Genetic Analysis of α_4 Integrin Functions in the Development of Mouse Skeletal Muscle

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Abstract. It has been suggested, on the basis of immunolocalization studies in vivo and antibody blocking experiments in vitro, that α_4 integrins interacting with vascular cell adhesion molecule 1 (VCAM-1) are involved in myogenesis and skeletal muscle development. To test this proposal, we generated embryonic stem (ES) cells homozygous null for the gene encoding the α_4 subunit and used them to generate chimeric mice. These chimeric mice showed high contributions of α_4-null cells in many tissues, including skeletal muscle, and muscles lacking any detectable ($<2\%$) α_4-positive cells did not reveal any gross morphological abnormalities. Furthermore, assays for in vitro myogenesis using either pure cultures of α_4-null myoblasts derived from the chimeras or α_4-null ES cells showed conclusively that α_4 integrins are not essential for muscle cell fusion and differentiation. Taking these results together, we conclude that α_4 integrins appear not to play essential roles in normal skeletal muscle development.

DEVELOPMENT of skeletal muscle is a multistep process. During embryogenesis, myogenic precursor cells in the somites form myoblasts that proliferate, migrate, and eventually exit the cell cycle and differentiate into postmitotic multinucleated myotubes. The formation of mature muscle involves a biphasic fusion process. Primary myoblasts first fuse with one another to form primary myotubes; secondary myoblasts then proliferate and progressively fuse to form secondary myotubes juxtaposed to the primary myotubes. Eventually, the myotubes, most of which are secondary myotubes, specialize as fast and slow contracting fibers and become striated and innervated (Kelly and Rubenstein, 1994).

In each stage of skeletal muscle development, cell–cell and cell–extracellular matrix adhesion are thought to play critical roles. Inhibition of in vitro myogenic differentiation by antibodies has been interpreted to suggest that adhesion molecules such as NCAM (Knudsen et al., 1990*a*) and N-cadherin (Knudsen et al., 1990*b*) are involved. Similarly, myogenic differentiation can be inhibited in culture with antibodies against α_4 integrins or their counterreceptor vascular cell adhesion molecule 1 (VCAM-1)[1] (Rosen et al. 1992). These adhesion receptors have also been implicated in secondary myotube formation based on their patterns of expression during development (Rosen et al., 1992; Sheppard et al., 1994). Finally, studies of expression patterns have also implicated $\alpha_7\beta_1$ integrin (Song et al., 1993) and M-cadherin (Cifuentes-Diaz et al., 1995) in secondary myogenesis.

Integrins are $\alpha\beta$ heterodimeric transmembrane glycoproteins that mediate cell adhesion to extracellular matrix by interacting with a number of extracellular matrix proteins such as fibronectin, laminin, collagen, and vitronectin. Integrins also mediate cell-cell adhesion by interacting with members of the immunoglobulin superfamily (Hynes, 1992). The integrin family so far has >20 known members, assembled from various combinations of 16 α subunits and 8 β subunits. Among these integrins, those containing the α_4 subunit have been implicated in skeletal muscle development and in other developmental and physiological processes such as hematopoiesis (Williams et al., 1991; Miyake et al., 1991; Papayannopoulou and Brice, 1992; Hemler, 1990; Arroyo et al., 1996), lymphocyte homing (Holzmann et al., 1989; Berlin et al., 1993), recruitment of inflammatory cells (Yednock et al., 1992), and metastasis (Qian et al., 1994). The integrin α_4 subunit can be assem-

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1. *Abbreviations used in this paper:* ES, embryonic stem; GPI, glucose phosphate isomerase; LIF, leukemia inhibitory factor; VCAM-1, vascular cell adhesion molecule 1.

bled into two integrin heterodimers, $\alpha_4\beta_1$ and $\alpha_4\beta_7$. Both $\alpha_4\beta_1$ and $\alpha_4\beta_7$ are fibronectin receptors that bind to a non-RGD peptide in an alternatively spliced V25 (or CS-1) region (Guan and Hynes, 1990; Mould et al., 1990; Ruegg et al., 1992). Both $\alpha_4\beta_1$ and $\alpha_4\beta_7$ also bind to VCAM-1, a member of the immunoglobulin superfamily (Chan et al., 1992).

To study the in vivo functions of α_4 integrins further, we have previously generated a null mutation in the gene encoding the α_4 subunit and shown that the mutation is recessive embryonic lethal. The resulting embryonic defects show that α_4 integrins have important functions in two cell–cell adhesion events during embryogenesis: allantois-chorion fusion during placental development, and epicardium-myocardium attachment during cardiac development (Yang et al., 1995). However, it was not possible to address the question of whether α_4 integrins play an essential role in skeletal muscle development, since the embryos deficient in α_4 integrins died at gestation days 11–13, whereas skeletal muscles form around gestation day 13.

To examine the role of α_4 integrins in muscle development, in this study we generated chimeric mice using α_4 integrin–deficient embryonic stem (ES) cells to circumvent the problem of embryonic lethality. We report that α_4 integrin–deficient ES cells are able to contribute to adult skeletal muscle in high proportion. Skeletal muscle derived primarily, if not exclusively, from α_4 integrin–deficient ES cells displays no obvious morphological abnormalities. In addition, we show that myoblasts and ES cells completely lacking α_4 integrins are both fully competent to differentiate into myotubes in vitro. These results suggest that, in contrast with the implication of previous reports, α_4 integrins are not essential for skeletal muscle differentiation during development.

Materials and Methods

Growth, Selection, and Differentiation of ES Cells Mutant in the α_4 Integrin Gene

The methods for growth of ES cells were described previously (George et al., 1993; George and Hynes, 1994). The D3 line of ES cells (Doetschman et al., 1985) was cultured on irradiated embryonic fibroblast feeder cells in ES cell medium, which contains DME (high glucose with 26 mM Hepes, pH 7.5, 14 mM sodium bicarbonate), 15% FBS (Intergen Co. Purchase, NY), 1× nonessential amino acids (GIBCO BRL, Gaithersburg, MD), and 0.1 mM β-mercaptoethanol, supplemented with 1,000 U/ml leukemia inhibitory factor (LIF) (ESGRO; GIBCO BRL).

A heterozygous α_4 integrin knockout ES cell line, 182 (Yang et al., 1995), was expanded and selected with 1 mg/ml or 1.5 mg/ml G418 (GIBCO BRL). After 7–9 d of selection, the drug-resistant colonies were picked and expanded on feeder cells. Half of the cells from each colony were frozen in 10% DMSO in FBS and half were lysed for extraction and analysis of DNA.

For differentiation, ES cells were trypsinized and resuspended in ES medium without LIF. The cell suspension was plated and incubated under standard tissue-culture conditions (the same for all the incubations below) for 30 min to remove the feeder cells. Unattached ES cells were removed from the plate, spun down, and resuspended in LIF-free ES medium at 1 × 10⁴ cells per ml. 10–μl drops, each containing ~100 ES cells, were put onto a cover of a large bacterial petri dish. The cover was placed onto a petri dish containing medium to maintain humidity, and the petri dish with the hanging drop ES cultures was incubated at 37°C. After 2 d the hanging drops of ES cells were washed into LIF-free ES medium in the petri dish and incubated for an additional 2 d. Embryoid bodies formed from the ES cells and were plated into 24-well plates. Each well contained 1 ml LIF-free ES medium and a coverslip coated with 0.1% gelatin (type

II swine gelatin; Sigma Chemical Co., St. Louis, MO). Two embryoid bodies were plated in each well. The embryoid bodies were incubated for 30 d with daily feeding with LIF-free ES medium. The differentiated ES cells were fixed in methanol for 5 min, and immunohistochemical staining was performed with an antibody against skeletal muscle myosin heavy chain (MY32; Sigma Chemical Co.), using the Vectastain Elite ABC kit from Vector Laboratories, Inc. (Burlingame, CA).

For generating chimeric mice, ES cells were prepared and injected into C57BL/6J blastocysts as described by George et al. (1993) and George and Hynes (1994). Blastocysts were manipulated as described (Bradley, 1987). Chimeric progeny were identified by coat color. The efficiencies of generation of chimeras from α_4-null, α_4-heterozygous, and wild-type ES cells were all equivalent.

DNA Extraction, Southern Blot, and PCR Analyses

ES cells or myoblast cells were washed with PBS (140 mM NaCl, 3 mM KCl, 4 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2) and lysed with lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.5, 5 mM EDTA. 0.5% SDS, and 0.25 mg/ml proteinase K) for 30 min at 37°C. The lysates were stored at −20°C. DNA was prepared from the lysates by phenol extraction and ethanol precipitation, and redissolved in 50 μl of 10 mM Tris (pH 7.5) and 0.1 mM EDTA. Southern blot analyses were carried out as described elsewhere (Church and Gilbert, 1984), using a 1.4-kb KpnI/PstI fragment 5' of the targeting vector as a probe for genotyping the ES cells (Yang et al., 1995).

PCR reaction conditions were essentially those described by the manufacturer of Taq polymerase (Perkin-Elmer Cetus Corp., Norwalk, CT), except that primers were annealed for 2 min at 55°C in each cycle and 40 amplification cycles were performed. The oligonucleotide primers for PCR were as follows: α_4 integrin wild-type primers are 5'-CACTAC-GATCGTCCGCTGTG-3' and 5'-GAAGTACAACAACAGCATCA-CCG-3'; Neo primers (for control) are (5'-GACAATCGGCTGCT-CTGA-3' and 5'-GCAGGCATCGCCATG-3'. The amplified PCR products were separated by electrophoresis on 1% agarose gels and analyzed by Southern blot hybridization (Church and Gilbert, 1984) using a probe for the α_4 integrin wild-type allele (a 950-bp PstI/BsmI fragment; see Yang et al., 1995). For controls, the blot was rehybridized with a 650-bp PstI/BamHI fragment containing the coding sequence of the neomycin gene (Yenofsky et al., 1990).

Glucose Phosphate Isomerase Analysis of Muscle

Glucose phosphate isomerase (GPI) analysis was performed on extracts of whole muscle and on extracts from muscle cryosections. For whole muscle analysis, muscles were dissected, frozen in liquid N₂, and stored at −80°C. The samples, each ~20 μl, were thawed and ground in 200 μl ddH₂O in Eppendorf tubes using a mini-pestle (Kontes Co., Vineland, NJ). Intact cells were then lysed by four cycles of freezing and thawing (5 min on dry ice, 5 min at 37°C). The suspension was spun at 3,000 rpm (700 g) for 10 s, and 0.5–1 μl of lysate, diluted to a final volume of 5 μl in ddH₂O, was used in the GPI assay.

For analysis of muscle cryosections, muscles were dissected, embedded in mounting medium (O.C.T. compound; Miles Laboratories, Elkhart, IN), and frozen in isopentane (2-methyl-butane, Aldrich Chemical Co., Milwaukee, WI) cooled in liquid nitrogen. The muscles were cryosectioned along their entire lengths. Every 500 μm, one 30-μm section was collected into a 0.65-ml microcentrifuge tube (cooled to −20°C in the cryostat) and stored at −80°C. The sections were thawed by the addition of 5–μl of ddH₂O to each tube, the tissue was subjected to repeated freeze/thaw cycles as above, and the entire sample was used in the GPI assay.

GPI assay was performed essentially as described by Bradley (1987). Briefly, samples were applied (with a Super Z applicator kit, 4088; Helena Laboratories, Beaumont, TX) to a cellulose acetate plate (Titan III 3023; Helena Laboratories) presoaked in 1× TG buffer (25 mM Tris, 200 mM glycine, ammonia-free, pH 8.5). The plate was then subjected to electrophoresis (Zip Zone chamber, 1283; Helena Laboratories) in 1× TG buffer at 150 V and 4°C for 1.5 h. The substrate mixture for staining the isozymes contained 2 ml 0.2 M Tris-HCl, pH 8.0, 0.1 ml 54.1 g/liter magnesium acetate, 0.1 ml 100 mg/ml fructose-6-phosphate (F3627; Sigma Chemical Co.), 0.1 ml 10 mg/ml NADP (Na₂ salt, N0505, Sigma Chemical Co.), 0.1 ml 10 mg/ml methylthiazolium tetrazolium (MTT) (M2128; Sigma Chemical Co.), 0.05 ml 2.5 mg/ml phenazine methosulphate (PMS), (P9625; Sigma Chemical Co.). Right before use, 8 μl glucose-6-phosphate dehydrogenase (G5885; Sigma Chemical Co.) and melted 1.5% agarose (in water) were added to the substrate mixture. The substrate mixture was poured onto

the plate, and the plate was left in the dark until the isozymes were visualized. The plate was photographed, and the photograph was used for densitometric analysis.

Densitometric analysis of the GPI assays was performed using 1D-multi-lane Scan of the IS-1000 Digital Imaging System (Alpha Innotech, San Leandro, CA). Percentage of the ES cell–derived (129/Sv) isoform of GPI was calculated as:

$$\frac{\text{area of the upper band} + 1/2 \text{ area of the middle band}}{\text{total area of the three GPI bands}} \times 100\%.$$

Histological Analysis of Muscle

For some muscles, both histological and biochemical analyses were performed on serial cryosections (Rando et al., 1995). In those muscles, for each section collected for GPI analysis, adjacent 10-µm serial sections were collected onto a gelatin-coated glass slide for histological analysis. The sections were stained with hematoxylin and eosin and mounted in Pro-Texx mounting medium (American Scientific Products, McGraw Park, IL). Microscopic analysis and photography were done using a microscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY).

Isolation of Wild-type and α_4 Integrin–deficient Myoblasts

Muscles from mature wild-type (C57BL/6J) and chimeric mice were dissociated to isolate pure populations of myoblasts (Rando and Blau, 1994). Briefly, the muscles were minced and enzymatically dissociated with a mixture of collagenase (class II, 1%; Boehringer Mannheim Corp., Indianapolis, IN) and dispase (grade II, 2.4 U/ml; Boehringer Mannheim Corp.). Primary cultures were plated and passaged in growth medium consisting of F-10 nutrient mixture (GIBCO BRL), 20% FBS (Hyclone Laboratories, Logan, UT), 2.5 ng/ml basic FGF (Promega Corp., Madison, WI), and penicillin (200 U/ml)/streptomycin (200 µg/ml) (GIBCO BRL). From these primary cultures, myoblasts were enriched to ~100% by preplating and growth in this selective growth medium (Rando and Blau, 1994). The cells were identified as myogenic by staining with an antibody to desmin (Kaufman and Foster, 1988).

From the myoblast cultures of chimeric mouse muscle, α_4 integrin–deficient myoblasts were purified by selection in 50 µg/ml G418 for a week to allow the growth only of those cells expressing the neomycin-resistance gene from the knockout vector. After G418 selection, an aliquot of the cells was analyzed by PCR and Southern blot analysis to confirm the efficacy of the selection process to eliminate all wild-type cells and to purify α_4 integrin–deficient cells. The myoblast cultures were maintained in G418 indefinitely to maintain selection pressure.

Analysis of Myoblast Differentiation In Vitro

For studies of differentiation and fusion, myoblast cultures were placed in differentiation medium (DME [GIBCO BRL], 2% horse serum [Hyclone Laboratories], and penicillin/streptomycin). In this medium, primary myoblast cultures differentiate and fuse into multinucleated myotubes. The fusion index, a measure of the propensity of the cells to fuse, was determined microscopically as the ratio of the number of nuclei in myotubes (elongated cells with greater than or equal to three nuclei) to the total number of nuclei in a field. 20 random fields at a magnification of 250 were analyzed for each cell type. The indices were calculated as the mean fusion index at each time, ± SD. For photography, the differentiated culture was fixed for 5 min in 4% paraformaldehyde, stained with 0.01% methylene blue in a solution of 50% ethanol, rinsed with water, stained with hematoxylin and Scott's water, and photographed without phase contrast.

Results

Generation of Chimeric Mice Derived from α_4 Integrin–deficient (α_4-Null) ES Cells

We have previously generated an α_4 integrin null mutation in a D3 ES cell line (Yang et al., 1995) by replacing the first coding exon of the α_4 integrin gene with a neomycin-resistance cassette, which carries a mutant form of the neomycin gene (Yenofsky et al., 1990). The ES cell lines carrying the α_4 integrin null mutation are heterozygous. To obtain ES cells homozygous for the mutation, one of the heterozygous ES cell lines was selected with G418 at a concentration (1–1.5 mg/ml) five times higher than that used for the original selection (Yang et al., 1995). The G418-resistant ES colonies were picked and screened by Southern blot analysis, and 91% of the drug-resistant colonies ($n = 46$) were homozygous for the α_4 integrin knock-out mutation (data not shown). Two independent α_4-null ES cell lines (4D20 and 4D47) were expanded and confirmed by Southern blot analysis to be homozygous for the α_4 integrin null mutation (Fig. 1). Further Southern blot analyses also showed that the vicinity of the mutated genomic region was not altered by the selection (data not shown).

These two α_4-null ES cell lines were used for producing chimeric mice by injecting the ES cells into blastocysts derived from wild-type C57BL/6J females. The chimeric mice had both black (from C57BL/6J) and agouti (from 129/Sv-derived ES cells) coat colors. As estimated from the agouti coat color, the contribution of α_4-null ES cells in the chimeric mice ranged from 20–100%. These chimeric mice appeared as healthy and active as control mice generated by injecting wild-type D3 ES cells or α_4-heterozygous ES cells (line 182) into wild-type C57BL/6J blastocysts. We have also observed germline transmission of the α_4-null allele from a male chimera. Thus, many cell types, including mesenchymal cells and sperm, can develop without α_4 integrins. The skeletal and cardiac muscle tissues from mice with 50–100% ES cell contributions were further analyzed.

α_4-Null ES Cells Were Able to Contribute to Chimeric Muscle Tissues

Contributions of α_4-null ES cells in skeletal and cardiac muscle tissues were examined using GPI markers (Fig. 2 A).
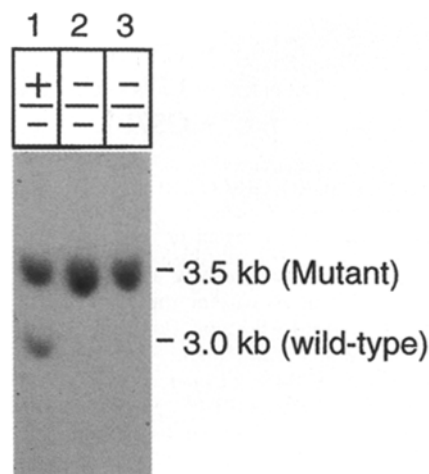

Figure 1. Southern blot analysis of ES cells homozygous for the α_4 integrin knockout mutation. DNA extracted from ES cells of various lines was digested with PstI. The 3.0-kb fragment was derived from the wild-type allele; the 3.5-kb fragment was derived from the mutated allele. (Lane 1), α_4-heterozygote, clone 4D10; (lanes 2 and 3) α_4-null, clones 4D20 and 4D47, respectively.

GPI has different isozyme forms, GPI-I^{aa} and GPI-I^{bb}, respectively, in 129/Sv and C57BL/6J strains, and these isozymes can be separated as distinct bands by electrophoresis on a cellulose acetate plate. Since GPI isozymes are dimers, a hybrid band (GPI-I^{ab}) is formed as a result of heterodimerization between the two isozymes in syncytial skeletal muscle tissues (Fig. 2 A, lanes 3–6). To quantitate the contribution of ES cells in the muscle tissues, the intensities of the GPI bands were determined by densitometric scanning, and the percentage of GPI-I^a isozyme (129/Sv) from each muscle sample was calculated. This analysis was performed on skeletal and cardiac muscle tissues of 29 chimeric mice including 18 α null (13 from line 4D20 and five from Line 4D47), four α_4 heterozygous (line 182), and seven wild-type (D3) ES cell derived. The results are summarized in Table I and show that the α_4-null cells derived from two independent ES cell lines were able to

Figure 2. GPI assays. (A) GPI assays on skeletal and cardiac muscle tissues from α_4-null ES cell–derived chimeric mice. (Lanes *1–7*) Skeletal muscle; (lane *8*) cardiac muscle. (Lane *1*) 129/Sv wild type; (lane *2*) C57BL/6J wild type; (lanes *3–8*) $\alpha_4^{-/-}$; $\alpha_4^{+/+}$ chimeras (lane *3*, CM6; lane *4*, CM11; lane *5*, CM39; lane *6*, CM40; lane *7*, CM45; lane *8*, CM45). (*Upper band*) GPI-I^{aa}; (*lower band*) GPI-I^{bb}; (*middle band*) GPI-I^{ab}. Notice that the GPI-I^{ab} and GPI-I^{bb} bands are not detectable in lane *7*, indicating that this chimeric muscle is >98% 129/Sv $\alpha_4^{-/-}$. (B) GPI assay of sections of skeletal muscle from a single $\alpha_4^{+/+}$; $\alpha_4^{-/-}$ chimera with a high percentage contribution of $\alpha_4^{-/-}$ cells. (Lanes *1–6*) Muscle sections from one $\alpha_4^{+/+}$; $\alpha_4^{-/-}$ chimeric muscle. Notice the GPI-I^{ab} and GPI-I^{bb} bands are not detectable in these lanes. (Lane *7*) Control chimeric muscle showing the bands of all isoforms. (*Upper band*) GPI-I^{aa}; (*lower band*) GPI-I^{bb}; (*middle band*) GPI-I^{ab}.

Table I. Percentage Contributions of $\alpha_4^{-/-}$ Cells in Chimeric Skeletal and Cardiac Muscles

	ES clone injected	Coat color*	Skeletal muscle†	Cardiac muscle†
$\alpha_4^{+/+}; \alpha_4^{-/-}$				
CM1	4D47	80	>98	29
CM6	4D47	90	85	51
CM11	4D20	70	89	49
CM12	4D20	70	>98	51
CM19	4D20	100	>98	64
CM21	4D20	95	>98	47
CM25	4D20	100	>98	57
CM39	4D20	95	87	59
CM40	4D20	90	88	55
CM41	4D20	90	97	51
CM45	4D20	100	>98	50
CM44	4D20	100	97	ND
CM73	4D47	100	67	ND
CM74	4D47	100	>98	ND
CM75	4D47	70	73	ND
CM28	4D20	100	>98	ND
CM51	4D20	100	>98	ND
CM52	4D20	95	>98	ND
$\alpha_4^{+/+}; \alpha_4^{+/-}$				
Control 1	182	100	76	50
Control 2	182	95	83	63
Control 3	182	70	50	42
Control 4	182	50	52	54
$\alpha_4^{+/+}; \alpha_4^{+/+}$				
Control 5	D3	90	72	51
Control 6	D3	80	86	45
Control 7	D3	90	72	21
Control 8	D3	100	58	10
Control 9	D3	95	76	57
Control 10	D3	90	60	51
Control 11	D3	90	58	ND

*Percentage of agouti coat color estimated by eye.
†Percentage contributions of $\alpha_4^{-/-}$ cells determined by densitometric scanning of GPI gels as described in the Materials and Methods.

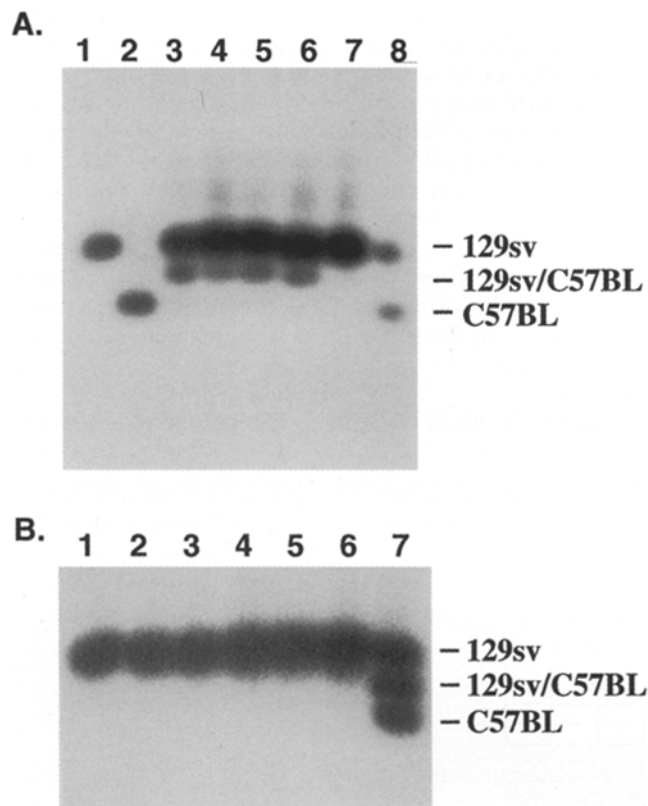contribute to both skeletal and cardiac muscles. The contributions of α_4-null cells to cardiac muscle were similar to those of α_4-heterozygous and wild-type ES cells, and the contributions of α_4-null cells to skeletal muscles appear even a bit higher than those observed with the α_4-heterozygous and wild-type ES cells. It is noticeable that the chimerism in skeletal muscle from the α_4-null cell-derived chimeras correlated fairly well with that of the coat colors of the mice; skeletal muscles from mice with a high percentage of agouti coat color had a high percentage of contribution from the α_4-null cells (e.g., Fig. 2 A, lanes 3–7). In eight skeletal muscle samples, GPI-I^{bb} and GPI-I^{ab} isozymes were completely undetectable (e.g., Fig. 2A, lane 7). To assess the sensitivity of the GPI assay, we performed a serial dilution of a muscle tissue sample from a wild-type mouse for GPI analysis, starting with the equivalent amount used for the chimeric muscle analysis; the GPI band was still detectable when the sample was diluted 1:50 (data not shown). Based on this observation, we conclude that the muscles in those chimeric mice in which the GPI-I^{bb} and GPI-I^{ab} isozymes were undetectable were composed of at least 98% α_4-null cells (e.g., Fig. 2 A, lane 7).

Skeletal Muscles Deficient in α_4 Integrins Exhibit No Gross Morphological Changes

Having observed that skeletal muscles of many of the chimeric mice appeared to have been derived predominantly, perhaps even exclusively, from the α_4-null ES cells, we analyzed the muscle histologically for signs of structural abnormalities. We chose mice whose coat color suggested that the muscle would likely have low or negligible contribution from wild-type cells, and confirmed this by GPI analysis of the muscle cryosections. Indeed, such an analysis demonstrated that in one muscle segment of 4 mm in length, no contribution from wild-type cells could be detected (Fig. 2 B). In muscle thus shown to be composed almost entirely of α_4-null cells, histologic examination revealed no gross abnormalities (see Fig. 4 A). In particular, myofiber alignment was not disrupted, fiber diameters were uniform and indistinguishable from wild-type muscle, no degenerating or regenerating fibers were seen, and no central nuclei were observed. Thus, myofiber formation is not noticeably disrupted by the absence of α_4 integrins.

α_4 Integrin–deficient Myoblasts Fuse As Well As Wild-type Myoblasts

To further assess the role of α_4 integrins in muscle cell differentiation, wild-type and α_4 integrin–deficient myoblasts were compared in an in vitro assay of myoblast fusion. Wild-type myoblasts were derived from primary muscle cell cultures from mature C57BL/6J mice. The α_4 integrin–deficient myoblasts were obtained by deriving primary myoblast cultures from chimeric mouse muscle, purifying the myogenic cells from those cultures, and then selecting α_4 integrin–deficient cells by growth in G418. The G418-resistant population was genotyped by Southern blot analysis (data not shown) and by PCR (Fig. 3 A), which should detect <1% contribution of wild-type cells, to confirm the absence of any wild-type cells in the population.
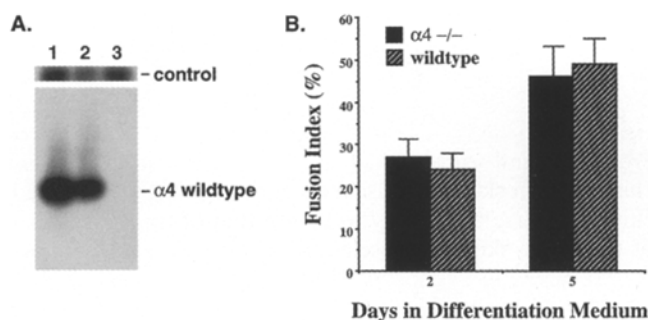

Figure 3. Differentiation of α_4 integrin–deficient myoblasts. (A) PCR analysis of the myoblast cultures. (Lane *1*) $\alpha_4^{+/+}$ (N-cadherin +/−); (lane *2*) α_4+/−; (lane *3*) $\alpha_4^{-/-}$. The PCR products were Southern blotted and hybridized with a probe for the α_4 integrin wild-type bands. The blot was then rehybridized with a probe for the control bands, which are PCR products from the Neo primers. Notice that the PCR product for the α_4 integrin wild-type allele was absent in lane *3*. (B) Histograms of the fusion index of $\alpha_4^{-/-}$ and $\alpha_4^{+/+}$ myoblasts at the same time points. Notice that the mutant and wild-type myoblasts fused into myotubes in a similar manner.

The two cell populations were differentiated in vitro, and the degree of fusion was assessed microscopically at two different times. The α_4 integrin–deficient myoblasts formed multinucleated myotubes when placed in differentiation medium, and myotube formation increased between day 2 and day 5 (Fig. 3 B and 4 B). No significant difference was observed between wild-type and α_4 integrin–deficient myoblasts in their abilities to differentiate and to fuse in vitro.

Differentiation of α_4-Null ES Cells into Myotubes

We also examined the ability of α_4-null ES cells to differentiate into myotubes. The wild-type and mutant ES cells were grown in suspension to produce embryoid bodies, and the embryoid bodies were plated on gelatin-coated coverslips to induce differentiation. Among the multiple cell types typical of such differentiating embryoid body cultures, skeletal myotubes were readily detectable. The differentiated cultures were stained with an antibody against skeletal muscle myosin heavy chain. 34.5% of the wells (10 out of 29) with α_4-null embryoid bodies and 33% of the wells (6 out of 18) with wild-type embryoid bodies had multinucleated myotubes. The myotubes formed by the α_4-null embryoid bodies (Fig. 5 B) were as well differentiated as those formed by the wild-type embryoid bodies (Fig. 5 A). This result shows that ES cells can differentiate into myotubes in the complete absence of α_4 integrins.

Discussion

We have previously generated a null mutation in the gene encoding the α_4 integrin subunit and shown that the mutation was embryonic lethal. To circumvent the problem of embryonic lethality and to study the roles of α_4 integrins in later stages of development, in this study we generated chimeric mice by injecting α_4-null ES cells into wild-type blastocysts. We addressed the question of whether α_4 integrins are essential in skeletal muscle development by examining the ability of α_4 integrin–deficient ES cells to participate in forming skeletal muscles. Our results showed that the α_4-null ES cells were able to contribute to skeletal muscle at high percentages, and the skeletal muscle tissues, where the proportion of α_4-null ES cells was >98%, appeared morphologically normal. These results show that skeletal muscle can differentiate normally in the apparent absence of α_4 integrins, suggesting that α_4 integrins are not essential for skeletal muscle development. Our in vitro studies also strongly support the view that α_4 integrins do not play an essential role during myogenesis. First, we showed that primary myoblasts deficient in α_4 integrins were able to differentiate to form myotubes; second, we showed that α_4-null ES cells can also differentiate into myotubes. These results clearly demonstrate that α_4 integrins are not essential for myogenesis in vitro.

A study on chimeric mice generated using ES cells lacking the β_1 integrin subunit has previously been reported by Fässler and Meyer (1995). They showed that β_1-null ES cell–derived chimeras with high percentage chimerism die at early embryonic stages, while those with low percentage chimerism (2–25%) can survive to adulthood. In these low percentage chimeras, β_1-null cells were able to contribute
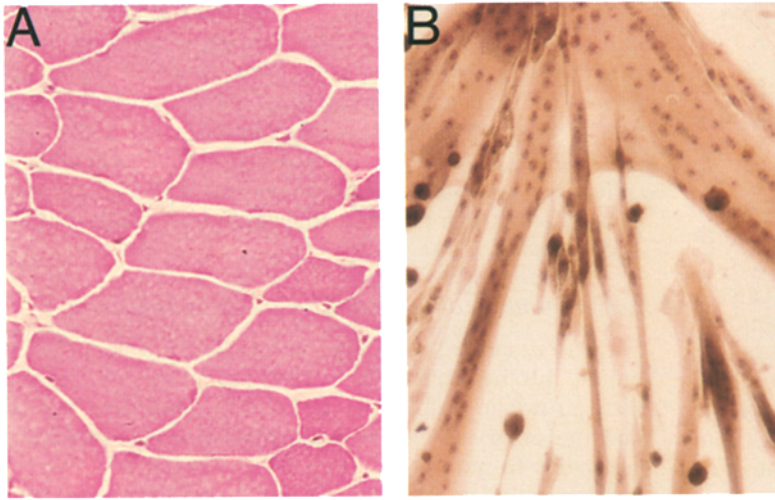
Figure 4. Morphology of α_4 integrin–deficient skeletal muscle (*A*) and $\alpha_4^{-/-}$ myotubes in culture (*B*). (*A*) Histological staining (hematoxylin and eosin) of a frozen section from the same series of chimeric muscle sections shown in Fig. 2 *B*, which had no detectable wild-type contribution. (*B*) Micrograph of well-formed myotubes differentiated from $\alpha_4^{-/-}$ myoblasts.

to most tissues, including skeletal muscle. However, because of the low percentage chimerism and the syncytial nature of skeletal muscle, these studies do not assess the essentiality of β_1 integrins for skeletal muscle development.

Our GPI analysis also showed that α_4-null ES cells are able to contribute to cardiac muscles at a percentage similar to that of wild-type ES cells, suggesting that α_4 integrins also may not play an essential role in cardiac muscle development. This is consistent with our previous study of α_4-null embryos, showing that α_4 integrins play an important role in the formation and maintenance of epicardium but do not play any essential roles in cardiac muscle formation (Yang et al., 1995). Moreover, we have observed high percentage contributions of α_4-null ES cells in the generation of agouti coat color, as well as germline transmission of the α_4-null allele from a male chimera. Thus, besides skeletal muscle, cardiac muscle, mesenchymal cells (responsible for the agouti coat color) and sperm, can also develop without α_4 integrins. Given the high efficiency of generation of chimeras with a high contribution of α_4-null cells, many other cell types clearly can develop normally without α_4 integrins.

Although our results suggest that α_4 integrins are not essential for skeletal muscle development, they do not rule out a possible nonessential involvement of α_4 integrins in this process. Immunohistochemical studies by Rosen et al. (1992) and Sheppard et al. (1994) have shown that the α_4 integrin subunit and VCAM-1, the counterreceptor of α_4 integrins, are localized on secondary myotubes and secondary myoblasts, respectively, suggesting a role for α_4 integrin–VCAM-1 interactions during secondary myotube fusion. Our results, however, did not reveal an essential role of α_4 integrins in this process. One possible explanation is that functional overlap may exist among members of the integrin superfamily. It has been shown that $\alpha_7\beta_1$ is expressed on secondary myoblasts, and there is an increased expression level of $\alpha_7\beta_1$ when these secondary myoblasts fuse into secondary myotubes (Song et al., 1992). It is possible that both $\alpha_4\beta_1$ and $\alpha_7\beta_1$ are involved in secondary myotube fusion. Functional overlap could also explain the discrepancy between our in vitro results and the observation by Rosen et al. (1992) that an anti–α_4 integrin antibody inhibited the ability of C2C12 myoblast cells to differentiate into myotubes. A related but distinct possibility
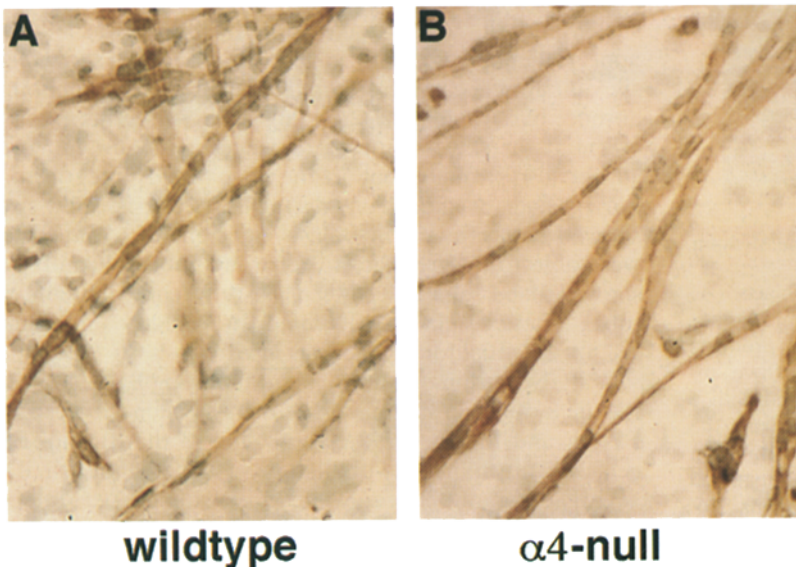

wildtype   **α4-null**

Figure 5. Differentiation of α_4 integrin–deficient ES cells. The differentiated wild-type (*A*) and $\alpha_4^{-/-}$ (*B*) cultures were stained with an antibody against skeletal muscle myosin heavy chain. Notice that the α_4 integrin–deficient ES cells differentiate into multinucleated myotubes as well as wild-type ES cells.

is that other adhesion receptors could compensate for the loss of α_4 integrins by upregulation of expression or function. Alternatively, the antibody blocking results could be misleading for a variety of reasons. The antibodies could be having effects above and beyond merely inhibiting the interactions of α_4 integrins and/or VCAM-1. Apart from the relatively trivial possibilities of cross-reaction with, or steric hindrance of, other adhesion receptors, it is known that some blocking antibodies to integrins can act as agonists themselves, leading to secondary consequences in addition to the blockage of adhesion. Such secondary consequences can include the transdominant inhibition of other integrins in the same cell (Diaz-Gonzalez, F., J. Forsyth, B. Steiner, and M.H. Ginsberg, manuscript submitted for publication).

Based on our studies of myogenesis in vitro and in vivo, we conclude that skeletal muscle differentiation can proceed in the absence of the α_4 integrins, although we cannot rule out the possibility that a small contribution ($<2\%$) of α_4 integrin–expressing cells in vivo that were not detected in our assays might nucleate skeletal muscle formation. The temporal expression of the α_4 integrins during development suggested a role for these molecules in the differentiation process. However, as determined by our assays, the processes of cell fusion and differentiation are not dependent on these integrins. The biological role of the α_4 integrins in myogenesis remains to be determined.

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