Partial Laminin α2 Chain Restoration in α2 Chain-deficient *dy/dy* Mouse by Primary Muscle Cell Culture Transplantation

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Abstract. Laminin-2 is a component of skeletal and cardiac basal lamina expressed in normal mouse and human. Laminin α2 chain (LAMA2), however, is absent from muscles of some congenital muscular dystrophy patients and the *dystrophia muscularis* (*dy/dy*) mouse model. LAMA2 restoration was investigated following cell transplantation in vivo in *dy/dy* mouse. Allogeneic primary muscle cell cultures expressing the β-galactosidase transgene under control of a muscular promoter, or histocompatible primary muscle cell cultures, were transplanted into *dy/dy* mouse muscles. FK506 immunosuppression was used in noncompatible models. All transplanted animals expressed LAMA2 in these immunologically-controlled models, and the degrees of LAMA2 restoration were shown to depend on the age of the animal at transplantation, on muscle pretreatment, and on duration time after transplantation in some cases. LAMA2 did not always colocalize with new or hybrid muscle fibers formed by the fusion of donor myoblasts. LAMA2 deposition around muscle fibers was often segmental and seemed to radiate from the center to the periphery of the injection site. Allogeneic conditionally immortalized pure myogenic cells expressing the β-galactosidase transgene were characterized in vitro and in vivo. When injected into FK506-immunosuppressed *dy/dy* mice, these cells formed new or hybrid muscle fibers but essentially did not express LAMA2 in vivo. These data show that partial LAMA2 restoration is achieved in LAMA2-deficient *dy/dy* mouse by primary muscle cell culture transplantation. However, not all myoblasts, or myoblasts alone, or the muscle fibers they form are capable of LAMA2 secretion and deposition in vivo.

LAMININ-2 belongs to the laminin family (Burgeson et al., 1994) and is present in basal laminae of cardiac and skeletal muscle cells (Ehrig et al., 1990). Some cells of mesenchymal origin, Schwann cells, and thyroid cells would participate in its secretion (Leivo and Engvall, 1988; Engvall, 1993; Andre et al., 1994). Laminin-2 is constituted by the assembly of three subunits called α2 chain (400 kD), β1, and γ1 chains (200 kD each). Laminins have cross-like three-dimensional structures which interact with cellular receptors such as integrins (Engvall, 1993). Laminin-2 interacts with the 156-kD dystrophin-associated glycoprotein (α-dystroglycan; Campbell, 1995). The laminins promote cell spreading, migration, proliferation, and/or differentiation, and laminin-2 also promotes neurite outgrowth and nerve-muscle interactions (Engvall et al., 1992). Laminin α2 chain (LAMA2)[1] also promotes Schwann cell migration in vitro and nerve regeneration in vivo (Anton et al., 1994). The gene coding for human α2 chain has been cloned and located on chromosome 6 in position q22/23. The cDNA is about 9.5 kb in length and encodes for a more than 3,100–amino acid protein (Vuolteenaho et al., 1994).

Hayashi et al. (1993) showed that the LAMA2 expression was reduced in muscle cell basal membranes of some Fukuyama-type congenital muscular dystrophy patients, and Tomé et al. (1994) showed that LAMA2 expression was deficient in 13 of 20 congenital muscular dystrophy (CMD) patients (i.e., 65%). Out of 25 CMD patients, Sewry et al. (1995) also showed that LAMA2 expression was undetectable in seven and reduced to trace amounts in five. Thus, 48% of CMD patients in this series were deficient for LAMA2 expression. The LAMA2 deficiency has been linked to the *Lama2* gene on chromosome 6 (Hillaire

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1. *Abbreviations used in this paper*: β-gal, β-galactosidase; CMD, congenital muscular dystrophy; IFN-γ, interferon γ; LAMA2, laminin-2 α2 chain; TA, Tibialis anterior.

et al., 1994), and a direct implication of the *Lama2* gene in the development of the LAMA2-negative CMD has been reported in man (Helbling-Leclerc et al., 1995). One LAMA2-negative CMD patient also presented LAMA2 mRNA deficiency (Hayashi et al., 1995). The LAMA2-deficient CMD patients usually have a more severe clinical phenotype than LAMA2-positive CMD patients. They regularly present white matter changes on brain imaging (Philpot et al., 1995). The identification of mutations on the *Lama2* gene could provide a useful tool in categorizing various CMD which show clinical heterogeneity (for review see Dubowitz and Fardeau, 1995; Helbling-Leclerc et al., 1995). The Fukuyama-type CMD would not be directly linked to the *Lama2* gene.

Three groups showed that the *dystrophia muscularis* (*dy/dy*) mouse model was deficient for LAMA2 expression (Arahata et al., 1993; Sunada et al., 1994; Xu et al., 1994*a*). In these studies, LAMA2 was undetectable in immunohistofluorescence in skeletal and cardiac muscle cell basement membranes and in peripheral nerve and some very low levels of LAMA2 mRNA could be detected only after RT-PCR amplification (Arahata et al., 1993; Xu et al., 1994*a*). The LAMA2 deficiency has been linked to the *Lama2* gene on chromosome 10 in *dy/dy* mouse (Sunada et al., 1994) and a mutation has been identified in the dy^{2J}/dy^{2J} mouse, an allelic variant of the LAMA2-negative *dy/dy* model (Xu et al., 1994*b*; Sunada et al., 1995). First described in 1955, the *dy/dy* mouse develops early signs of muscular dystrophy (Michelson et al., 1955). The progressive and lethal dystrophy is characterized first by ataxia, convulsive head movements, spasmodic hind leg flexion, and flaccid extension, followed by progressive muscle atrophy and paralysis extending to axial musculature and anterior legs. At the late stages, the posterior leg locomotion is lost and the mouse develops marked kyphosis. The size, number and diameter of muscle fibers, are greatly variable in *dy/dy* mice, but always less than in normal mice. The muscles are invaded by connective tissues. The neural system of the *dy/dy* mice also show many abnormalities. The Schwann cell basal lamina are patchy, and the myelination of the peripheral nervous system is variably defective (Bradley and Jenkison, 1973; Madrid et al., 1975; Montgomery and Swenarchuk, 1977). The life span of the *dy/dy* mouse is rarely more than six months, and the disease is of autosomal recessive transmission.

The milder allelic variant of the *dy/dy* mouse, the dy^{2J}/dy^{2J} mouse model, express a truncated protein (Xu et al., 1994*b*; Sunada et al., 1995). This model would not truly reflect at least the majority of the human CMD because of the expression of a truncated but partially functional LAMA2. Muscle regeneration was investigated in the dy^{2J}/dy^{2J} mouse following muscle tissue, G8 clonal muscle cells, and primary fetal or newborn muscle cell transplantations (Law, 1982; Law et al., 1988*a*,*b*). The aetiology and molecular basis of this neuromuscular pathology were unknown at the time of these experiments and the use of this animal model seems now partially inadequate. Actually, G8 myoblasts did not form hybrid muscle fibers (Law et al., 1988*a*), and some models were not compatible for minor antigens (Law et al., 1988*b*). Some, but few animals showed behavioral improvements 2 to 7 mo after transplantation (Law et al., 1988*a*).

Myoblast transplantations have been performed in the *mdx/mdx* mouse, which is a biochemical model of Duchenne muscular dystrophy. Transplantations of cultured muscle cells promoted myoblast fusion, formation of new and/or hybrid muscle fibers in vivo, and intracellular dystrophin restoration in *mdx/mdx* mice. One of the major drawbacks of myoblast transplantation experiments is the host immune response against donor cells (Kinoshita et al., 1994*b*; Guérette et al., 1995). Immunosuppressive treatments, immunodeficient or immunocompatible animals, were used to overcome this problem (Partridge et al., 1978, 1989; Grounds et al., 1980; Watt et al., 1984; Morgan et al., 1990, 1992, 1993, 1994; Huard et al., 1994; Kinoshita et al., 1994*a*,*b*; Pavlath et al., 1994; Asselin et al., 1995; Vilquin et al., 1995*a*,*c*). Our group has obtained the best success either using FK506 immunosuppression to control nonhistocompatible myoblast transplantation, or using fully compatible host/donor combinations (Kinoshita et al., 1994*b*; Vilquin et al., 1995*c*).

In the present study, LAMA2 restoration in the *dy/dy* mouse after transplantation of cultured primary muscle cells was evaluated with respect to age at transplantation, type of transplantation, and muscle pretreatment. In addition, pure myoblast cultures were used to study the role of myoblasts in LAMA2 deposition in vivo.

Materials and Methods

Animals

The C57BL/6J *dy/dy* mice (Jackson Laboratories, Bar Harbor, ME) were used as recipients for myoblast transplantation. The C57BL/6J +/+ normal newborn mice (Jackson Laboratories) were used as compatible donors for some myoblast transplantations.

The transgenic TnI-LacZ1/29 mice (Tn-LacZ, gift from K. Hasting, McGill University, Montréal, Canada) contain the LacZ gene under the control of the quail fast troponin I promoter, thus, myotubes and muscle fibers express a cytoplasmic β-galactosidase (β-gal) protein (Hallauer et al., 1993; Kinoshita et al., 1994*a*,*b*). β-gal expression is not restricted to the nucleus in this model. Both male and female parents are heterozygous animals, and newborn mice were used as donors for some immunologically noncompatible myoblast transplantations.

The H-2K^b-tsA58 transgenic mice carry the thermolabile tsA58 mutant of SV40 large T antigen under the control of the H-2K^b promoter (Jat et al., 1991). Interferon γ (IFN-γ) increases the transcription of this promoter. The thermolabile protein is functionally active at 33°C but not at 39°C. These characteristics facilitate the derivation of conditionally immortalized cell lines (Morgan et al., 1994). An homozygous H-2K^b-tsA58 male mouse (Charles River Lab., Wilmington, MA) was crossed with a heterozygous transgenic Tn-LacZ female mouse. Offsprings were all heterozygous for the H-2K^b-tsA58 transgene and some were heterozygous for the Tn-LacZ transgene. Thus, newborns were tested for β-gal expression, and only β-gal positive animals were used for the establishment of myogenic cell lines (see below).

Primary Cell Cultures

Mouse primary myoblast cultures were obtained from biopsies from newborn skeletal muscle as previously described (Vilquin et al., 1995*c*). The cell suspension was cultured in 199 medium (GIBCO BRL, Gaithersberg, MD) supplemented with 15% FBS (GIBCO BRL) and antibiotics. Cells were harvested at 70% confluence, that is, 2 d after plating, either for immediate grafting or for freezing until grafting. These cultures were not pure and contained several cell types, with 30–40% being commited myoblasts as assessed by desmin immunostaining (personal results).

Cultures were also obtained from newborn transgenic Tn-LacZ. Because parents were both heterozygous, newborns were individually tested for the expression of β-gal using X-gal (see below). Only β-gal–positive newborns were used for primary myoblast cultures.

a. Noncompatible primary muscle cell transplantation

d -3	d -1	d 0	d 21, 27 or 39
↑	↑	↑	↑
Irradiation (when indicated)	Notexin (when indicated)	Cell transplantation (noncompatible Tn-LacZ) FK506 immunosuppression	Mouse sacrifice Muscle examination (LAMA2, β-gal)

b. Compatible primary muscle cell transplantation into male recipients

d -3	d -1	d 0	d 77
↑	↑	↑	↑
Irradiation (when indicated)	Notexin (when indicated)	Cell transplantation (compatible C57BL6J +/+)	Mouse sacrifice Muscle examination (LAMA2, β-gal)

c. Conditionally immortalized myogenic cell line transplantation

d 0	d 14, 21 or 28
↑	↑
Cell transplantation: Left leg: myogenic clones or colonies (noncompatible Tn-LacZ/H-2K^b-tsA58) Right leg: myogenic clones or colonies plus primary muscle cells (compatible C57BL6J +/+) FK506 immunosuppression	Mouse sacrifice Muscle examination (LAMA2, β-gal)

Figure 1. Transplantation models used in this study.

Different batches of primary cell cultures have been used for this work. The mice transplanted with these batches have been gathered under the letters A, B (β-gal cells), and H (histocompatible cells).

Establishment of Permanent Myogenic Cell Lines from (TnI-LacZ1/29) × (H-2K^b-tsA58) Offsprings

Primary muscle cell cultures were started using the β-gal–positive offsprings. Following preplating, the cells were plated at 50 cells/cm^2 in gelatin-coated wells and grown in DME (GIBCO BRL) supplemented with 20% FCS and 2% chick embryo extract (GIBCO BRL) at 33°C in 10% CO_2. Mouse recombinant IFN-γ (Genzyme Co., Cambridge, MA) was added at the final concentration of 20 U/ml (Morgan et al., 1994). Colonies of typical myogenic morphology were subsequently cloned in 96-well plates at the limit dilution of one cell per well. The myogenicity of colonies and clones was assessed by desmin immunostaining and by the ability to fuse and form myotubes in vitro. Fusion was obtained by reducing the FCS concentration to 5% and growing the cells at 37°C in 5% CO_2 in the absence of IFN-γ.

Permanent Fibroblast Cell Lines

Permanent fibroblast cell lines S3T3 and BLK CL.4 were both from American Type Culture Collection (Rockville, MD).

Cell Transplantation

On the day of transplantation, the cells were harvested by trypsinization or thawing, washed three times in HBSS (GIBCO BRL), and concentrated as pellets. Cell viability was assessed using trypan blue staining. The *Tibialis anterior* (TA) muscles were exposed and injected with approximately 4×10^6 (primary muscle cell cultures), or 10^6 (myogenic cell lines No. 24, MB7, MB27) or 3×10^6 (myogenic cell line MB3, fibroblast cell lines S3T3, and BLK CL.4) viable cells suspended in 10 μl of HBSS. When the role of myoblasts in LAMA2 restoration was explored by clonal cell transplantation, the left TA of the mouse received only myogenic clones, while the right TA received both myogenic clones and a histocompatible primary muscle cell culture (i.e., obtained from normal C57BL6J +/+ newborn mice). Some protocols included γ-irradiation and/or notexin treatment of the muscles before cell transplantation: three days before transplantation, one or both hind legs of the *dy/dy* mice were Cobalt-irradiated (20 Gy). This level of irradiation has been shown to block host myoblast proliferation and to favor donor myoblast implantation (Wirtz et al., 1982; Morgan et al., 1990; Wakeford et al., 1991); one day before transplantation, one or both TA were exposed and injected with 10 μl of notexin venom (5 μg/ml), which has been shown to trigger muscle fiber degeneration without damaging myoblasts (Harris et al., 1975). FK506 immunosuppression was started on the day of transplantation in noncompatible grafting models (2.5 mg/kg/d i.m.; Fujisawa Co, Osaka, Japan; Kinoshita et al., 1994*b*). The transplantation schedules are presented in Fig. 1.

Muscle Collection

At the times indicated, the TA muscles were collected and immersed in a sucrose solution (Tremblay et al., 1993). Muscles were embedded, frozen in liquid nitrogen, and serially sectioned at 8 μm. Adjacent serial sections were thus spaced by 8 μm, while the series of sections were separated by 180 μm.

β-gal Histochemistry

Differentiated conditionally immortalized myogenic cells and muscle cryostat sections were fixed with 0.25% glutaraldehyde for 3 min and incubated overnight at room temperature with 0.4 mM X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Boehringer Mannheim, Laval, Canada) (Kinoshita et al., 1994*a*). Preliminary experiments indicated that the X-gal enzymatic histochemistry was as sensitive as three-step immunohistochemistry using monoclonal anti-β-galactosidase antibodies to localize muscle fibers formed by the fusion of donor myoblasts (not shown). Newborns originating from Tn-LacZ mice were tested for β-gal expression using the X-gal reagent, except that no fixation was necessary and incubation of a small muscle piece was performed at 37°C for 1 h.

LAMA2 Immunohistochemistry

Muscle sections were fixed in acetone at −20°C for 10 min and then nonspecific Ig binding was blocked with 10% FBS in PBS for 30 min. The rabbit polyclonal anti–mouse LAMA2 (Xu et al., 1994*a*) was used 1/300 in PBS containing 1% FBS for 2 h at 37°C. The second antibody was a biotinylated goat anti–rabbit Ig (1/100 in PBS containing 1% FBS for 1 h; Dako, Copenhagen, Denmark). The next step was incubation with streptavidin-HRP or streptavidin-FITC in some cases (1/200 in PBS containing 1% FBS; Dako). Binding was revealed with DAB (0.5 mg/ml; Sigma Chemical Co., St. Louis, MO) and 0.015% hydrogen peroxide. Slides were mounted in PBS-glycerol. Immunoperoxidase-positive and -negative fibers were counted by microscopic examination of each muscle on the section with the most positive fibers.

Desmin Immunocytochemistry

Conditionally immortalized myogenic cells were grown on 2% gelatin-coated plates and allowed to fuse in 5% FBS at 37°C. The cells were fixed and permeabilized with methanol at −20°C. Nonspecific binding was blocked using 10% FBS in PBS for 30 min. The cells were incubated with a mouse anti–desmin antibody (1/50 in PBS containing 1% FBS for 1 h; Dako). The second antibody was FITC-conjugated rabbit anti–mouse IgG (1/100 in PBS containing 1% FBS for 1 h; Dako).

Results

Primary Muscle Cell Culture Transplantation under FK506 Immunosuppression

β-gal Expression. Primary muscle cell cultures from transgenic mice expressing β-gal under the control of a muscle-specific promoter were able to develop inside the TA muscles of *dy/dy* mice immunosuppressed with FK506. Some myoblasts fused together or with host muscle fibers to form new or hybrid muscle fibers expressing β-gal. These β-gal expressing fibers were not numerous and they were not dispersed throughout muscles (low magnification, Fig. 2). They were presumably located only near the injection sites. As seen in Table I, the percentage of β-gal–positive fibers was rarely higher than 5%. These low percentages were obtained whatever the age of the recipient mice at the time of transplantation, a slight but not significant in-

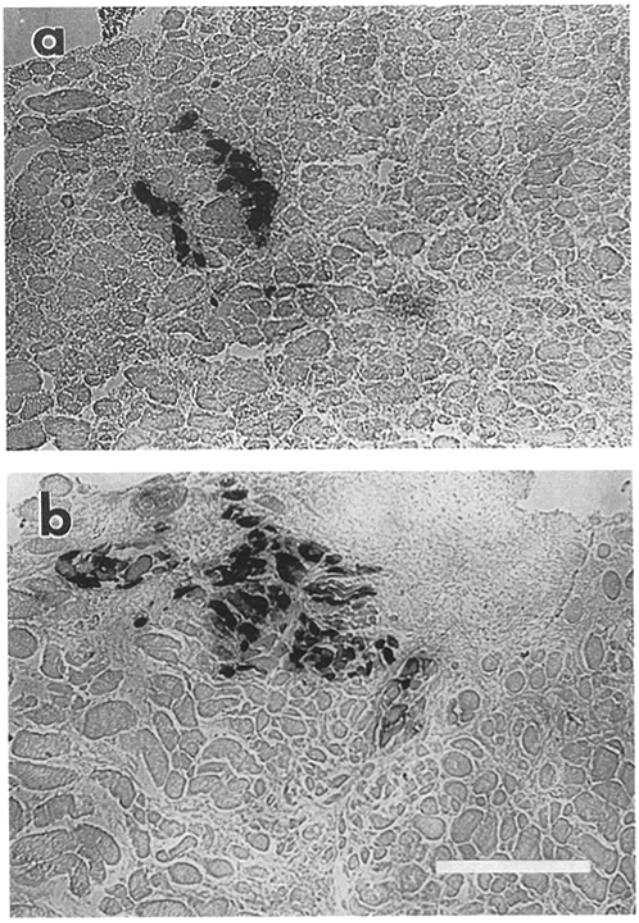

Figure 2. β-gal–positive muscle fibers following noncompatible transgenic primary muscle cell culture transplantation in *dy/dy* mice. X-gal staining by histochemistry. The new (donor/donor) or hybrid (host/donor) β-gal expressing fibers are less numerous when cells are injected in older animals without muscle pretreatment (*a*) than when cells are injected in younger animals after γ-irradiation and notexin pretreatment (*b*). Bar, 260 μm.

crease was observed when animals were kept longer after transplantation.

LAMA2 Expression. As previously reported, LAMA2 was absent from muscles of *dy/dy* mice (Arahata et al., 1993; Sunada et al., 1994; Xu et al., 1994*a*,*b*) and was never expressed in muscles only injected with HBSS (Fig. 3 *a*). LAMA2 surrounded normal mouse muscles (Fig. 3 *b*). LAMA2 was expressed around some muscle fibers following Tn-LacZ primary muscle cell culture transplantation. The total and relative numbers of LAMA2-positive fibers, however, depended on the age of the mouse at transplantation and on the pretreatment of the muscle (Table I). The percentage of LAMA2-positive fibers was low (mean ± SD, 6.4 ± 4.4) when older animals (i.e., more than 3 mo), were used as recipients (Fig. 3, *c* and *d*). The percentage of LAMA2-positive fibers was higher (15.9 ± 9.0) when younger animals (i.e., 6 wk old) were used as recipients (Fig. 3, *e* and *f*). Notexin alone, or γ-irradiation alone, did not increase the number of LAMA2-positive fibers. The combination use of notexin and γ-irradiation increased the percentage of LAMA2-positive fibers in transplanted muscles (27.8 ± 14.9). There was an overall but not significant increase in the percentage of LAMA2-positive fibers with the duration of the experiment (Table I, the mice in group A all received the same myoblast preparation).

Colocalization of β-gal and LAMA2. The Tn-LacZ primary muscle cultures originate from mice with normal LAMA2 expression. Thus, both the normal gene for LAMA2 and the reporter gene coding for β-gal are present in the donor cells. LAMA2 and β-gal were characterized on serial sections only spaced by 8 μm. As shown in Table I, the percentages of LAMA2-positive and β-gal–positive fibers were comparable when old animals (>3 mo) were used for transplantation. LAMA2 and β-gal labeling were localized in the same clusters of fibers (Fig. 4). Some LAMA2-positive fibers, however, were negative for β-gal expression, and some β-gal–positive fibers were negative for LAMA2 expression on serial sections (Fig. 4, *g* and *h*). Most of the

Table I. Outcome of Transplantation of Transgenic Primary Muscle Cell Culture in FK506-immunosuppressed Animals

Mouse No.	Age at Tx	Days after Tx*	Leg	Muscle treatment‡	Total number of fibers	β-gal–positive	LAMA2-positive	Percent β-gal–positive	Percent LAMA2-positive
	d								
A1	92	21	Left	None	1,655	53	85	3.2	5.1
A2	92	21	Left	None	1,187	88	97	7.4	8.2
A3	92	21	Left	None	1,165	28	45	2.4	3.9
			Right	Notexin	875	5	55	0.6	6.3
A4	92	21	Left	None	774	15	24	1.9	3.1
A5	92	39	Left	Notexin	920	58	128	6.3	13.7
A6	92	39	Left	None	1,125	103	165	9.1	14.7
A7	92	39	Left	None	1,277	25	47	1.9	3.7
B1	45	27	Left	γ + Notexin	932	3	162	0.3	17.4
			Right	None	1,454	70	204	4.8	14
B2	45	27	Left	γ + Notexin	1,161	51	511	4.4	44
			Right	None	1,375	58	355	4.2	25.8
B3	45	27	Left	γ + Notexin	1,505	85	285	5.6	18.9
			Right	None	1,468	48	118	3.3	8
B4	45	27	Left	γ	502	24	97	4.8	19.3
			Right	Notexin	895	8	115	0.9	12.8
B5	45	27	Left	γ	651	2	101	0.3	15.5
			Right	Notexin	953	21	193	2.2	20.3

Cultured cells were injected in one or both TA at the ages indicated, with or without muscle pretreatment. At the times indicated, the mice were sacrificed. The β-gal- and LAMA2-positive fibers were counted on the best section. *Tx, transplantation. ‡Notexin indicates notexin injection and γ, γ ray irradiation.

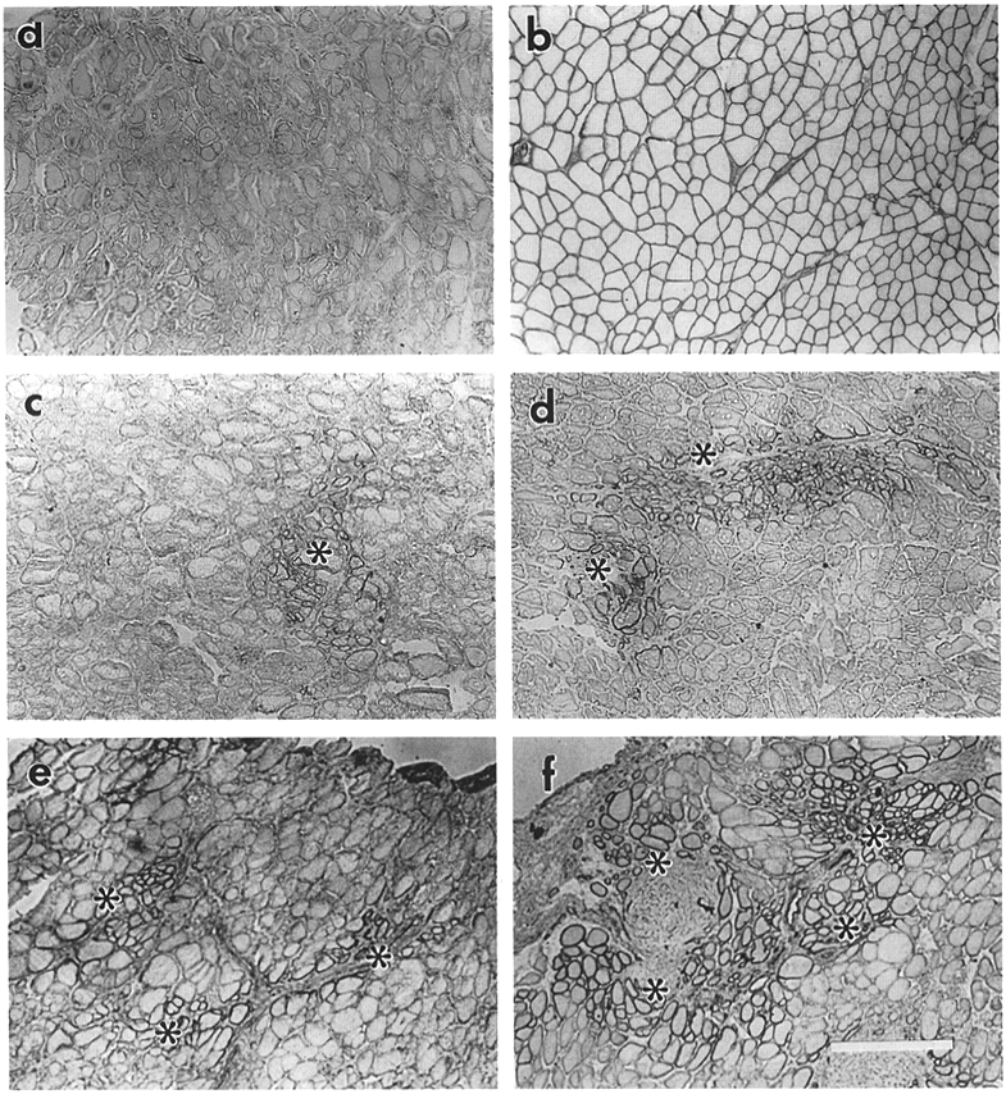

Figure 3. Localization of LAMA2 following noncompatible transgenic primary muscle cell culture transplantation in *dy/dy* mice. Immunoperoxidase labeling. LAMA2 is not present in non-injected or sham-injected *dy/dy* muscles (*a*) whereas it surrounds all the muscle fibers in normal animals (*b*). The percentage of LAMA2-positive fibers is low when cells are injected in older animals without pretreatment (*c*) or notexin pretreatment alone (*d*). The use of younger animals increases the percentage of LAMA2-positive fibers without pretreatment (*e*) or after γ-irradiation and notexin pretreatment (*f*). Bar, 260 μm.

smallest fibers were β-gal positive. These results indicated either that β-gal and LAMA2 expression were not regulated similarly, or that the same type of cells were not responsible for their expression.

When experiments were designed in younger animals (i.e., 6 wk old), the LAMA2-positive fibers were much more abundant than β-gal–positive fibers (Table I). Surprisingly, the pattern and intensity of β-gal and LAMA2 expression greatly differed. Some LAMA2-positive clusters of fibers were totally devoid of β-gal (Fig. 4, *a–f*). The β-gal–positive fibers were restricted to smaller areas than LAMA2-surrounded fibers. LAMA2-positive fibers were observed over long distances throughout the muscle, i.e., more than 1,000 μm, whereas no β-gal was observed in most of these fibers over all length of LAMA2 expression (Fig. 4, *a–f*).

Long-term Histocompatible Transplantations

Transplantation of histocompatible, isogenic primary mouse muscle cells in 6-wk-old male *dy/dy* mice led to long-term LAMA2 expression (i.e., 11 wk) in the transplanted muscles of all the animals. The percentage of LAMA2-positive muscles was greater when muscles were treated using notexin and γ-irradiation before transplantation (14.75 ± 6.4 without pretreatment versus 41.2 ± 14.3 with pretreatment). Notexin alone (12.75 ± 1.2 positive fibers), or irradiation alone (19.25 ± 6.9 positive fibers), followed by transplantation, was not sufficient to increase dramatically the percentage of LAMA2-positive fibers (Table II, Fig. 5, *a* and *b*). Given the small number of *dy/dy* mice available for these experiments, however, no statistical analysis was performed.

LAMA2 seemed to have spread centrifugally from the injection site (Fig. 5, *e* and *f*). While LAMA2 completely surrounded normal mouse muscle fibers, its localization was frequently incomplete and disrupted in transplanted *dy/dy* muscles, especially in case of the largest fibers (Fig. 5, *g* and *h*). Thus, LAMA2 deposition seemed to be localized (Fig. 5, *f* and *g*). LAMA2 deposition also presented important variations in transplanted mice as compared to normal mice. Most of the smallest fibers were totally surrounded by LAMA2 (Fig. 5 *g*).

The use of γ-irradiation and notexin induced some damages into the muscles (Fig. 4, *a–f*; Fig. 5 *b*), that were more pronounced in the long-term experiments. As previously

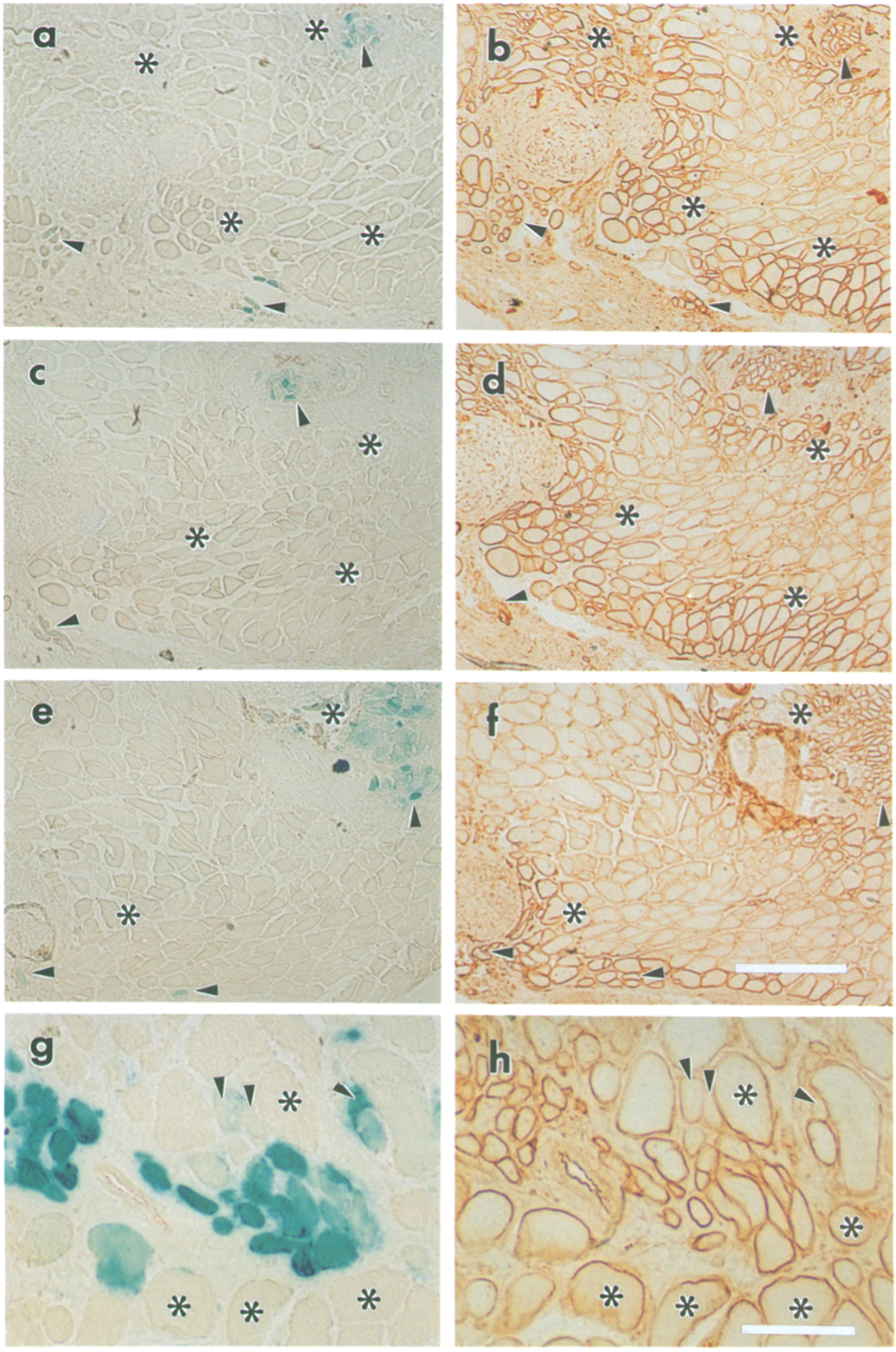

Figure 4. β-gal and LAMA2 coexpression in serial sections following noncompatible transgenic primary muscle cell transplantation in *dy/dy* mice. X-gal histochemistry (*left*) and LAMA2 immunohistochemistry (*right*). β-gal–positive clusters (*a*, *c*, and *e*, *arrowheads*) are less numerous than LAMA2-positive clusters (*b*, *d*, and *f*, *asterisks*). On low magnification pictures, β-gal–positive fibers are also LAMA2-positive, whereas LAMA2-positive are not always β-gal positive; *a* and *c* are spaced by 180 μm; *a* and *e* are spaced by 1,080 μm. At higher magnification of new or hybrid fibers (*g* and *h*), some LAMA2-positive fibers (*h*, *asterisks*) do not express β-gal, whereas some β-gal–positive fibers (*g*, *arrowheads*) do not express LAMA2. Bars: (*a–f*) 20 μm; (*g* and *h*) 80 μm.

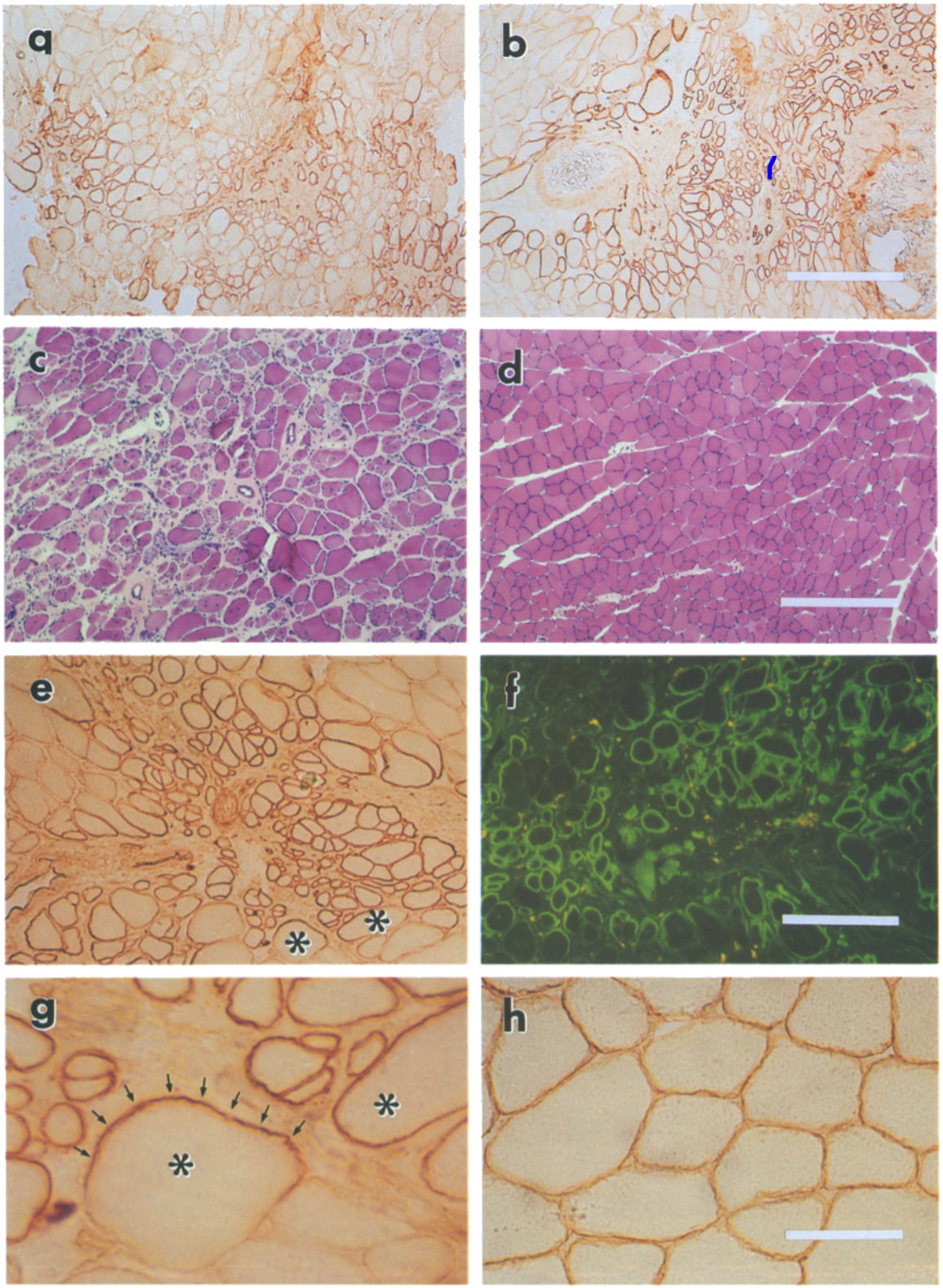

Figure 5. LAMA2 expression pattern in long-term histocompatible transplantation model. LAMA2 immunoperoxidase labeling (*a*, *b*, *e*, *g*, and *h*), hematoxylin-eosin staining (*c* and *d*), FITC immunostaining (*f*). The percentage of LAMA2-positive fibers is higher after γ-irradiation and notexin pretreatment (*b*) than without pretreatment (*a*). Note that γ-irradiation and notexin pretreatment induce muscle damages (*b*) that are particularly evident in long-term experiments. Noninjected and nonpretreated muscle of 4-mo-old *dy/dy* mouse, however, shows extensive fibrosis, infiltration, and the muscle fiber size, shape and distribution are variable (*c*). Compare to the TA muscle from a normal mouse (*d*). The pictures *e* and *f* show that LAMA2 is present at the injection sites in a centrifugal manner, that is, cells at the periphery are less positive than cells in the center, or positive in part of their perimeter. In *g* (high magnification of *e*, *asterisks* locate the same fibers), LAMA2 is only present at the side of the fiber facing the center of injection site (*arrows*). The thickness of the immunolabeling for LAMA2 is variable, as compared to normal mouse muscle (*h*). Bars: (*a–d*) 290 μm; (*e* and *f*) 115 μm; and (*g* and *h*) 55 μm.

Table II. Outcome of Long-term Histocompatible Primary Muscle Cell Transplantation without Immunosuppression

Mouse No.	Age at Tx*	Days after Tx	Leg	Muscle treatment‡	Total Number of fibers	LAMA2-positive	Percent LAMA2-positive
	d						
H1	45	77	Left	γ	1,238	178	14.4
			Right	Notexin	1,655	225	13.6
H2	45	77	Left	γ	1,396	336	24.1
			Right	Notexin	1,475	175	11.9
H3	45	77	Left	γ + Notexin	1,007	517	51.3
			Right	None	942	182	19.3
H4	45	77	Left	γ + Notexin	711	221	31.1
			Right	None	1,280	130	10.2

Cell cultures were injected in both legs after muscle pretreatment. The mice were killed 11 weeks after transplantation, and LAMA2-positive fibers were counted on the best section. *Tx, transplantation. ‡Notexin indicates notexin injection, and γ, γ ray irradiation.

described in the *mdx/mdx* mouse model, the *dy/dy* muscle weight was significatively decreased by 30% (mean weight, no pretreatment, 17 ± 2 mg; γ-irradiation and notexin 12 ± 2 mg; $P < 0.004$). It should be noted, however, that *dy/dy* muscles already show extensive fibrosis and cellular infiltration in the absence of irradiation and notexin pretreatment (Fig. 5, *c* and *d*).

Conditionally Immortalized Myoblast Culture Transplantation under FK506 Immunosuppression

Characterization of Immortalized Myogenic Cell Lines. The problem of tumorigenicity was frequently reported upon the use of the classical C2 mouse myoblast cell line (Wernig et al., 1991; Morgan et al., 1992). Thus, Morgan et

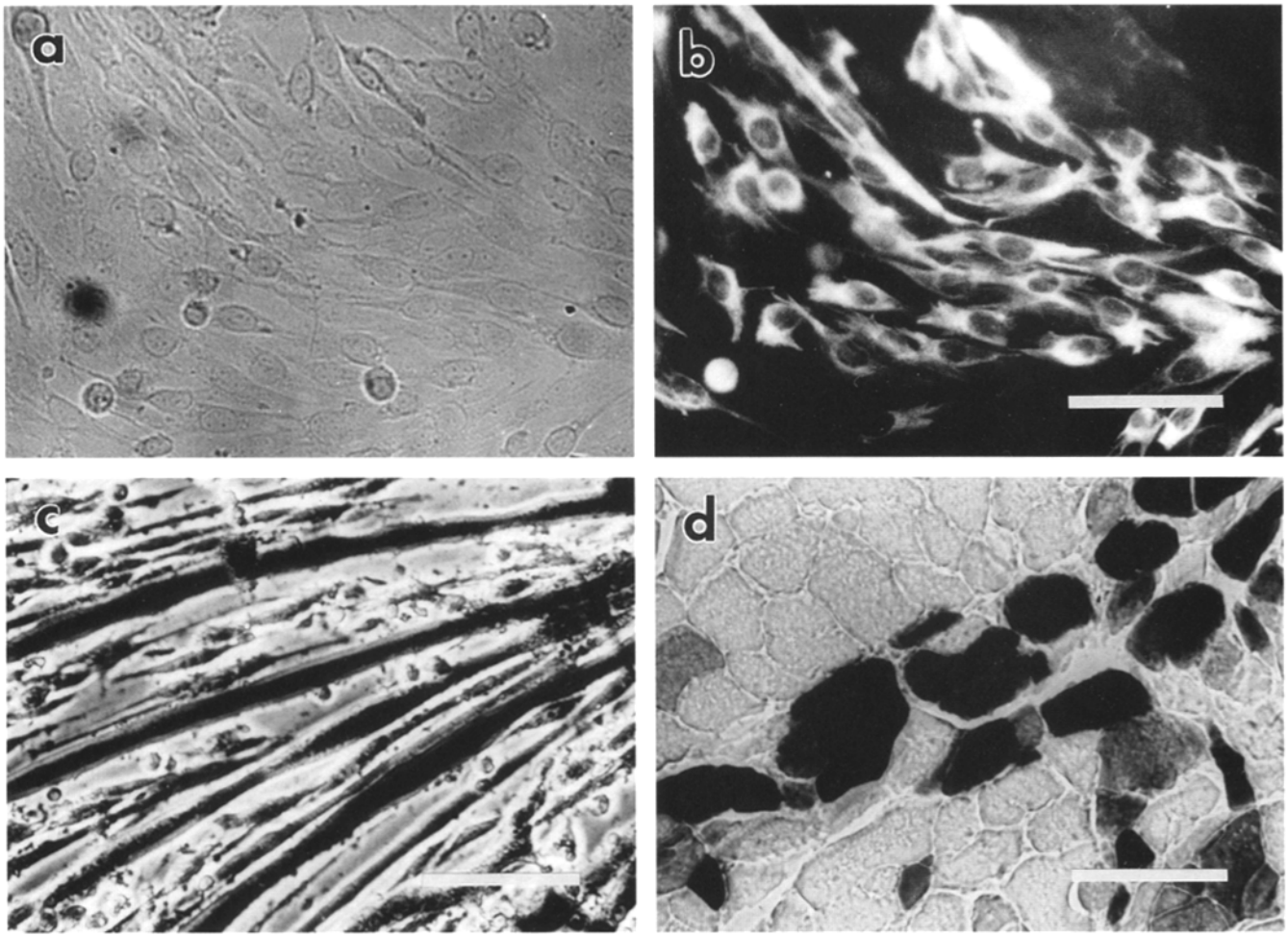

Figure 6. In vitro and in vivo characterization of conditionally immortalized myogenic cells. Most of the myogenic cells (*a*, clone MB7) express desmin (*b*, clone MB7) after some hours upon passage into differentiation medium. 3 d after passage in differentiation medium, these cells fuse and differentiate into myotubes which express the β-gal transgene (*c*, clone MB27). The myogenic cells participate in the formation of new or hybrid muscle fibers when injected into immunodeficient SCID mice (*d*, 1 mo after cell transplantation of clone MB27). Bars: (*a* and *b*) 30 μm; (*c*) 45 μm; and (*d*) 100 μm.

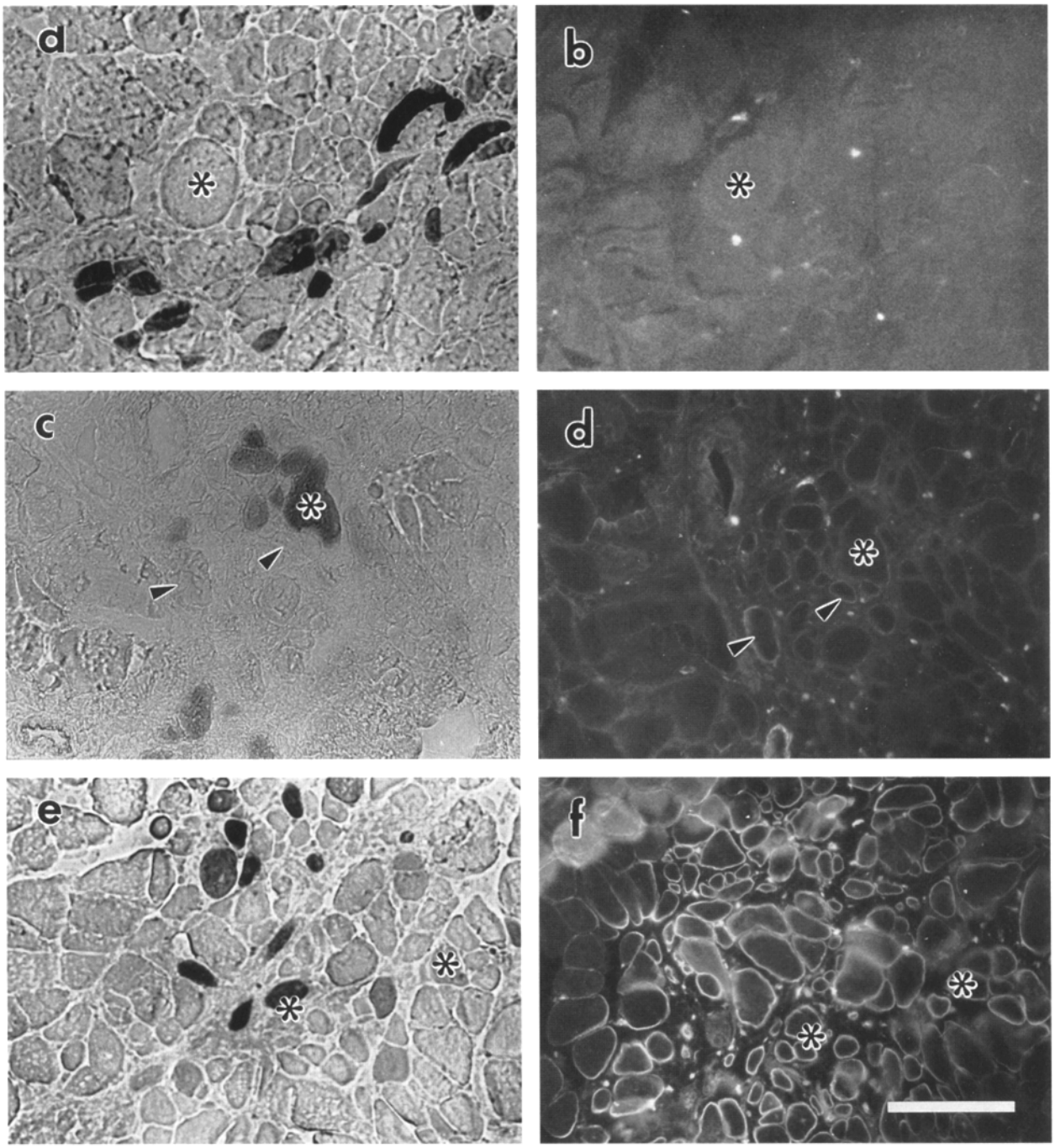

Figure 7. Injections of pure myogenic cells into *dy/dy* muscles. X-gal histochemistry (*a*, *c*, and *e*) and LAMA2 FITC immunostaining (*b*, *d*, and *f*). When injected into left *dy/dy* TA, myogenic clone MB27 is able to form β-gal–positive new or hybrid fibers (*a*). These fibers are not surrounded by LAMA2 on an adjacent section (*b*, asterisk locate the same fiber). When clone MB3 is injected into *dy/dy* muscles, β-gal–positive new or hybrid fibers are formed (*c*); a very faint LAMA2 labeling could be observed around two fibers (*d*, *arrowheads*) out of which only one is β-gal positive. When clone MB27 is injected together with compatible primary muscle cell culture in the right TA, β-gal–positive fibers are formed (*e*) and LAMA2 expression is observed at the injection site (*f*) around β-gal–positive and −negative fibers on adjacent sections (*asterisks* locate the same fiber). Bar, 100 μm.

al. (1994) developed a technique to obtain pure, immortalized myoblast clones with the ability to form new muscle fibers after transplantation into immunodeficient and dystrophin-deficient *nu/mdx* mice, without inducing tumors. The transgenic myoblast cultures developed in the present study have the additional advantage that differentiated cells express β-gal under the control of a specific muscle promoter. This allows the rapid identification of new or hybrid fibers formed by the fusion of these myoblasts in vitro or in vivo in any mouse model.

The muscle cells isolated as colonies or single clones from newborn muscles of (Tn-LacZ) X (H-2K^b-tsA58) mice were able to grow and proliferate at 33°C in a 10% CO_2 atmosphere when stimulated by murine IFN-γ. When

Table III. Characteristics of the Conditionally Immortalized Myogenic Cells

In vitro characterization				Injection in left leg*		Injection in right leg‡	
Cell culture	Passage No.	Desmin expression	β-gal expression	LAMA2-positive	β-gal–positive	LAMA2-positive	β-gal–positive
				n§	n‖	n§	n‖
MB3	9	pos (>95%)	myotubes	0 (1)¶	0 (1)	ND	ND
MB7	7	pos (>95%)	myotubes	0 (3)¶	3 (3)	2 (2)	2 (2)
Colony No. 24	4	pos (>90%)	myotubes	0 (1)	0 (1)	1 (1)	1 (1)
MB27	8	pos (>90%)	myotubes	0 (3)	3 (3)	3 (3)	3 (3)

Myogenic cells were selected in vitro based on desmin expression and the ability to form β-gal–positive myotubes in differentiation medium. The cells were injected in FK506-immunosuppressed, 4–8-wk-old *dy* mice with or without addition of primary muscle cell culture, 2, 3, or 4 wk later the mice were sacrificed. β-gal and LAMA2 expression were investigated on adjacent sections. Typical β-gal and LAMA2 expression are presented in Fig. 6.

*Only the myogenic clone or colony injected. ‡Myogenic clone or colony injected together with primary muscle cell culture. §Number of mice expressing LAMA2; (*n*) total number of mice in one experiment. ‖Number of mice expressing β-gal; (*n*) total number of mice in one experiment. ¶2or 3 fibers were faintly surrounded. ND: not done.

shifted to differentiation medium the muscle cells fused together and formed giant myotubes. These cells, either myotubes or myoblasts alone, expressed the intracellular filament desmin, which is an early marker of myoblasts (Fig. 6, *a* and *b*) (Lin et al., 1994). The myotubes and some early differentiated myoblasts expressed also β-gal (Fig. 6 *c*). The cells were able to fuse and to form new or hybrid muscle fibers expressing β-gal in vivo in immunodeficient SCID mice without forming solid tumors (Fig. 6 *d*). The colony No. 24 (two cells in the original cloning well) and the clones MB3, MB7 and MB27 were selected based on their in vitro myogenic characteristics and were injected into *dy/dy* muscles.

Lack of LAMA2 Expression Despite Muscle Fiber Formation after Myoblast Clone Transplantations. 2, 3, and 4 wk after injection of conditionally immortalized myoblast clones into left TA of *dy/dy* mouse, some β-gal positive fibers were formed (Fig. 7, *a* and *c*, respectively, clones MB27 and MB3, and Table III). The number of β-gal positive fibers was low possibly because the muscles were not pretreated with notexin and irradiation. On adjacent sections, however, essentially no LAMA2 could be detected in the same TA muscle, neither surrounding β-gal–positive fibers or β-gal–negative fibers (Fig. 7 *b*, clone MB27). LAMA2 was also absent from the series of sections preceeding and succeeding β-gal–positive sections, thus indicating that a putative difference in the restriction domain of LAMA2 and β-gal was not responsible for the lack of LAMA2 expression in this model. A very faint labeling could be observed around some, but not all muscle fibers (Fig. 7 *d*, clone MB3) and some of these faintly positive fibers were β-gal negative. Pure myoblasts were thus able to fuse together or with host muscle fibers and to trigger β-gal expression, but in most of the cases they did not trigger LAMA2 formation or extracellular deposition at least at levels detectable by immunohistochemistry or immunohistofluorescence (Table III). The right *dy/dy* TA of the same mouse, which was injected with a classical histocompatible primary cell culture together with the same myogenic clone MB27, however, contained many LAMA2-surrounded muscle fibers and/or β-gal-positive fibers (Fig. 7, *e* and *f*). The β-gal and LAMA2 proteins were not always located in and around the same fibers.

Lack of LAMA2 Expression after Fibroblast Permanent Cell Lines Transplantations. Permanent fibroblast cell lines S3T3 and BLK CL.4 (both from American Type Culture Collection) were transplanted into *dy/dy* mice but did not induce LAMA2 production (data not shown). These fibroblast lines, however, may not be representative of all cell types obtained in a primary muscle cell culture.

Discussion

Overall Transplantation Success

Three transplantation models were used in this study. In the first one, nonhistocompatible primary muscle cells expressing the β-gal under control of a muscular promoter were transplanted into *dy/dy* mice. This allogenic transplantation required an efficient immunosuppression, which was obtained using FK506 (Kinoshita et al., 1994*b*; Vilquin et al., 1995*b*). The use of β-gal expressing cells showed the exogenous origin of the labeled muscle fibers and allowed the comparison between LAMA2 and β-gal localization after transplantation. In the second model, histocompatible syngeneic primary cultures from normal littermates were transplanted into *dy/dy* mice. This model did not require immunosuppression and allowed the study of muscle regeneration over longer time. In the third model, pure but nonhistocompatible myoblasts were transplanted into one leg of FK506-immunosuppressed *dy/dy* mice, whereas the other leg received histocompatible primary culture as a control of LAMA2 expression. This model allowed the direct comparison of the outcome of transplantation between pure myoblast and primary muscle cells and allowed the investigation of the potential role of myoblasts in LAMA2 deposition.

Cell transplantation allowed the restoration of a structural, extracellular protein. In these immunologically controlled models the transplantation of primary muscle cells led to LAMA2 expression in variable amounts in all animals. The number of LAMA2-surrounded fibers was shown to depend on the age of the animal at the time of transplantation and on the muscle pretreatment. The highest numbers of LAMA2-surrounded fibers were obtained when young animals (6 wk old) received histocompatible cells after γ-irradiation and notexin pretreatment of the muscle. Irradiation was shown to hamper myoblast proliferation and thus reduce normal muscle regeneration, and to favor extensive fibrosis in *dy/dy* (Wirtz et al., 1982) and *mdx/mdx* (Wakeford et al., 1991) mice. This treatment greatly increased donor myoblast permeation into host degenerating fibers and the formation of new or hybrid dystrophin-positive fibers in *mdx/mdx* mice (Morgan et al.,

1990). In our models, it is thus likely that irradiation and notexin necrosis initially favor donor cell development in vivo over the *dy/dy* recipient cells.

In the histocompatible model LAMA2, which is the only known difference between *dy/dy* host mice and +/? donor mice, is not sufficient to trigger efficient acute rejection of LAMA2-expressing cells. Interestingly, the mice did not produce antibodies directed against LAMA2 (not shown). Chronic rejection was not assessed in this study, but the overall highest numbers and percentages of LAMA2-surrounded fibers were observed after the longest survival time (i.e., 11 wk after grafting). LAMA2 is undectable in immunohistochemistry but very low amounts of LAMA2 mRNA are detected in untreated *dy/dy* mice by RT-PCR (Arahata et al., 1993; Xu et al., 1994*a*). The reason why the transplanted animals are tolerant to LAMA2 is not known.

The intensity of β-gal staining and the number of β-gal–positive fibers after transgenic Tn-LacZ culture transplantation was low as compared to results obtained in other, non *dy/dy* mice strains under the same FK506 immunosuppressive treatment. In a previous report, up to 90% of *mdx/mdx* TA muscle fibers could express β-gal only one month after transplantation (Kinoshita et al., 1994*b*). The present results suggest that muscle cell transplantation is less efficient in *dy/dy* mouse than in *mdx*. Actually, more than 1,000 dystrophin-positive fibers are frequently obtained in only 2 mo after histocompatible primary muscle culture transplantation in *mdx/mdx* mice (Vilquin et al., 1995*c*), whereas it is difficult to obtain more than 300 to 400 LAMA2-surrounded muscle fibers in the same time in *dy/dy* mice. The humoral and cellular immune reactions against β-gal and MHC proteins are very efficiently controlled by FK506 in various transplantation models, animals, or mouse strains (Kinoshita et al., 1994*b*, 1995; Asselin et al., 1995; Vilquin et al., 1995*b*,*c*). Thus, immune rejection is not likely to be the cause of the relatively poor cellular engraftment in immunosuppressed *dy/dy* mouse as compared to *mdx/mdx* mouse. The *dy/dy* muscle fibers are smaller than normal muscle fibers and they show important size variation within a single muscle (Michelson et al., 1955; see also Fig. 5, *c* and *d*). This may reflect trophic problems in *dy/dy* muscles related to the absence of LAMA2 in basal lamina that could explain the variation in regeneration efficiency between the *mdx/mdx* and the *dy/dy* models. First, the initial lack of LAMA2 could hamper myoblast migration or alignment either with other myoblasts or with host muscle fibers, because basal lamina is known to play important roles in the control of migration, proliferation and differentiation of various cell types (Engvall et al., 1992). Thus, the lack of LAMA2 could hamper regeneration. Muscle regeneration could depend on the presence of a pre-existing complete basal lamina. Second, an impaired muscle innervation due to the absence of LAMA2 around peripheral nerve fibers or neuromuscular junctions (Leivo and Engvall, 1988; Sunada et al., 1994; Xu et al., 1994*a*) could induce a muscle atrophy. Third, the myoblast proliferation and/or migration could be impaired by the abundant connective tissues, present especially in the oldest *dy/dy* mice. Taken together, these problems may explain why muscle regeneration was relatively poor following transplantation, whatever the pretreatment used.

LAMA2 and β-gal Colocalization after Transplantation of Transgenic Primary Cells

In transplanted muscle, LAMA2 was generally more widely distributed than β-gal. This could indicate differences in the regulation of expression of these two proteins, that LAMA2 but not β-gal can diffuse extracellularly, or that the cell type responsible for LAMA2 expression is not the cell type responsible for β-gal expression. Although the *β-gal* gene in the TnI-LacZ1/29 mice is under the control of the promoter of the quail fast troponinI, the β-gal was reported to be expressed in all fiber types by Hallauer et al. (1993). Moreover, transgenic β-gal myoblast transplantation into the TA of *mdx/mdx* mice under FK506 immunosuppression was reported to lead to the strong expression of β-gal in as much as 90% of the fibers within one month, thus probably including both fast and slow muscle fibers (Kinoshita et al., 1994*b*). β-gal expression is restricted to skeletal muscle cells as an intracellular protein, and its nuclear domain is ~1,000 μm (Kinoshita, I., personal results). In contrast, laminins and likely LAMA2 are secreted outside of the cells, and may have a relatively long half-life in vivo (Engvall, 1993) as components of muscle cells basal lamina. Thus, the fate of β-gal and LAMA2 in vivo is probably different. Whereas β-gal expression requires myoblast fusion and formation of hybrid or new muscle fibers, the developmental mechanisms of LAMA2 expression are still unclear.

β-gal and LAMA2 did not always colocalize in and around the same segments of muscle fibers. On serial sections β-gal–positive segments of fibers could be LAMA2 negative and vice versa. Thus, either myoblasts and muscle fibers do express both β-gal and LAMA2, or these proteins are expressed by several or only partially overlapping different cell types, or by cells at different stages of differentiation. If muscle cells express both LAMA2 and β-gal, then β-gal–positive fibers should be surrounded by LAMA2, even on short muscle fiber segments. This was not always observed. The other hypothesis implies that myoblasts and muscle fibers should express β-gal, whereas other cells should secrete LAMA2. Depending on the presence and development of myoblasts, or LAMA2-secreting cell, or both cell types in the vicinity of host muscle fibers after transplantation of primary muscle cell culture, β-gal–positive, or LAMA2 positive, or both β-gal– and LAMA2-positive fibers should be observed.

LAMA2 Expression Pattern

LAMA2 generally deposited continuously around small caliber muscle fibers, whereas this deposition was frequently discontinuous around normal diameter muscle fibers, even two months after transplantation (Fig. 5, *e* and *f*). In contrast, dystrophin was expressed along the complete inner circumference of muscle fibers by some weeks after transplantation, and dystrophin disruption was rarely observed one or several months after transplantation in the *mdx/mdx* or SCID mouse models (Partridge et al., 1989; Morgan et al., 1990, 1993, 1994; Huard et al., 1994; Kinoshita et al., 1994*b*; Vilquin et al., 1995*c*). The expression of LAMA2 seemed to occur in a radial manner from the site of injection. This observation suggests that competent cells could proliferate and diffuse, or that the secre-

tion product may diffuse, thus producing a gradient of LAMA2 expression that would progressively accumulate around the most proximal muscle fibers.

Role of Myoblasts in LAMA2 Restoration in dy/dy Mice

Cloned myogenic cells formed new or hybrid β-gal–positive muscle fibers following transplantation into *dy/dy* mice. Small and normal caliber β-gal–positive fibers were obtained already 2 wk after transplantation. Small-caliber fibers were likely entirely of donor origin. Normal-caliber fibers probably originated from the fusion of donor myoblasts with host muscle fibers undergoing a segmental degeneration-regeneration process at the site of injection. The β-gal–positive fibers, however, were very rarely surrounded by LAMA2, thus indicating that myoblasts and muscle fibers formed by them were not able to secrete LAMA2, at least to levels detectable by immunohistochemistry and immunohistofluorescence. As a control for LAMA2 expression, primary cells from mixed cultures injected in the contralateral leg were able to trigger LAMA2 deposition around new and hybrid muscle fibers within 2 wk. Thus, LAMA2 may be secreted by some competent cells present in primary culture, but not in cloned myoblasts. Another hypothesis which cannot be ruled out yet, is that none of the myogenic clones used in this study was able to produce LAMA2 in vivo, despite their ability to express desmin, to express β-gal under control of a muscular promoter, to form myotubes in vitro and to form muscle fibers in vivo. Indeed, some distinct populations of satellite cells have been described in regenerating rat skeletal muscle (Rantanen et al., 1995). The secretion of LAMA2 could be dependent on cell differentiation status and could be regulated in myogenic cells, as are other muscle proteins and myogenic factors (Rantanen et al., 1995). This hypothesis would be supported by the very faint labeling observed around 2 or 3, but not all, muscle fibers following clone MB3 and clone MB7 transplantation, and is currently under investigation.

Future Prospects

The transplantation models and results presented in this article should prove useful to study in vivo interactions between muscle cells and extracellular matrix. The critical steps for normal and pathologic muscle regeneration, that is, myoblast proliferation, alignment or fusion, or the stability of nerve–muscle regulations, should be discriminated.

The identification of the cell type responsible for LAMA2 secretion and its relation with other muscle constituents are important future issues, and could help to categorize clinical cases of CMD and develop new strategies for the correction of muscular diseases by gene complementation. Long-term experiments should also be designed to improve the transplantation results. The feasability of myelination restoration in the peripheral nervous system of the *dy/dy* mouse should be assessed (Bradley and Jenkison, 1973; Sunada et al., 1995). Alternatively, the transducibility of the LAMA2-secreting cell type by different gene vectors (adenoviruses, retroviruses, herpesviruses) should indicate if it could constitute a good target candidate for in vivo or ex vivo gene therapy of CMD.

Pancreatic islet cells, embryonic neurons, myoblasts, hematopoietic bone marrow stem cells transplantations are currently investigated as potential avenues for the treatment of inherited or acquired diseases. Only further studies will indicate if some extracellular matrix pathologies could be rescued by cell transplantation.

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