Pax 3, and Pax 7 (28). With the appearance of expression of desmin and m-cadherin, as well as MyF5/MyD, the myogenin-negative myoblast stage ensues. With subsequent expression of myogenin, P21, and MRF4, the myogenin-positive myoblast stage develops. With subsequent expression of myosin, the myogenin-positive myoblasts fuse with each other to form multinucleated myofibers (28).

While most of the myoblast nuclei after fusion become myonuclei of myofibers, a certain percent of the above-mentioned myogenic cells do not fuse and remain in the so-called "satellite position" (i.e., reside in the space between the basal lamina and plasmalemma of the myofibers) (41). These satellite cells appear to be heterogeneous. Thus, during preparation of myoblast cultures by the dissociation procedure of mature myofibers, the resultant myoblast fraction will contain not only genuine myoblasts but some residual embryonic stem cells as well (36,47).

Besides embryonic myogenesis, myoblasts participate in postnatal new muscle formation in work hypertrophy of muscle fibers as well as regeneration of muscle fibers after necrosis (48). In these situations, they have a replication limit in vitro and in vivo (about a total of 50 doublings) and as they reach that stage (i.e., approaching senescence) their myogenicity declines (50). By contrast, the embryonic stem cells are immortal in vitro but their myogenicity is variable and depends on the expression of Pax 3 and Pax 7 (21,23,26,45,53).

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However, their participation in postembryonic myogenesis (i.e., regeneration) and work hypertrophy is unclear.

Intramuscular injection of suspensions of myogenic cells has been used for therapeutic purposes in skeletal and cardiac muscle diseases (20,25). The earliest attempts were myoblast transfers, which consisted of injecting suspension of cultured myoblasts (obtained by the dissociation technique using normal muscles of patients' fathers) into skeletal muscles of the patients with Duchenne muscular dystrophy (DMD) (19). In DMD, muscle fibers are damaged and destroyed by a genetically determined deficiency of dystrophin. The injected myogenic cells, presumably containing not only true myoblasts but also myogenic stem cells, were expected to fuse with the patient's muscle fibers and their nuclei to be established as new myonuclei. Such muscle fibers would now be capable of expressing some dystrophin and be, more or less, protected from damage. In this mode, myoblast transfer can be considered as a means of cell-mediated gene transfer (44). If the injected myoblasts fuse with each other and establish new innervated muscle fibers, the procedure can be considered a tissue replacement strategy.

The greatest problem with the above-described myoblast transfer was that a large proportion of the injected cells died in situ before they had a chance to fuse with host fibers or with each other (42). The precise cause of this phenomenon is still not clear. However, if the injectable myogenic cells were enriched before injection or even before culturing for the stem cell contingent, the therapeutic efficiency of the myogenic cell transfer was markedly enhanced (18,36). It was discovered that the expression of the transcription factor Pax 3 in the transferable cells is a prerequisite for good myogenicity (26, 39,45).

In the present experiments, we set out to determine if murine embryo-derived embryonic stem cells (ESCs) (17) would have even a better capacity for myogenic differentiation and myogenicity as well as in vivo survival capacity than the adult muscle-derived stem cells (14,16). While the therapeutic use of embryonic stem cells is controversial (1,4,5,51), if the in vivo survival capacity and myogenicity of ESCs without oncogenicity is significantly better than that of adult myogenic stem cells, further consideration for their therapeutic use could be justified (27).

MATERIALS AND METHODS

ESC Culture

The products for cell culture specified below were purchased from Gibco (Invitrogen, Grand Island, NY) except when specifically noted. Embryonic stem cells (ESCs) (Stemcell Technologies Inc., Vancouver, BC) is a murine stem cell line derived from 129/Sv mouse strain (22,40). They were maintained on mouse embryonic fibroblast feeder cells (Specialty Media, Phillipsburg, NJ) in gelatin-coated tissue culture dishes in DMEM medium (Specialty Media, Phillipsburg, NJ) containing 15% ESC-qualified fetal bovine serum (FBS) (Stemcell Technologies Inc., Vancouver, BC), plus 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM monothioglycerol (Sigma, St. Louis, MO), and 1000 U/ml recombinant mouse leukemia inhibitory factor (LIF, Chemicon, Temecula, CA). Culture medium was changed daily and passage of cells was performed every second day to maintain the undifferentiated state of the ESCs.

ESCs were validated by positive immunostaining with antibodies to SSEA antigen (Chemicon, Temecula, CA) irradiated F9 terato-carcinoma cells or with antibodies to OCT-4 human recombinant antigen (Chemicon).

Stable Transfection of ESCs With LacZ and Isolation of the Transfected Clones

ESCs were transfected with the construct: pEF1α-LacZ (Invitrogen, Grand Island, NY). Plasmid DNA was linearized prior to transfection into ESCs. For transfection, 2×10^6 ESCs were electroporated with 30 μg DNA in 800 μl electroporation buffer (Specialty Media) using a Gene-pulser (Bio-Rad, Mississauga, ON) with a pulse of 220 V and 960 uF. After electroporation, the cells were plated on mitomycin-C-inactivated embryonic feeder cells in culture medium and were allowed to grow for 24 h. For selection, 250 μg/ml G418 was added to the culture medium that was changed daily until clones of gentamycin-resistant cells could be identified (7-10 days post G418 selection). Single colonies were picked under microscopic control and plated on 96-well tissue culture plate for growth and identification.

Formation of Embryoid Bodies (EB)

Undifferentiated ESCs were cultured on gelatin-coated tissue culture dishes without feeder cells. ESCs were harvested with 0.25% trypsin-EDTA and the dissociated cells were suspended. Aliquots of 600 cells/20 μl in primary differentiation medium (PDM) [Iscove's modified Eagle medium supplemented with 15% FCS, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomysin, 0.5 mM ascorbic acid, 5 μg/ml insulin transferring (Roche, Sandhofer Strasse, Germany), 450 μM α-monothioglycerol] were placed onto the lids of petri dishes filled with PBS and EB were allowed to form in hanging drops.

Myogenic Differentiation of ESCs In Vitro

Two days after being cultured in PDM medium, EB were transferred to bacteriological petri dishes for sus-

pension culture for an additional 4 days in serum-free PDM medium (15% knockout serum replacement instead of FBS) supplemented with 5 ng/ml activin (R&D Systems, Minneapolis, MN). The 6-day-old EB were cultured 4 more days in serum-free PDM medium without activin. The 10-day-old EB were plated onto 100 different tissue culture plates coated with 0.1% gelatin in PDM medium and allowed to attach and spread. Myogenic differentiation was tested by evaluation of the cultures for the presence of multinucleated (twitching) myotubes and demonstrating histochemically the presence of desmin, and embryonic myosin heavy chain as well as molecular analysis for myf5, myogenin, and myoD (see below).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis for Myogenic Gene Expression

Total RNAs were isolated from ESC or EB outgrowth (EBs/ESCs) by using RNeasy Mini kit (Qiagen, Mississauga, ON) and RT-PCR were performed using Qiagen One-Step RT-PCR kit according to the manufacturer's instructions with the following primers: 5′-GGA GATCCTCAGGAATGCCATCCGC-3′, 5′-TGCTGTTC TTTCGGGACCAGACAGG-3′ for myf5; 5′-GACAG GACAGGACAGGGAGG-3′, 5′-GCACCGCAGTAGA GAAGTTGT-3′ for myoD; 5′-ACCAGGAGCCCCAC TTCTAT-3′, 5′-GTCCCCAGTCCCTTTTCTTC-3′ for myogenin; 5′-TCACTGTGCCTGAACTTACC-3′, 5′-GGAACATAGCCGTAAACTGC-3′ for tubulin; and 5′-GGCGTTCTCTTTGGAAAGGTGTTC-3′ for OCT-4. DNA primers for each gene were derived from different exons to ensure that the PCR product represents the specific mRNA species and not genomic DNA. Thermocycling parameters for RT-PCRs were: 50°C for 30 min, 95°C for 15 min, 94°C for 1 min, 65°C for 1 min, 72°C for 1 min, for 30 cycles. All samples were adjusted to yield equal amplification of tubulin. The amplified products were separated by electrophoresis on 1% agarose gels and stained with ethidium bromide.

Injection of ESCs Into ATM of Mice

Mature mice (4–6 weeks old) from the following strains were used ($n = 18$ in each or a total of 72): isogenic 129/Sv, CD1, mdx, and immunosuppressed mdx. Immunosuppression was attained by SC injections of tacrolimus (Prograf®) 2.5 mg/kg/day starting 3 days before ESC injections and continuing daily for 30 days until euthanasia. The ATM were injected with 25 μl of 10 μm cardiotoxin (Sigma, St. Louis, MO) 48 h before ESC injection to enhance necrosis/regeneration, which is supposed to enhance the take of the injected ESCs.

The ESCs were trypsinized by 0.25% trypsin and resuspended at 1×10^6 cells/30 μl in sterile PBS for transplantation. The EB were allowed to settle down by gravity and suspended in 100 EB/30 μl. Aliquots (30 μl) were directly injected to the right ATM (nine in each group) using a 25-gauge needle for ESCs or 21-gauge needle for EBs. PBS was injected into the contralateral nine ATM (in each group) as control. All animals received humane care in compliance with institutional guidelines.

Immunocytochemical Staining of Aliquots to Confirm Their ESC or EB/ESC Status of Injectable Cells

ESCs or EBs/ESCs were fixed with 4% paraformaldehyde for 20 min at room temperature. After rinsing with PBS, the cells were permeabilized using 0.4% Triton X-100 and then incubated in blocking buffer: 5% goat serum in PBS/BSA solution (1% BSA, 0.1% Triton X-100 in PBS). The cells were then exposed to each of the following primary antibodies: SSEA (Chemicon, 1:50); OCT-4 (Chemicon, 1:25); Pax 3 (R&D Systems, 1:100); Pax 7 (R&D Systems, 1:10); desmin (Novocastra, Newcastle, UK, 1:50); and pan-myosin (DSHB, Iowa City, IA, 1:100) diluted in PBS/BSA solution in a humidified chamber at room temperature for 1 h. After washing with PBS, the cells were treated with secondary Cy3-conjugated secondary antibody 1:300 diluted in PBS/BSA solution for 15 min. The cells were washed in PBS and mounted with Fluoromount G (Southern Biotechnology) and examined with a Leica fluorescence photomicroscope.

Immunohistochemistry of Tissue Sections

Injected ATM were embedded in Tissue Tek Oct and snap frozen in isopentane precooled in liquid nitrogen. Serial 5-μm transverse cryosections were collected at 100-μm intervals throughout the entire muscles. The sections were fixed for 2 min in acetone at room temperature. The endogenous mouse immunoglobulins were blocked by incubation with an affinity purified goat IgG (Fab fragment, Jackson Immuno Research). The sections were incubated for 60 min with each of the following primary antibodies to: SSEA (1:50); polyclonal anti-dystrophin (1:20) (generous gift by Dr. Paul Holland, McGill University, Montreal, QC); desmin (1:50); and embryonic myosin heavy chain (1:50) diluted in PBS containing 0.1% bovine serum albumin (BSA). After rinsing in PBS, sections were incubated with Cy3-conjugated goat anti-mouse IgG diluted (1:1000) in PBS with 3% BSA. After a further rinse with PBS, the sections were mounted with glass coverslips and examined using a fluorescence photomicroscope. Negative controls were obtained by incubation without applying the primary antibody.

β-Gal Staining

ESCs or EB/ESCs were fixed with glutaraldehyde for 3 min followed by a wash with PBS twice for 2 min.

The sections were overlaid with 2% β-Gal solution [400 μg/ml X-Gal (5-bromo-4-chloro-3 indolye-β-galactosidase) (Sigma, St. Louis, MO), 1 mmol magnesium chloride, and 5 mmol/L potassium-ferriferro-cyamide in 0.1 mmol/L PBS] and incubated at 37°C overnight.

Immunoblot Analysis for Pax 3

For immunoblot analysis, ESCs or EBs/ESCs or muscle homogenates were resuspended into 100 μl of sample buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02% bromophenol blue, 10 μM PMSF, 2.5 U aprotinin/ml, 0.5 μg leupeptin/ml) and boiled for 4 min. The nuclear DNA was sheared by 20 passages through a 22-gauge needle and the protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL). Total protein (25 μg) was separated on a 10% SDS-PAGE and blotted on a nitrocellulose membrane. Before loading the samples, 5% β-mercaptoethanol was added and the samples were boiled for 3 min. The blots were blocked with 5% milk overnight, then incubated with a monoclonal antibody against Pax 3 (R&D Systems) followed by a peroxidase-conjugated affinity-purified goat anti-mouse IgG (Fab specific, Sigma). The signal was then visualized by Enhanced Chemiluminescence (ECL, Amersham).

Teratomas

ESCs were trypsinized or EBs/ESCs washed twice, suspended in 100 μl PBS, and then injected SC (1×10^7 cells or 1000 EBs) on the neck region as well as IM (1×10^6 cells or 100 EB). After 30 days, tumors were surgically removed and frozen in ice-cold isopentane. Cryostat sections (10 μ thick) were prepared and stained with H&E and with all other immunostains as noted for the ATM.

RESULTS

Validation of ESC Cytochemical Characteristics and Pax Status

ESCs are pluripotent cells derived from the inner cell mass of the blastocyst stage of preimplantation embryos (3). In situ, they have the ability to self-renew, giving rise to new pluripotent stem cells, and to differentiate into all specialized cell types found in the adult mouse. ESCs of the 129/Sv mouse strain were obtained from Stem Cell Technologies, Vancouver, BC. The cell line is a good model for studying stem cell differentiation (40). ESC cultures contained undifferentiated colonies with dome-shaped and smooth, sharp-looking edges. ESCs are defined on the basis of cell surface antigens such as SSEA-1 and OCT-4 (15) (Fig. 1).

To investigate the fate of ESCs, a special cell line (ESC/LacZ) stably marked with LacZ was established. This stable cell line expressing LacZ proved that the promoter EF1α was effective in ESCs (54). A number of stable G418-resistant ESC colonies were generated by electroporation of linearized pEF1α-LacZ plasmid DNA into ESCs. Single colonies were selected to further identify these cells by β-galactosidase staining. As shown in Figure 2, blue cells (about 70% of total) could be found among the cells when the positive colonies were dissociated. These results showed that the LacZ marker was successfully introduced to a large population of ESCs.

Pax genes play key roles during development. Pax 3 and its paralogue Pax 7 have been implicated in the specification of cells that will enter the myogenic program (38,47). We investigated by immunocytochemistry and by immunoblot whether Pax 3 and Pax 7 expression in ESCs occurs. The ESCs were cultured four days and then stained with Pax 3 and Pax 7 antibody. Extremely rare positive cells were found (Fig. 5). The Western blot results also demonstrated that there was no Pax 3 expression in the ESCs (Fig. 3). The Pax 7 antibody was not suitable for Western blot analysis.

Characterization of Embryoid Bodies

A more powerful ESC differentiation model system was also used by the production of EBs (9). EB formation has been utilized in mouse to initiate differentiation into a variety of cell types including cardiomyocytes, neurons, endothelial cells, and hepatocytes (9). Our ESCs and ESCs/LacZ can form EBs in hanging drops culture, as shown in Figure 4.

To investigate whether EB-derived cells can undergo myogenic differentiation in vitro, the EBs were dissociated and stained for SSEA, Pax 3, and Pax 7. The results are shown in Figure 5. The lack of SSEA staining indicated the cells in EB-derived ESCs no longer had ESC-staining characteristics but a few Pax 3- or Pax 7-positive cells indicated that some cells entered myogenic program. However, the amount of Pax 3 protein present in protein extracts from ESC- or EB-derived ESCs was too low to be detected by Western blot analysis (Fig. 3).

Myogenic Differentiation of ESCs In Vitro

The myogenic basic helix–loop–helix transcription factors myf5, myoD, and myogenin play important roles in skeletal muscle cell development, each with unique functions, but being interchangeable in determining myogenic lineage formation (49). To test whether a possible myogenic differentiation of ESCs and EBs/ESCs is predictable by augmented mRNA levels of myogenic genes myf5, myoD, and myogenin, they were studied by RT-PCR analysis in ESCs and EBs/ESCs (Fig. 6). Undifferentiated ESC from the different clones did not show skeletal-specific transcripts. However, expression

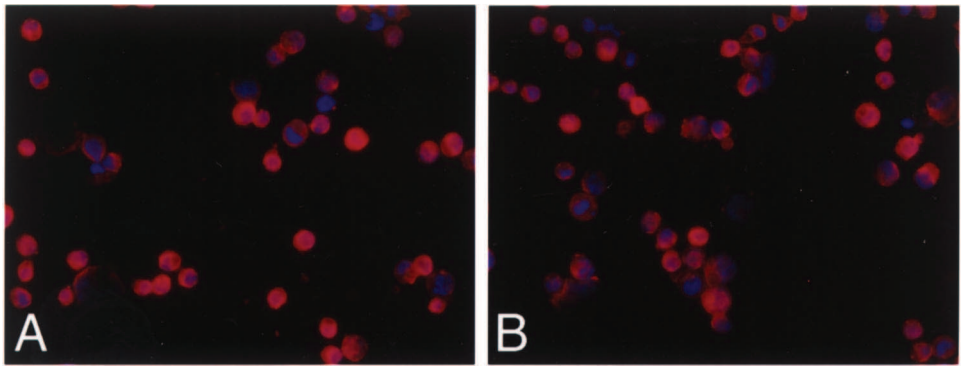

Figure 1. (A) Approximately 80% of ESCs showed SSEA-1 immunostaining. (B) About the same SSEA-1 percentage of ESCs are OCT-4 positive. Red fluorescence indicates SSEA-1 or OCT-4 positivity and blue color (Hoechst) marks nuclei. Original magnification 200×.

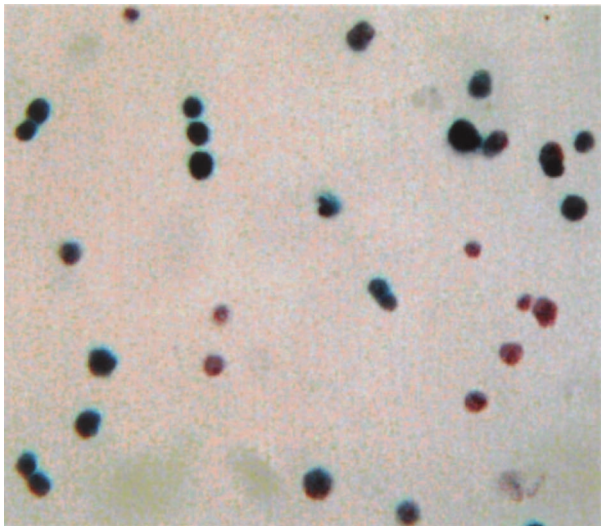

Figure 2. Approximately 70% of cultured ESCs, stably transfected with an EF1-LacZ construct, show β-galactosidase positivity (blue cells). The red staining cells (about 30%) are from H&E counterstain and are β-galactosidase negative. Original magnification 200×.

of myf5 in EBs/ESCs was detectable at day 5 while myoD and myogenin mRNAs were first expressed at 10 days.

ESCs and EBs/ESCs were examined for myogenic cells by phase contrast microscopy in 100 and 50 dishes, respectively. In day 10 culture, in about 95% of the dishes, the EBs/ESCs showed 1–3 multinucleated myotubes, and these myotubes began to contract around day 10. Immunofluorescence studies of EBs/ESCs for desmin and myosin heavy chain are shown in Figure 7, revealing that rare EBs/ESCs can differentiate into desmin- or myosin-positive myotubes.

Myogenic Differentiation of ESCs and EBs/ESCs in Nonisogenic Animals In Vivo

Possible myogenic differentiation of ESCs and EBs/ESCs in vivo is best demonstrable by implantation of these cells into muscles of mdx mice. To this end, suspensions of ESCs or activin-induced EBs were injected into ATM of adult intact ($N = 18$) as well as immunosuppressed mdx ($N = 18$) mice. The injected muscles were examined 10, 30, and 90 days after injection. The sections were immunostained with a polyclonal anti-

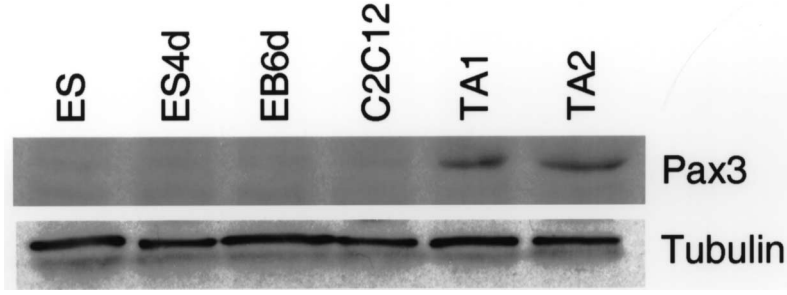

Figure 3. Pax 3 expression profile shown on immunoblot in ESCs, EBs, C2C12 cells, and two samples of normal ATM. None of the ESCs or EBs showed a signal but the ATM did. Our antibodies for Pax 7 were not suitable for immunoblot application.

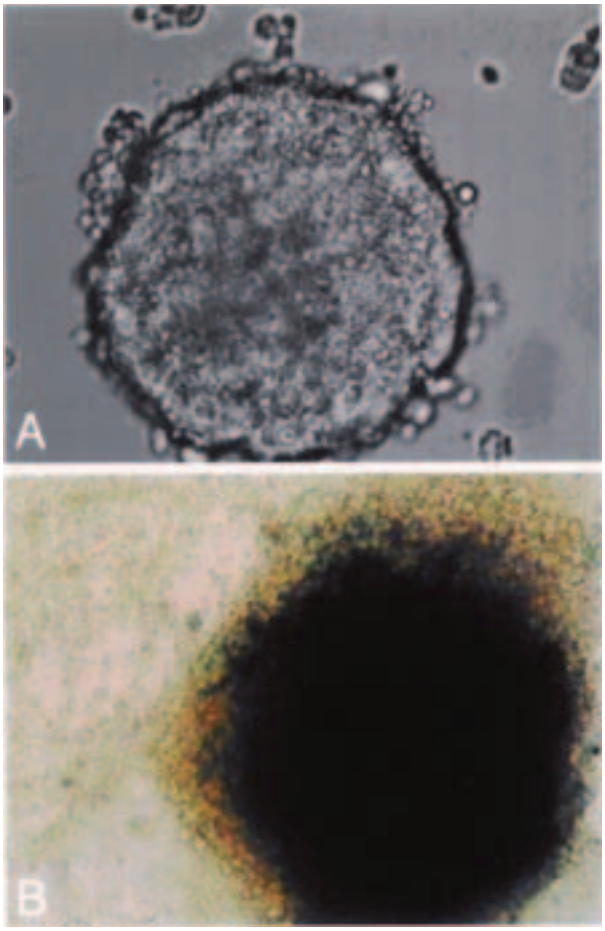

Figure 4. Intact EB and EB outgrowths (EB/ESC). (A) EB in hanging drop prepared under phase contrast microscopy (original magnification 100×).(B) EB with peripheral outgrowths at 5 days showing β-galactosidase stain. Only a portion of the outgrowth cells remains LacZ expressing (original magnification 100×).

dystrophin and anti-myosin heavy chain (MHC) and anti-desmin antibody. There were only 3 of the 18 intact and immunosuppressed mdx mice that showed very small groups or scattered dystrophin-positive muscle fibers. Some of these may have been revertant muscle fibers (41) (data not shown).

We also analyzed the injected muscles for the possible presence of undifferentiated ESCs or EBs. The specially marked ESC line (ESCs/LacZ) was used to check the fate of the injected ESCs in vivo. First, we injected the ESCs/LacZ into ATM of intact mdx mice and 30 days postinjection the mice were euthanized and the sections were stained for SSEA and LacZ (Fig. 8). Very rare collections of SSEA-positive (Fig. 8A) or LacZ-positive (Fig. 8B) cells were detected in 3 of 18 injected muscles. The results showed that some of the injected cells stayed together without spreading near the injection sites in big clusters in the mouse ATM and some of them expressed LacZ, demonstrating that they corresponded to the injected ESCs (Fig. 8B). EBs/ESCs/lacZ showed similar picture of survival in vivo (data not shown).

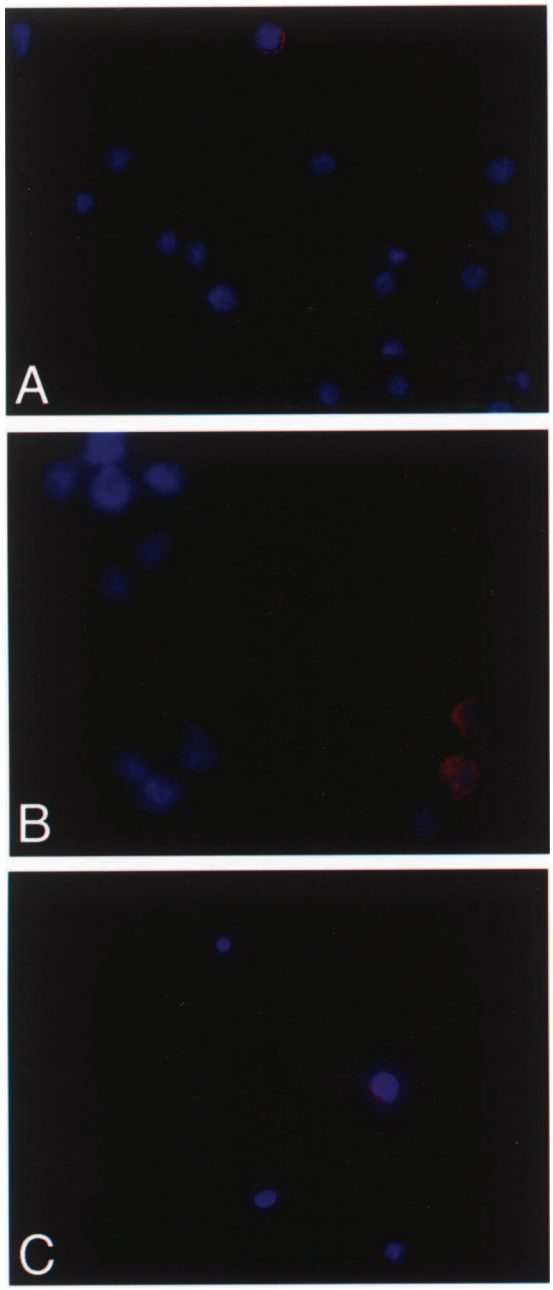

Figure 5. Cells detached from EBs (ESCs/EBs) show only traces of SSEA-1 at the peripheral cytoplasm (A); Pax 3 (B) and Pax 7 (C) staining is in the nuclei. The cells were also stained with Hoechst (blue) to show the nuclei of cells. Original magnification 400×.

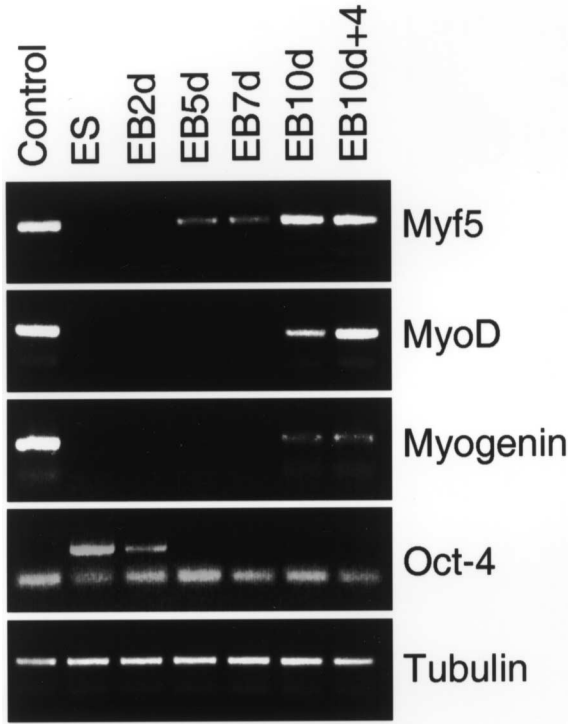

Figure 6. Expression of muscle-specific molecules in cultured ESCs (10 days) and EBs (5, 7, and 10 days) shown by RT-PCR. Note that ESCs do not express the three molecules (myf5, myoD, or myogenin) but EBs show increasing expression of myf5 (from day 5) and myoD and myogenin (at day 10). OCT-4 expression was present in the ESCs sample and in the early stage of EBs.

Teratoma Formation in Isogenic (129/Sv) or Immunosuppressed Nonisogenic (mdx) Mice After Injection of ESCs or EBs/ESCs (32)

In most injected ATM, by 30 days postinjection, large tumors formed in the injected muscles but not in the control muscles injected by heat-denatured cells (Fig. 9). [Similar tumors developed after SC injection of these cells into the subcutaneous tissue of the cervical region (data not shown).] The tumors contained varied cell types related to all germinal cell layers (glands, cartilage, epithelium, etc.) (Fig. 9B, C), indicating their teratomatous nature. In the midst of some teratomas, small pockets or scattered, differentiated small-caliber skeletal muscle fibers were present showing dystrophin (Fig. 9D). The host muscles contained some necrotic muscle fibers adjacent to the teratomas (Fig. 9B).

DISCUSSION

Several types of myogenic progenitor cells have been identified in embryonic as well as postnatal tissues (2,29,37). The focus of recent attention is concentrated on myogenic progenitor cells active in the postnatal stage because these cells have a key role in regeneration of muscle fibers after segmental necrosis in disease (29) as well as for their potential therapeutic value in cell therapy of genetic muscle diseases such as Duchenne muscular dystrophy (19,20). Although ESCs should prominently be considered as probable myogenic progenitor cells for possible application in cell therapy of muscle diseases, published studies in this field are very scarce (17).

ESCs, derived from the inner cell mass of preimplantation blastocysts, are pluripotent and, in situ, able to self-renew, and have the capacity to generate any cell type of the developing embryo (9,11). Because of these characteristics, they have the potential to be useful as an approach for cell therapy of certain human diseases (30,33). When transferred to suspension cultures, murine ESCs began to differentiate into multicellular aggregates of differentiated and undifferentiated cells, termed embryoid bodies (EBs), which resemble early postimplantation embryos (22). By in vitro differentiation via EBs into endodermal, ectodermal, and mesodermal cells, ESCs should offer a suitable model to study terminal differentiation into specialized cells of the cardiogenic, neurogenic, and myogenic lineages (22).

The ESCs studied in our experiments have been validated as true stem cells by several means. These included the presence of SSEA and OCT-4 markers and their ability to form EBs. Furthermore, as expected, they showed striking pluripotentiality in vivo by teratoma formation in mice that were isogenic with those that were the source of our ESCs (129/Sv) or in immunosuppressed nonisogenic (mdx) animals. The latter features verified that ESCs or their immediate derivatives express histocompatibility antigens, which have important therapeutic implications.

The fundamental aim of our experiments was to demonstrate if myogenic differentiation of ESC- or EB-derived ESCs would occur in vitro and in vivo and characterize important molecular features during such process.

The in vitro studies revealed that ESC- or EB-derived ESC cultures contained only very rare examples of contracting myotubes showing dystrophin in desmin immunostaining. Similar findings were also reported by Odorico et al. (33). Nunes et al. (31) reported that this lack of significant myogenic differentiation is in keeping with a lack of Pax 3 and Pax 7 expression by immunocytochemistry or Western blot (Pax 3 only) as well as absent mRNA for myf5, myoD, and myogenin in ESCs. The presence of myf5 and myoD, but not myogenin, mRNA in some ESC/EB cultures is curious because in these cultures myogenic differentiation was not greater than in those ESC cultures not showing such features. We

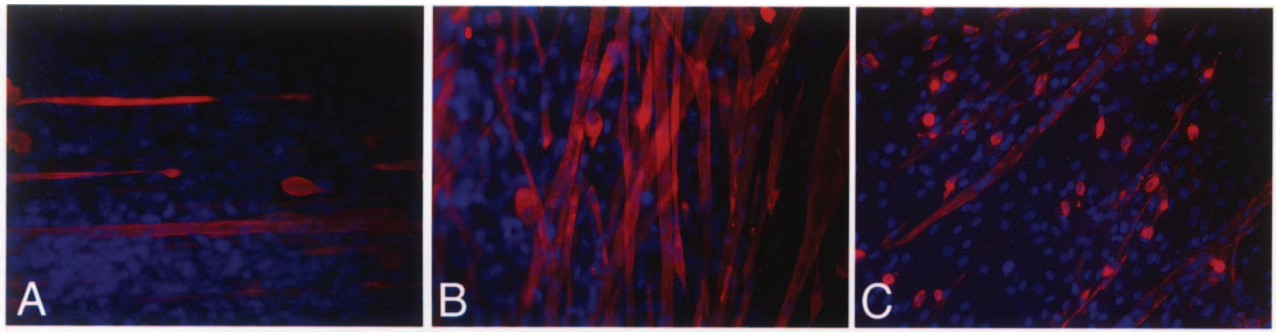

Figure 7. Myotubes derived from ESCs/EBs after 7 days of culture in differentiation medium. (A) Immunostaining with myosin heavy chain antibody (embryonic isoform) (red) and counterstaining with Hoechst (blue). Original magnification 200×. (B) Immunostaining with desmin antibody (red) and counterstaining with Hoechst (blue). Original magnification 200×. (C) C2C12 myotubes and myoblasts show desmin positivity (red).

also tried extra efforts to make ESCs undergo myogenic differentiation in vitro by adding various factors such as DMSO, 5-azacytidine, IGFII, LiCl, or IL-4 to the differentiation medium without differentiation occurring (data not shown). Furthermore, conditioned medium from the mouse myoblast cell line C1C12 was used for culturing ESCs or the ESCs were cocultured with C2C12 cells, but none of these measure produced improved myogenic differentiation of ESCs (data not shown). This is at variance with the findings of Nunes et al. (31), who managed to induce myogenicity of human ESCs in vitro by coculturing them with human myoblasts.

Concerning in vivo myogenic differentiation of ESCs or EBs/ESCs, our findings were different in isogenic (129/Sv) or immunosuppressed nonisogenic (mdx) animals versus nonisogenic immunocompetent animals (mdx). In the latter model, there were only very few cells (in 3/30 injected ATM) showing ESC markers such as SSEA and LacZ, implying poor survival of the injected ESCs or EBs/ESCs. In such muscles, dystrophin- and desmin-positive muscle fibers were exceedingly rare up to 3 months postinjection (3/30 muscles). In fact, we could not be certain if these were not revertant fibers (46). In such animals teratoma formation was absent.

By contrast, in ATM of isogenic or immunosuppressed nonisogenic (mdx) mice, large tumors were noted in the ESC- or EB-injected muscles but not in control-injected contralateral ATM. This was an additional proof that our ESCs were true pluripotent stem cells. By histology, these tumors were typical teratomas containing cell types pertaining to all three germinal layers. It is of interest that in the midst of such teratomas, there were clusters of skeletal muscle fibers showing dystrophin and myosin heavy chain positivity. These muscle fibers were clearly part of the teratomas and not revertant fibers. However, large clusters of muscle cells outside of the teratomas were not observed, indicating that even in a permissive immunological milieu, selective myogenic differentiation of ESCs or EBs/ESCs did not occur. It is of interest that teratoma formation is not specific for

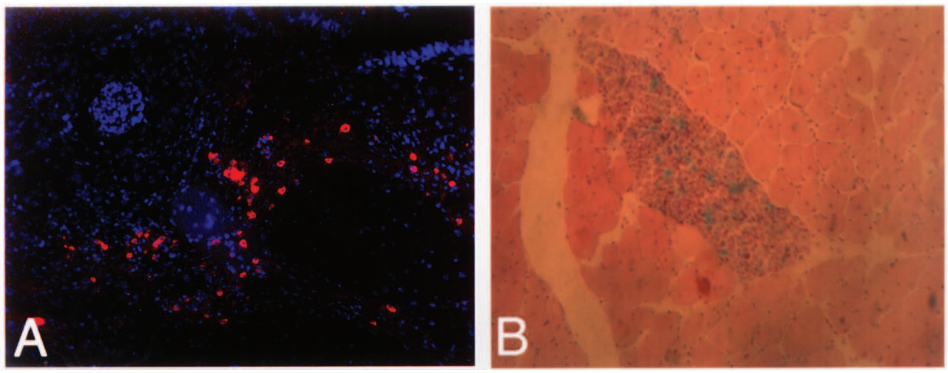

Figure 8. ATM of mdx muscle injected with ESCs/LacZ 30 days without immunosuppression shows faint SSEA-positive cells (A; red fluorescence) and β-galactosidase-positive cells (B, blue signal). The section in (A) was counterstained with Hoechst, which stains the nuclei in blue.

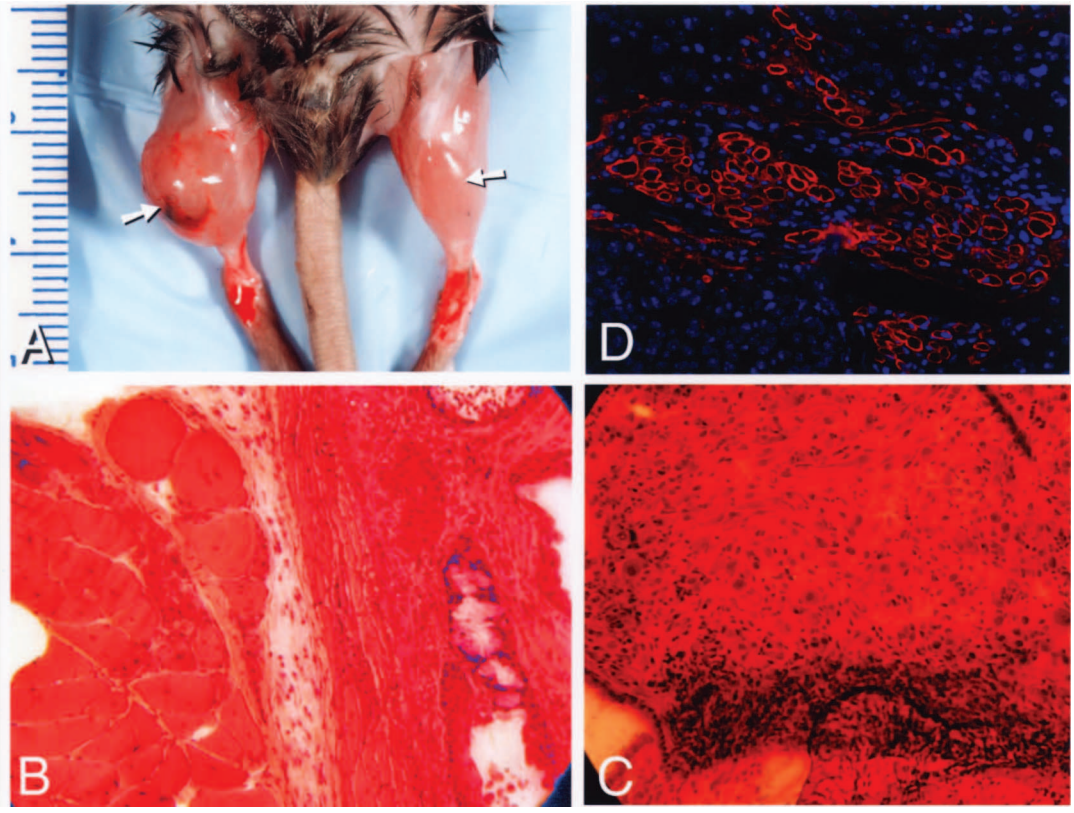

Figure 9. (A) Large teratoma (arrows) in the right ATM of immunosuppressed mdx mouse 30 days after injection of ESCs. This mouse received daily injections of 2.5 mg/kg tacrolimus SC. The left ATM injected with PBS showed no teratoma. (B) Microscopic picture of the teratoma. On the right, a large space lined with column epithelium as well as numerous pleomorphic cells are shown. On the left, there is a portion of the host muscle showing foci of necrosis and regenerating muscle fibers. (C) Other regions of the teratoma showing pleomorphic cells. (D) A rare cluster of dystrophin-positive muscle fibers in the midst of a teratoma. Blue color marks counterstaining with Hoechst.

muscle, because it occurred in subcutaneous tissues after injection of ESC into such sites.

To ascertain that the failure of in vivo myogenic differentiation is not a peculiarity inherent to our ESC line, we performed similar experiments with another ESC line (R1 or EB derived from R1/ES cells) (52) with similar negative results. Furthermore, we have ample evidence that the failure of myogenic differentiation was not due to technical flaws. This evidence includes a large number of similar experiments performed on different occasions and our controls, using in situ myogenic cells derived from adult muscle, showing vigorous myogenic differentiation in situ and in vivo.The experiments reported in this study indicate that in our experimental conditions, either in vivo or in vivo, ESCs or EBs/ESCs failed to undergo selective myogenic differentiation even under permissive immunological circumstances where indiscriminate differentiation amounting to tumor formation did occur.

While the results of our studies can be considered as "negative," they have important theoretical and practical implications. Our findings should not be interpreted as a discouragement from further experimentation to determine how ESCs can be used as a tool for cell therapy of muscle disease by obviating the rejection of ESCs by nonisogenic hosts and/or negating teratoma formation in isogenic hosts. In this respect, encouragement was provided by a recent paper by Chang and Cotsarelis (7) reporting the experimental transformation of cutaneous cells into ESCs. This approach has the advantage of easy availability of isogenic ESCs from and for isogenic hosts. The latter custom-designed approach would spare the ESCs from rejection. However, the presence or absence of tumerogenicity in such ESCs is still unclear, but could easily be established. If the tumorigenicty of such ESCs persists, further study should be undertaken in two areas: proper adjustment of the gene expression profile of ESCs and/or appropriate modification of the muscle environment (10).

The apparent failure of robust activation of the appro-

priate gene array that could have lead to myogenic differentiation in our ESCs did not occur in our experimental conditions. We have not derived precise molecular information that could explain this situation (27).

To gain such possible information, it would be of interest to compare the gene expression profile of the robustly myogenic stem cells derived from adult muscle (ASC) with those of ESCs as well as the gene expression profile of ESCs in embryos during early development when proper differentiation of ESCs does occur. For example, whether expressing CD34 or CD133 by ASC is a clue for their robust myogenicity in postnatal mdx muscle (6,18,36).

Our results are partially in keeping with those of Smith (43) showing that although some EBs can differentiate into skeletal muscle cells in vitro, transplantation of EBs into postnatal muscle, without any measure to direct development along a specific pathway, leads to a failure of tissue-specific differentiation.

For proper interpretation of the results of our study, we must consider the possibility that survival and appropriate differentiation as well as antigenicity of ESCs in vivo may be different in different species and organs (32,54). For example, Coleman et al. demonstrated that murine ESCs injected in dysfunctional cochlea of guinea pigs resulted in survival and functionally appropriate differentiation of some ESCs for 28 days (8).

The desirability of further relevant exploration in ESC biology is evident because a great deal of often uncritical promotion is under way in the public domain for the use of ESCs for the therapy of various serious human disorders (24,35). In fact, this issue has become a politically hot topic (12,13,34,35).

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