

Differentiation and integrity of cardiac muscle cells are impaired in the absence of β_1 integrin

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SUMMARY

Cellular interactions with substrata of the microenvironment are one of the major mechanisms for differentiation and morphogenesis. Many of these interactions are mediated via the β_1 integrin subfamily of cell surface receptors, which are believed to transduce signals upon cell adhesion. We have used β_1 integrin-deficient embryonic stem cells to test their ability to differentiate into cardiac muscle cells. We show here by several approaches that β_1 integrin is important for normal cardiogenesis. First, the *in vitro* differentiation of β_1 integrin-deficient embryonic stem cells into cardiac muscle cells is retarded. This is demonstrated by the delayed expression of cardiac muscle-specific genes and action potentials. Second, the specification of cardiac precursor cells into pacemaker-, atrial- and ventricular-like cells is significantly impaired in β_1 integrin-deficient cells. The occurrence of atrial- and ventricular-like

cells is reduced and transient. Only cells exhibiting pacemaker-like action potentials of high frequency and arrhythmias survive. Third, the sarcomeric architecture is incomplete and disarranged in the absence of β_1 integrin. Fourth, β_1 -deficient embryonic stem cells can contribute to the developing heart in chimaeric mice but many areas with β_1 -null cells contain cell debris. The number of β_1 -null cells decreases from prenatal to postnatal stages and is lost completely in 6-month-old hearts.

Thus, we conclude that interactions with the extracellular matrix via β_1 integrin is necessary for differentiation and the maintenance of a specialized phenotype of cardiac muscle cells.

Key words: β_1 integrin, Embryonic stem cell, Cardiomyocyte, Differentiation, Sarcomere

INTRODUCTION

Substrata of the cellular microenvironment, such as collagens, fibronectins and laminins, specifically interact with cells via integrin receptors and thereby influence a variety of cellular processes including shape and migration of cells, gene expression and differentiation (Hynes, 1992; Adams and Watt, 1993). Integrins are widely expressed and dynamically regulated during development (Bronner-Fraser et al., 1992; Sutherland et al., 1993; Ziober et al., 1993; Hirsch et al., 1994; Carver et al., 1994). Antibody perturbation assays revealed that integrin-matrix interactions are important for the formation of myotubes from myoblasts (Menko and Boettiger, 1987; Rosen et al., 1992), as well as for the differentiation of keratinocytes (Adams and Watt, 1990), neural crest cells (Bronner-Fraser, 1986; Lallier et al., 1994) and epithelial cells (Sorokin et al., 1990; Streuli et al., 1991; Kadoya et al., 1995). In the present study, we further demonstrate the importance of β_1 integrins for cardiac muscle cell differentiation and specialization.

Integrins constitute a large family of cell surface receptors, which can bind to extracellular matrix proteins and cell counter receptors (Hynes, 1992). They are composed of non-covalently associated α and β subunits. Fifteen α subunits and eight β subunits have been identified so far. The β_1 integrin can associate with at least ten different α subunits thereby forming the largest subfamily of integrin receptors. Besides extracellular matrix proteins, β_1 integrins were also shown to interact with cell surface ligands, such as vascular cell adhesion molecules 1 (VCAM-1) and fertilin, a sperm surface receptor (Almeida et al., 1995). To directly access the function of β_1 integrins, we and others have recently established mice (Fässler and Meyer, 1995; Stephens et al., 1995) and several embryonic stem (ES) cell lines (Fässler et al., 1995) that carry a null mutation in the β_1 integrin gene (β_1 -null ES cells). A lack of β_1 integrin expression in mice leads to embryonic lethality shortly after implantation. β_1 -null ES cells are associated with altered cell shape, impaired adhesion to extracellular matrix proteins and a change in gene expression. Despite these defects, upon injection

into host blastocysts, β_1 -null ES cells can migrate and differentiate in the context of normal cells (Fässler and Meyer, 1995). Most tissues including the fetal and adult heart contain β_1 -null cells. This finding was surprising because cardiac muscle cells express high levels of β_1 integrin, both during heart development and in adult mature tissue (Sheppard et al., 1994). Furthermore, a null mutation in the α_4 integrin gene leading to the loss of a single member of the β_1 integrin subfamily has been reported to be associated with defects in the development of epicardium and coronary vessels resulting in severe cardiac hemorrhage (Yang et al., 1995).

Previous experiments proposed a functional role of a β_1 integrin-related subunit (β_{PS}) in the formation of the sarcomeric cytoarchitecture of *Drosophila* skeletal muscle cells (Volk et al., 1990), as well as of β_1 integrins in the myofibrillogenesis of neonatal rat cardiomyocytes (Hilenski et al., 1992). However, the specific function of β_1 integrins for mammalian cardiogenesis, development of functional properties and cardiac specification remains unclear.

Using different *in vivo* and *in vitro* approaches with ES cells carrying a homozygously inactivated β_1 integrin gene (Fässler et al., 1995), we show in the present report that the differentiation of β_1 -null ES cells into cardiomyocytes is severely impaired. Whereas wild-type ES cells readily differentiate into sinusnodal-, atrial- and ventricular-like cells (Maltsev et al., 1993, 1994), differentiation of β_1 -null ES cells is delayed, and specialized cardiac cell types appear reduced and only transiently. Furthermore, we provide evidence that the organization of the sarcomeric architecture in cardiomyocytes both *in vivo* and *in vitro* is crucially dependent on the presence of β_1 integrin.

MATERIALS AND METHODS

Generation of β_1 integrin-deficient chimaeric mice

ES cell lines D3 (Doetschman et al., 1985) and R1 (Nagy et al., 1993) were used for generating one β_1 integrin heterozygous (G119) and two β_1 integrin-null ES cell clones, G201 and G110, respectively. The insertion of a β -galactosidase-neomycin (*geo*) fusion DNA in frame with the ATG of the β_1 integrin leads to the inactivation of the gene (Fässler et al., 1995; Fässler and Meyer, 1995). Cell lines are free of mycoplasma contamination.

Blastocysts were isolated at day 3.5 p.c. from C57BL/6 mice, injected with 15 G201 ES cells as described by Bradley (1987) and transferred into the uterus of pseudopregnant recipient (C57BL/6 \times DBA)F₁ females (2.5 days p.c.). Embryos were isolated at E10.5, fixed for 30 minutes in 4% paraformaldehyde and stained overnight for LacZ activity (Fässler et al., 1995).

Tissue sections were prepared from four 6-week- and three 6-month-old male chimaeric mice, which were transcardially perfused with PBS followed by 4% paraformaldehyde. After a short postfixation and equilibrium in 30% sucrose, the tissues were embedded in OCT compound (Miles), frozen in dry ice-pentane and stored at -80°C . Tissues were cut at 10–20 μm on a Leitz cryostat and collected on gelatin-coated slides. Staining of embryos and tissues for LacZ activity followed published protocols (Fässler et al., 1995).

Counterstaining with Eosin was performed according to standard histological techniques with minor modifications. Xylol was replaced with HistoClear (National Diagnostics, Atlanta, USA) and the sections were covered with Canada Balsam and coverslipped.

Cell culture and differentiation

ES cells were grown on a feeder layer of mitomycin C-treated embryonic fibroblasts in Dulbecco's modified Eagle's medium

(DMEM) supplemented with 15% heat-inactivated fetal calf serum (Gibco, Germany), 2 mM L-glutamine (Gibco), 5×10^{-5} M β -mercaptoethanol (Sigma, Germany) and nonessential amino acids (Gibco), as described (Wobus et al., 1991). Since β_1 -null ES cells adhere poorly to the fibroblast feeder layer, β_1 -null ES cells were grown without feeder cells in medium supplemented with 5 ng/ml recombinant human leukemia inhibitory factor (LIF). LIF was prepared from *E. coli* strain JM109 transformed with plasmid pGEX2T-LIF58 (a gift from M. Strauß, Berlin) and isolated according to the methods described by Smith and Johnson (1988) and Gearing et al. (1989).

For differentiation, cells were cultured as aggregates (embryoid bodies) in DMEM containing 20% FCS in hanging drops (Wobus et al., 1991): 600 ES cells were cultured in 20 μl medium hanging from the lid of the culture dish for 2 days and subsequently up to 37 days in suspension using bacteriological Petri dishes (Greiner, FRG). For immunofluorescence, embryoid bodies were cultured 9 to 10 days in suspension and plated. Single contracting cells were isolated by collagenase treatment (see below), plated on gelatin-coated glass coverslips, fixed and assayed.

Single cell preparation

Single cardiomyocytes for immunofluorescence and patch-clamp analysis were isolated from embryoid bodies by a modified procedure of Isenberg and Klöckner (1982; see Maltsev et al., 1993, 1994). The following solutions were used (in mM):

(1) Low Ca^{2+} -medium: NaCl 120, KCl 5.4, MgSO_4 5, sodium pyruvate 5, glucose 20, taurine 20, Hepes 10/NaOH, pH 6.9, at 24°C .
(2) Enzyme medium: Low Ca^{2+} medium supplemented with 1 mg/ml collagenase (collagenase B, Boehringer Mannheim, Germany) and 30 μM CaCl_2 .

(3) KB medium: KCl 85, K_2HPO_4 30, MgSO_4 5, EGTA 1, Na_2ATP 2, Na pyruvate 5, creatine 5, taurine 20, glucose 20, pH 7.2, at 24°C .

Pulsating areas of 20 embryoid body outgrowths plated from 10 to 35 days of cultivation were mechanically isolated under the microscope, washed in low Ca^{2+} -medium during 30 minutes at room temperature and incubated in enzyme medium for 15–20 minutes at 37°C . The dissociation of tissue was completed in KB medium at 37°C for about 10 hours. Isolated cells were resuspended in DMEM supplemented with 20% FCS and incubated at 37°C . Single cardiomyocytes attached to the surface were either immunostained or patch clamped within 2 days after isolation. The developmental stages of the cardiomyocytes (in days) include the time of differentiation as embryoid bodies and the time of cultivation after isolation.

Immunofluorescence assay

Contracting cells were rinsed 2 times with PBS and fixed with methanol:acetone (3:1) at -20°C . Fixed cells were treated for 1 hour with 10% goat serum in PBS followed by an incubation with the primary antibodies anti-myosin heavy chain (MF-20, Bader et al., 1982), anti-sarcomeric actin (5C5: Sigma, Germany) or anti- α -actinin (polyclonal antibody 653, a gift from D. Fürst, Potsdam) for 1 hour at 37°C in a humidified chamber, respectively. After washing in 0.05% Tween-20 in PBS cells were incubated for 45 minutes at 37°C with fluorescently labeled anti-mouse IgG FITC, anti-mouse IgM FITC and anti-rabbit IgG FITC (Dianova, Hamburg) in the case of MF-20, 5C5 and the polyclonal α -actinin antibody, respectively. After washing 3 times with PBS, slides were embedded in Tris-glycerin buffer (Walter et al., 1984) or Vectashield mounting medium (Vector, Burlingame, USA), coverslipped and analyzed with the fluorescence microscope Optiphot-2 (Nikon, Düsseldorf, Germany).

Detection of cardiac-specific transcripts by RT-PCR analysis

A total number of 15 embryoid bodies cultivated for 3, 7, 10, 14, 21 and 37 days were collected in lysis buffer (4.0 M guanidinium thiocyanate, 0.1 M Tris-HCl pH 7.5, 1% β -mercaptoethanol). Skeletal muscle and heart tissue of 16-day-old murine embryos served as a

control. The cell extract was affinity purified for poly(A)⁺ mRNA according to Sheardown (1992). Poly(A)⁺ mRNA was reverse transcribed and amplified using oligonucleotide primer complementary and identical to transcripts of the following genes (oligonucleotide sequences are given in brackets in the order antisense-, sense-primer followed by the annealing temperature used for PCR, the size of the amplified fragment in basepairs and a reference for the gene): The cardiac-specific genes encoding α -cardiac myosin heavy chain (5'-CTGCTGGAGAGGTTATTCTCG-3', 5'-GGAAGAGTGAGCG-GCCATCAAGG-3'; 64°C; 301 bp; Mahdavi et al., 1984), β -cardiac myosin heavy chain (5'-TGCAAAGGCTCCAGGTCTGAGGGC-3', GCCAACACCAACCTGTCCAAGTTC-3'; 64°C; 205 bp; Mahdavi et al., 1984), myosin light chain isoform 2V (5'-TGTGGGTCACCT-GAGGCTGTGGTTCAG-3', 5'-GAAGGCTGACTATGTCCGGGA-GATGC-3'; 60°C; 189 bp; Lee et al., 1992), atrial natriuretic factor (5'-TGATAGATGAAGGCAGGAAGCCGC-3', 5'-AGGATTGGA-GCCAGAGTGGACTAGG-3'; 64°C; 203 bp; Seidman et al., 1984), the genes encoding the cardiac-specific (5'-GTTCTGAAGG-AGGTGTGCTGGACG-3', 5'-AAAGGCAGTCCCATGCCGG-3'; 62°C; 183bp; Chaudhari and Beam, 1992) as well as the skeletal muscle-specific α_1 -subunit of the L-type calcium channel (5'-GAT-CACCAGCCAATAGAAGACC-3', 5'-GGCGAGGTCATGGACG-TGGACG-3'; 62°C; 200 bp; Chaudhari and Beam, 1992) and the housekeeping gene β -tubulin (5'-GGAACATAGCCGTAAGTGC-3', 5'-TCACTGTGCCTGAACTTACC-3'; 64°C; 317 bp; Wang et al., 1986) used as an internal standard. To exclude contamination of genomic DNA used as a source for amplified products, complementary and identical primers were chosen to span at least two exons on genomic level. The reverse transcription and amplification reactions were carried out with rTth-Polymerase (Perkin Elmer, USA) following the protocol supplied by the manufacturer. The products of the reverse transcription reactions were denatured for 2 minutes at 95°C, followed by 40 cycles of amplification: 40 seconds denaturation at 95°C, 40 seconds annealing at 60 to 64°C (depending on the primer used, see above) and 40 seconds elongation at 70°C. One fifth of each RT-PCR reaction was electrophoretically separated on 2% agarose gels.

RNAs from three independent experiments were analyzed and RT-PCR with all primers was done twice for each RNA probe isolated. Cardiac muscle and water were included as positive and negative controls, respectively.

Electrophysiological measurements

The whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) was used throughout the study. Action potentials were recorded by the L/M EPC-7 patch-clamp amplifier (List Electronics, Germany). The patch pipettes were prepared from glass capillaries (Leighton Buzzard, UK) and ranged from 1 to 3 MOhm when filled with an internal solution containing (in mM): KCl 50, K aspartate 80, MgCl₂ 1, EGTA 10, MgATP 3, Hepes/KOH 10 (pH 7.4 at 37°C). The external solution contained (in mM): NaCl 140, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1, glucose 10, Hepes/NaOH 5 (pH 7.4 at 37°C). After GOhm seal-formation, the membrane patch under the micropipette was disrupted by gentle suction to establish the whole-cell configuration. The membrane capacity (C_m) and series resistance were on-line compensated. Action potentials were recorded under current-clamp conditions. The action potential duration was measured at the 10% level of its amplitude. The results are presented as mean \pm s.e.m. for n cells. Classification as well as criteria of various action potentials are described by Maltsev et al. (1993; see also Sperelakis and Pappano, 1983). In short, action potentials of pacemaker-like cells were characterized by their high rhythmicity of the spontaneous activity and specific hormonal regulation. Atrial-like and ventricular-like cells were characterized by the absence or rare spontaneous activity as well as by the low resting potentials. Atrial-like and ventricular-like action potentials were differentiated by their different shape and hormonal modulation.

Electron microscopy

Embryoid bodies were fixed in a 100 mM Hepes/Pipes buffer (pH 7.35) containing 1.75% paraformaldehyde, 2% glutaraldehyde and 15% picric acid for 1 hour at room temperature (RT). Afterwards embryoid bodies were treated with 100 mM Hepes/Pipes buffer containing 1% tannic acid for 30 minutes at RT and finally osmified with 0.5% OsO₄. Prior to embedding in Epon resin (Agar Scientific, Stansted, UK), embryoid bodies were dehydrated in a graded series of ethanol. For light microscopy semithin sections were stained with toluidine blue.

Heart tissue was obtained from 6-week-old chimaeric animals, which were killed by cervical dislocation and subsequently transcardially perfused with a 0.1 M cacodylate buffer containing 2% paraformaldehyde and 2% glutaraldehyde at a perfusion pressure of 60 cm H₂O. Since none of the β_1 integrin antibodies showed binding after the fixation procedure used for electron microscopy with our protocol, areas containing β_1 -null cells were identified by staining for LacZ activity. Hearts were removed, fixed for additional 4 hours in the same fixative and then assayed for LacZ activity overnight at 30°C following standard protocols (Fässler et al., 1995). Cardiac tissue was cut into small pieces and fixed in 100 mM cacodylate buffer (pH 7.3) containing 2% osmium tetroxide for 2 hours at 4°C. Tissue pieces containing LacZ-positive areas were rinsed three times in cacodylate buffer, block stained for 8 hours in 70% ethanol containing 1% uranyl acetate, dehydrated in a series of graded ethanol and embedded in Araldite (Serva, Germany). Ultrathin sections (30-60 nm) obtained with a diamond knife on a Reichert ultramicrotome were placed on copper grids and examined with a Zeiss EM 902A electron microscope.

RESULTS

β_1 -null ES cells differentiate via embryoid bodies into beating cardiac muscle cells

Northern blot analysis demonstrated the absence of β_1 integrin mRNA in two independent β_1 -null ES cell clones (Fig. 1), one derived from D3 (G201) and one from R1 ES cells (G110). Both mutant ES cell lines together with the parental cell lines or β_1 integrin heterozygous cell lines (G119) were differentiated in aggregates of 600 ES cells. Whereas wild-type ES (D3, R1) cell-derived embryoid bodies showed differentiation of spontaneously, rhythmically contracting cardiomyocytes (see Wobus et al., 1991; Maltsev et al., 1993, 1994, unpublished data for R1), β_1 -null embryoid bodies at terminal stages are characterized by frequent and arrhythmic beating. The temporal pattern of differentiation of wild-type and β_1 integrin-deficient cardiomyocytes was completely different. Whereas wild-type cells showed beating cardiomyocytes maximally up

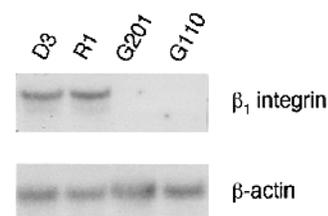


Fig. 1. Northern blot analysis of wild-type (D3, R1) and β_1 -null (G201, G110) ES cells indicates the absence of β_1 integrin mRNA in β_1 -null ES cells. Total RNA was isolated from undifferentiated ES cells grown in the presence of LIF but without embryonic feeder cells, gel separated, transferred onto a nylon membrane and hybridized with oligolabeled mouse β_1 integrin cDNA. β -actin was used to control loading of RNA samples.

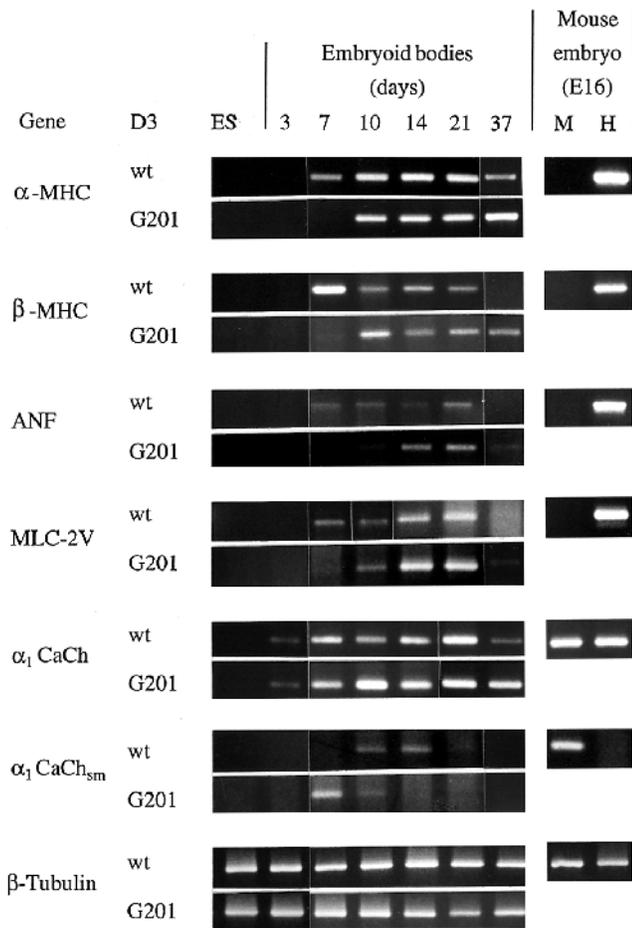


Fig. 2. RT-PCR analysis of cardiac-specific gene expression during in vitro differentiation of line D3 (wt) and β_1 -null (G201). D3-derived embryoid bodies already showed expression of the genes encoding α - and β -cardiac myosin heavy chain (α -MHC, β -MHC), atrial natriuretic factor (ANF) and myosin light chain isoform 2V (MLC-2V) at day 7 of cultivation whereas, in G201-derived embryoid bodies, these genes were delayed expressed between days 10 and 21. At the terminal differentiation stage (37 days) transcripts of the gene encoding β -MHC were not detectable in wt cells but present in G201 cells, whereas transcripts of ventricular (MLC-2V)- and atrial (ANF)-specific genes were almost completely absent at the terminal differentiation stage in both D3 and G201. The first cardiac-specific transcripts of the gene coding for the α_1 -subunit of the L-type Ca^{2+} channel were detected at day 3 in both lines ($\alpha_1\text{CaCh}$ = muscle- and cardiac specific, $\alpha_1\text{CaCh}_{sm}$ = muscle-specific transcripts). Embryonic stem (ES) cells showed no activity. Primers specific for the house-keeping gene β -tubulin were used as an internal standard, and skeletal muscle (M) and heart (H) from a day 16 p.c. mouse embryo as positive controls.

to 26 days, β_1 integrin-deficient arrhythmically beating cardiac-like cells were still present at 37 days of culture. This different developmental pattern was explained by the expression pattern of cardiac-specific genes (Fig. 2) and action potentials (Fig. 3).

Altered expression of cardiac-specific genes in β_1 integrin-deficient cardiac muscle cells

To compare the differentiation potential of wild-type (wt) and

β_1 -null ES cells, RT-PCR analysis was performed to determine the expression of cardiac muscle-specific genes. Total RNA was isolated from undifferentiated ES cells and embryoid bodies cultured for 3, 7, 10, 14, 21 and 37 days and assayed by RT-PCR. As a positive control, total RNA derived from skeletal and cardiac muscle of an E16 embryo was used (Fig. 2). Undifferentiated wt ES cells showed no cardiac-specific transcripts (Fig. 2).

Wt D3 embryoid bodies already showed expression of α - and β -cardiac myosin heavy chain (α - and β -MHC), myosin light chain isoform 2V (MLC-2V) and atrial natriuretic factor (ANF) genes after 7 days in culture (data not shown for R1 cells). In contrast, in β_1 -null embryoid bodies (G201, G110), these genes were never expressed before day 10 (data not shown for G110 cells). The α - and β -MHC mRNA appeared delayed at day 10 in β_1 -null embryoid bodies. β -MHC expression disappeared at day 37 in wt ES cells, but not in β_1 -null cells. The expression of the ventricular-specific MLC-2V gene was expressed in wt embryoid bodies at day 7 and increased throughout the culture period, but was delayed and significantly expressed between days 10 and 21 in β_1 -null embryoid bodies. MLC-2V transcripts disappeared almost completely at day 37 in both wt and β_1 -null embryoid bodies. Similarly, the expression of the gene encoding ANF is delayed in β_1 -null cardiomyocytes and has nearly completely disappeared at day 37 in both wt and β_1 -null embryoid bodies.

In summary, all analyzed cardiac-specific genes were expressed by wt and β_1 -null cells, but expression of most genes was delayed in β_1 -null cells. Furthermore, although beating cardiac-like cells were present in β_1 integrin-null embryoid bodies at day 37, expression of genes characteristic for specialized cardiac cells, such as MLC-2V and ANF were almost completely absent at this terminal differentiation stage in β_1 -null cardiomyocytes (for comparison see Fig. 3). During differentiation of wild-type cells, beating cardiomyocytes had nearly completely disappeared by day 26, and transcripts of ANF and MLC-2V were not found at stages later than day 26.

In contrast to the delayed and transient transcription of genes encoding sarcomeric proteins and of ANF in β_1 -null cells, the transcripts encoding α_1 -subunits of L-type Ca^{2+} channels ($\alpha_1\text{CaCh}$) were detected early at day 3 (Fig. 2). From day 7 onward, the expression was strong and remained constant in embryoid bodies derived from both wt and β_1 -null ES cells until day 21. Because cardiac and skeletal muscle cells differentiate from D3 ES cells (Doetschmann et al., 1985; Miller-Hance et al., 1993), we discriminated the expression of cardiac and skeletal muscle-specific subunits of the α_1 L-type Ca^{2+} channel by using primer pairs specific for cardiac and skeletal muscle ($\alpha_1\text{CaCh}$, Fig. 2) and for skeletal muscle ($\alpha_1\text{CaCh}_{sm}$, Fig. 2), respectively. Because of the weak expression of the skeletal muscle-specific $\alpha_1\text{CaCh}_{sm}$ transcripts in wt and β_1 -null embryoid bodies, the $\alpha_1\text{CaCh}$ transcripts are obviously cardiac-specific.

Specification of cardiomyocytes is disturbed in the absence of β_1 integrin

The action potential pattern and the expression and regulation of cardiac-specific ion channels of wt D3 ES cell-derived cardiomyocytes has been previously described (Maltsev et al., 1993, 1994). The cellular differentiation in vitro leads to the specification of functional atrial, ventricular and sinusodal

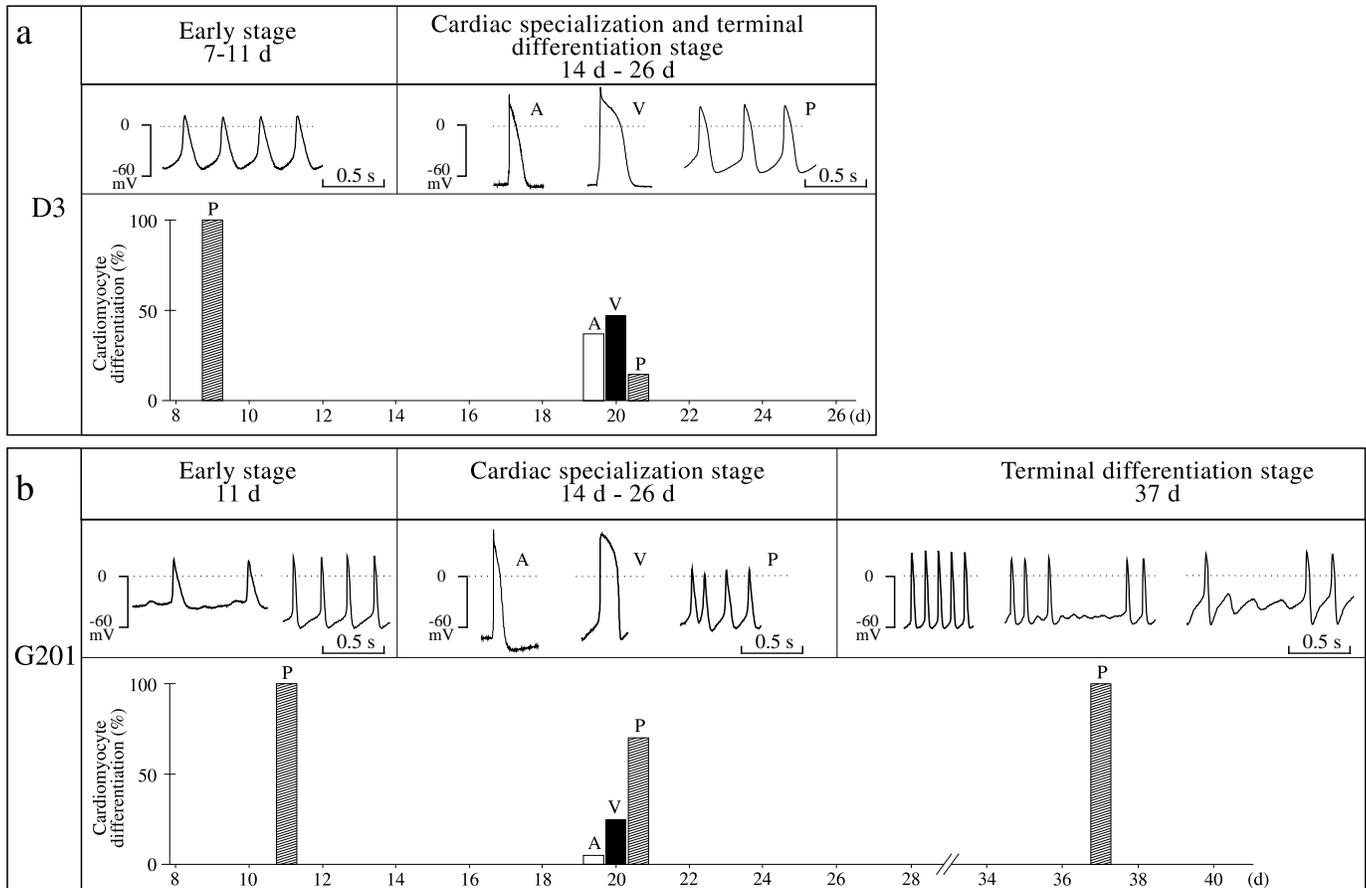


Fig. 3. Electrophysiological data of D3 wild type (a) in comparison to the action potential patterns of β_1 -null G201 cells (b). In line G201, the first beating cells exhibiting pacemaker-like action potentials appeared with a delay around day 11 (b; early stage). The number of specialized cardiac cells with atrial- and ventricular-like action potentials was significantly reduced in G201 cells during a cardiac specialization stage (14-26 days). These specialized cells disappeared at terminal differentiation stage (37 days) leaving cells with pacemaker-like action potentials of high frequency and irregular and asynchronous shape in β_1 -null cells. The atrial- and ventricular-like action potentials were evoked by pulse current stimulation in current-clamp, all others were generated spontaneously.

cells. A summary of the percentage of wt cells displaying action potentials of the particular types is presented in Fig. 3a. Early cardiac precursor cells showed pacemaker-like action potentials at early stage (day 7, 100%). At the cardiac specialization and terminal differentiation stage (from days 14 to 26), the action potential pattern differentiated to sinusnodal- (14%), atrial- (38%) and ventricular-like (48%) shapes. Cardiomyocytes isolated from R1 ES cells showed a similar distribution (unpublished).

To test whether these functional properties were affected by the absence of β_1 integrin, electrophysiological characteristics of action potentials were studied in cardiomyocytes derived from β_1 -null embryoid bodies (Fig. 3b). The action potential pattern was analyzed at different times representative of three stages of development: (i) an early stage at day 11, (ii) a stage of cardiac specialization between days 14 and 26 and (iii) a terminal differentiation stage at day 37. The development of action potentials is schematically illustrated in Fig. 3b, and changes in frequency of generation, amplitude, duration and upstroke velocity of action potentials are given in Table 1.

Wild-type D3-derived cardiomyocytes of the early differentiation stage and β_1 -null cardiomyocytes from 11 days

embryoid bodies exhibited pacemaker action potentials of various frequency characteristic for embryonic cardiomyocytes. Figs 3a and 3b (early stage) show examples of typical action potential patterns of early pacemaker cells with various frequency, which were not classified as specialized action potentials because of the lack of expression of ion channels characteristic for atrial, ventricular or sinusnodal cells (see Maltsev et al., 1993, 1994, for classification of action potential pattern and expression of ion channels of wild-type D3-derived cardiomyocytes). At the stage of cardiac specialization (14 to 26 days), three major types of action potentials were found in β_1 -null embryoid bodies. In addition to a decreasing population of pacemaker-like action potentials (69%), with a pattern similar to that of the early differentiated cells, two further types of spontaneous action potentials became obvious: 25% of the cells at this differentiation stage exhibited spontaneous action potentials of long duration with an enhanced plateau phase of about 200-800 ms (see Table 1 for mean value) representing ventricular-like cardiomyocytes. Approximately 6% of the cells displayed atrial-like action potentials of very short duration of 56.4 ± 12.4 ms ($n=5$) triggered from a stable resting potential of about -70 mV (Fig.

Table 1. Biophysical properties of action potentials in G201-derived cardiomyocytes of different developmental stages and percentage of cells exhibiting various types of action potentials at the particular stage

Developmental stage (d)	Type of action potentials	Amplitude (mV)	Duration (ms)	Upstroke velocity (V/s)	Frequency (Hz)	Min./rest. membrane potential (mV)	Number of cells	
							(n)	(%)
10-11	Early pacemaker	69.2±7.6	115.2±18.6	4.9±1.3	1.8±0.5	-49.0±4.8	6	100
14-24	Pacemaker of intermediate stage	73.1±2.7	82.0±4.7	9.3±1.2	5.4±0.7	-51.3±1.4	55	69
	Fetal ventricular type	97.2±3.1	218.9±19.5	12.1±1.6	2.5±0.3	-56.8±2.5	20	25
	Atrial-like type	121.8±3.7	56.4±12.4	170.0±39.8	0	-73.1±2.0	5	6
36-37	Pacemaker of terminal stage	85.5±4.7	63.7±6.4	15.6±2.8	6.6±0.9	-53.5±2.5	14	100

Results are presented as mean ± s.e.m.

(Early stage, $n=6$; stage of cardiac specialization, $n=80$; terminal stage, $n=14$).

3b). At the stage of terminal differentiation (37 days), cardiomyocytes from β_1 -null ES cell-derived embryoid bodies exclusively generated pacemaker-like action potentials with a higher frequency and shorter duration compared to those of the early stage. Generation of action potentials in these terminally differentiated cells was irregular or arranged in bursts. The majority of cells (71%) exhibited arrhythmias which were only rarely seen in the wild-type ES cell-derived cardiomyocytes. Cardiac-like cells isolated from β_1 integrin-deficient R1 cells (=G110) showed similar electrophysiological characteristics (not shown).

β_1 integrin is crucial for regular sarcomere organization in vitro

Sarcomeric organization was studied on isolated cardiomyocytes from embryoid bodies plated at day 10 and subsequent cultivation as single cardiomyocytes. These cardiomyocytes were immunostained for sarcomeric myosin heavy chain (MF-20), sarcomeric actin (5C5) and α -actinin (Fig. 4) during various developmental stages. Whereas cardiomyocytes from wt embryoid bodies displayed spindle- and triangular-like shapes and contained an intact sarcomeric cytoarchitecture with a sarcomeric length varying from 1.9 to 2.1 μm (Fig. 4a-

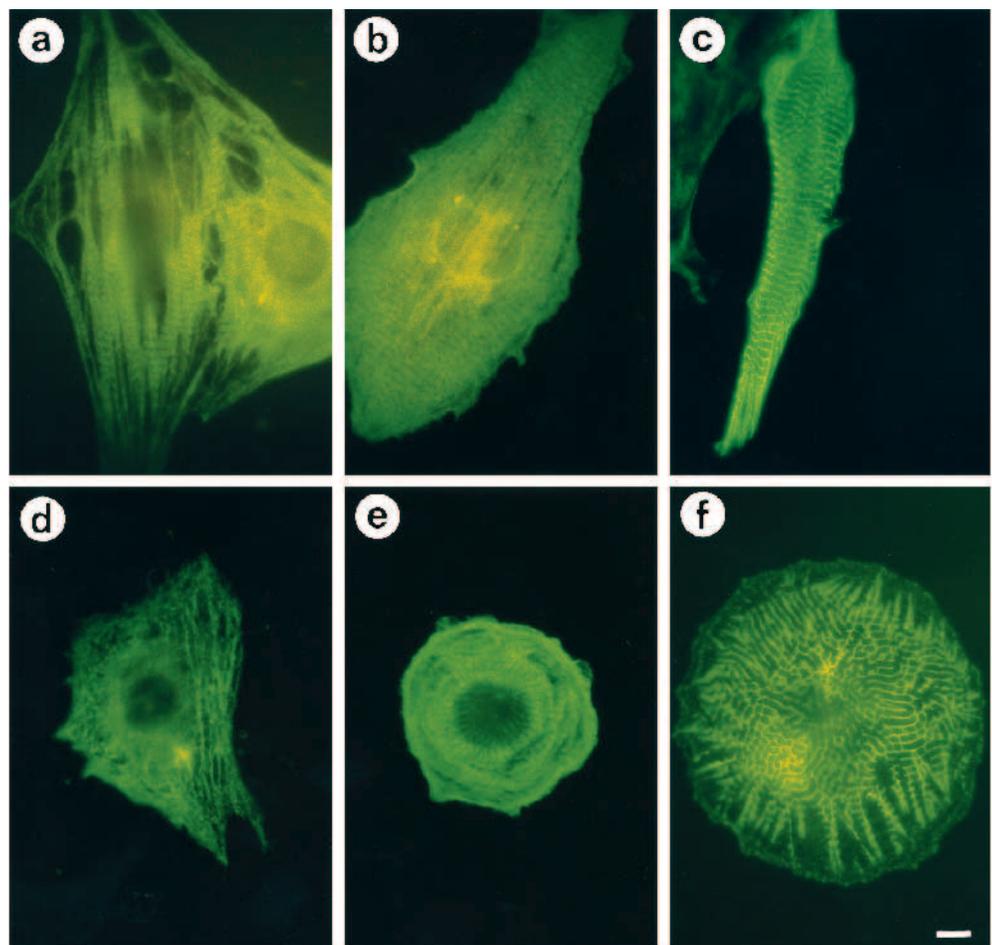


Fig. 4. Immunofluorescence studies with monoclonal antibodies against sarcomeric myosin heavy chain (MF-20; a,d), sarcomeric actin (5C5; b,e) and α -actinin (c,f) on single cell preparations of cardiomyocytes of wild-type D3 (a-c) and G201 (d-f) cells revealed that sarcomeric structures were affected in G201-derived cardiomyocytes. Whereas wild-type D3 cardiomyocytes (a-c) contain highly organized sarcomeres (sarcomeric length 2 μm), in β_1 integrin-null cardiac-like cells, a disordered network of tubular and fibrillar structures was found especially evident at the cardiac specialization stage (d,e). Pacemaker-like cells of the terminal differentiation stage showed sarcomeric-like structures, but of irregular orientation and organization (f). Bar, 10 μm .

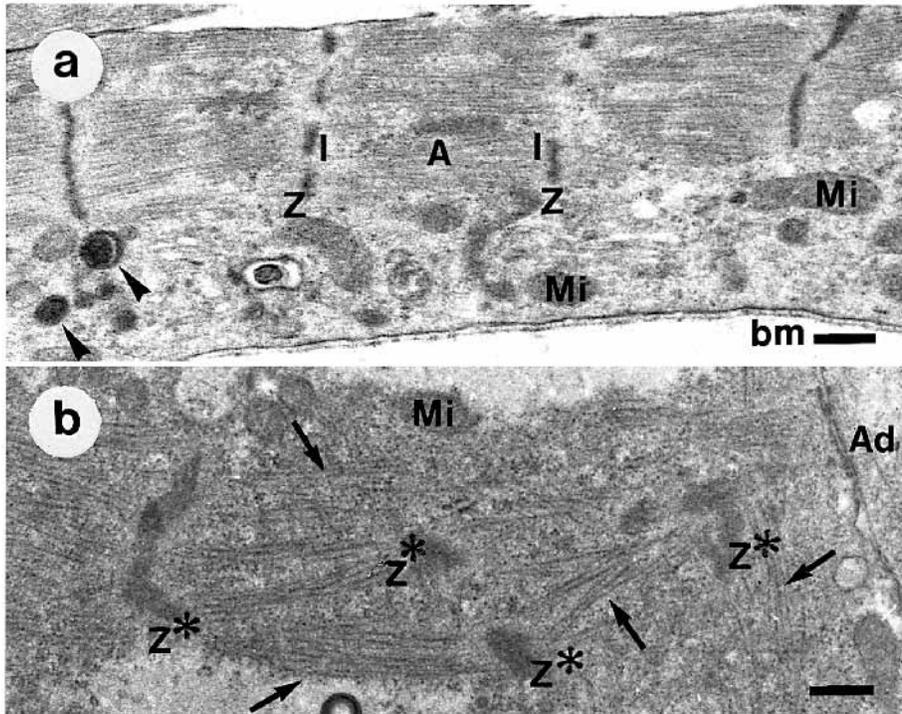


Fig. 5. Transmission electron microscopy of contracting areas of wild-type D3 (a) and G201 (b) embryoid body outgrowths. (a) Wild-type cardiac cells show developing sarcomeres with well-organized structure including Z-line (Z), I-band (I) and A-band (A). The myofilaments recognizable in the A-band are oriented in parallel. (b) In β_1 -null cardiac cells (G201) a typical sarcomeric organisation is not seen. Myofilament bundles (arrows) not arranged in parallel and dense areas of Z-line material (Z*) are existing in β_1 -null cells. bm, basal membrane; Ad, adherence junction; Mi, mitochondrion; arrowhead, atrial granule. Bar, 450 nm.

c, see also Maltsev et al., 1993), cardiac myocytes from β_1 -null embryoid bodies were mainly small and round (Fig. 4d-f). Cardiac cells in embryoid body outgrowths derived from β_1 -null ES cells and immunostained at day 18 (Fig. 4d), 24 (Fig. 4e) or 32 (Fig. 4f) of differentiation showed dramatically affected sarcomeric organization. The metameric structure of sarcomeres was significantly reduced and/or completely disorganized in β_1 -null-derived cardiomyocytes from the cardiac specialization stage (Fig. 4d,e). Whereas in some cells staining of myosin, actin or α -actinin was diffusely distributed in the cytoplasm, others showed organized sarcomeric proteins in a very primitive sarcomere-like structure (Fig. 4d). In other β_1 -

null cells, signals for the sarcomeric proteins were predominantly arranged in the periphery of the cells (Fig. 4e) and showed a disturbed pattern with irregular shaped and partially condensed Z-line protein (Fig. 4e). Sarcomeric-like structures in cardiac-like cells from terminal stages were radially oriented and disorganized forming networks of Z-line-protein as seen in Fig. 4f.

To analyze the defective sarcomeres of β_1 -null cardiac muscle cells at the ultrastructural level ultrathin sections were prepared from 10- or 15-day-old wt and β_1 -null embryoid bodies and evaluated by electron microscopy. Wt cardiomyocytes develop a regular arranged sarcomeric structure,

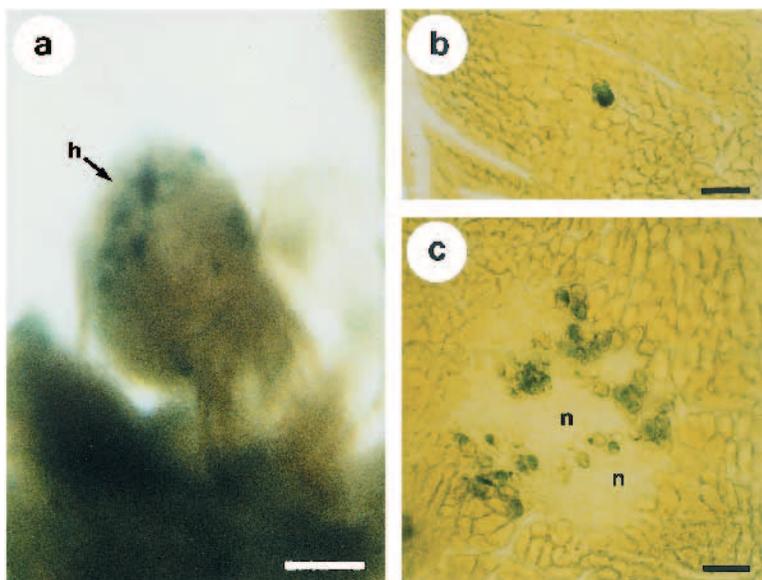


Fig. 6. In vivo staining of β_1 integrin-knockout cardiac muscle cells. β_1 -null cells were injected into normal blastocysts and transferred into foster mice. At 10.5 day of gestation, whole embryos were collected, fixed and stained for LacZ expression. (a) Many blue-positive areas were present in the embryonic heart (h). Adult mature heart tissue contains either single cells (b) or small nests of cells (c) which express LacZ. Several small nests contained cell debris (=n). Bar, 60 μ m.

exhibiting straight Z-lines and clear distinguishable I- and A-bands (Fig. 5a). In all β_1 -null embryoid bodies, detailed inspections of contractile areas revealed that sarcomeric-like structures were poorly developed and, when present, showed non-parallel myofilament orientation (Fig. 5b). Z-lines in β_1 -null cells are irregular in shape and size and interact partially with actin or with myosin filaments. Beside these defects in the sarcomeric architecture, all cells analyzed had regularly structured mitochondria with electron-dense matrix indicating that the cells were vital and were properly fixed.

Analysis of heart tissue in β_1 -integrin knockout chimaeric mice

To compare in vitro cardiac differentiation in embryoid bodies affected by a β_1 integrin deficiency with in vivo conditions, we analyzed β_1 integrin-knockout chimaeric mice.

To test whether β_1 integrin knockout cells can contribute to heart tissue both cell lines (G201 and G110) were injected into wild-type C57BL/6 blastocysts and transferred into foster mice. Out of 27 embryos isolated from decidual swellings at E10.5, 12 developed normally and were assayed for LacZ activity (indicating β_1 -null cells). Out of 12 embryos analyzed 9 contained visible staining for LacZ activity in the hearts (Fig. 6a). The three remaining embryos were clearly chimaeric, but showed no contribution of β_1 -null cells to the heart when inspected as whole mounts. A more detailed inspection of the LacZ-positive areas showed small nests of blue cells throughout the whole organ (Fig. 6a). This discontinuous pattern of β_1 -null cells seen in the heart was also present in most other tissues with the exception of the tissue under the apical ectodermal ridge, which contained LacZ-positive cells evenly distributed in the anterior/posterior and ventral/dorsal axis (Fig. 6a). Chimaeric animals generated from R1-derived β_1 integrin-deficient G110 cells showed a similar contribution (see Fässler and Meyer, 1995; unpublished data).

To test whether β_1 -null cells are still present in the adult heart, mature heart tissue of four chimaeric 6-week-old mice and three 6-month-old mice was analyzed histologically. Whereas cardiac tissue of prenatal chimaeric mice exhibited a large amount of larger LacZ-positive areas, in postnatal cardiac tissue LacZ-positive areas were diminished. Out of four 6-week-old adult hearts analyzed three contained LacZ-positive cells, which were distributed as single cells (Fig. 6b) or in small nests over the entire organ (Fig. 6c). In several areas containing β_1 -null cells, a considerable amount of cell debris could be observed (Fig. 6c). LacZ-positive areas were lost in 6-month-old chimaeric mice. In contrast, chimaeric mice made with heterozygous β_1 integrin mutant ES cells contained many more LacZ-positive areas, which did not contain necrotic cells (data not shown).

We tested whether sarcomeres are also altered in vivo, and we analyzed mature, adult heart tissue of chimaeric mice. Fig. 7a and b shows representative electron micrographs of heterozygous β_1 integrin mutant cells. No abnormalities could be observed. Fig. 7c shows an intact cardiac muscle cell adjacent to a β_1 -null cardiomyocyte. Whereas the wt cell (right) exhibited a regular arrangement of myosin and actin filaments and Z-bands, the β_1 -null cell (left) showed irregular deposition of myofibrils and large electron-dense areas containing components of sarcomeres. Most of the filament bundles present in β_1 -null cells contained thick myosin filaments stabilized by

normal M-bands (Fig. 7e). Thin filaments, however, were extremely scarce and, when present, were irregularly anchored in the Z-line (Fig. 7d). Typical I-bands were either not visible (Fig. 7d) or were completely absent (Fig. 7e).

In summary, loss of β_1 integrin causes an altered sarcomeric architecture in cardiac muscle cells, both in vitro and in vivo. Furthermore, β_1 -null cells can contribute to chimaeric heart tissue, although the number of cells is high in the prenatal period and decreases continuously in the adult heart.

DISCUSSION

In the present paper, we analyzed the consequences of the loss of β_1 integrin on cardiac muscle cell differentiation and on sarcomere integrity. The contribution of β_1 -null cells to cardiac cell lineage was particularly interesting since evidence has been provided that β_1 integrin is coordinately regulated during heart development (Carver et al., 1994; Terracio et al., 1991). Our results indicate that the absence of β_1 integrin results in an aberrant differentiation of cardiac muscle cells.

The delayed occurrence of fibrillating cardiomyocytes in β_1 -null embryoid bodies is accompanied by a retarded and transient expression of cardiac muscle-specific genes. In contrast to the genes coding for sarcomeric proteins, those encoding L-type Ca^{2+} channels were transcribed earlier (day 3) both in wt and in β_1 -null cells. Obviously, L-type Ca^{2+} channels are already present in a mesodermally committed state of differentiation, which leads to the development of cardiac cells (see Kleppisch et al., 1993).

The retarded development of sarcomeric proteins indicates that β_1 integrin-ECM interactions trigger signals that are important for the induction of the differentiation program that leads to specified cardiac muscle. A lack of β_1 integrins may deprive cells of information present in the microenvironment which is changing continuously throughout the development (Borg et al., 1982) and transduced to the cell interior by β_1 integrins. Although we found a delayed expression of cardiac marker genes in the β_1 -null embryoid bodies, these results demonstrate for the first time that induction of cardiac muscle-specific gene expression is possible even in the absence of β_1 integrins.

The retarded differentiation is confirmed by the action potential pattern of β_1 -null cardiomyocytes which profoundly differed from that of wt cardiomyocytes: action potentials that were typical for specialized cardiac muscle cells, such as pacemaker-, atrial- and ventricular-like cells occurred significantly delayed in β_1 -null cardiac muscle cells. The differentiation pattern of wild-type and β_1 integrin-null ES cells was found to be completely different: whereas, in wild-type cells, the stage of cardiac specialization and terminal differentiation is between day 14 and 26 (see Fig. 3), in β_1 integrin-deficient cells only the cardiac specialization stage corresponds to this time period, whereas the terminal differentiation stage corresponded to the stage around day 37. Furthermore, only a minor number of cells was able to elicit specialized action potentials for a short culture period. The functional impairment of cardiogenesis in the absence of β_1 integrin is most obvious at the terminal differentiation stage of in vitro differentiation. The absence of atrial and ventricular action potentials in the late culture period fits well with the disappearance of *ANF* and ven-

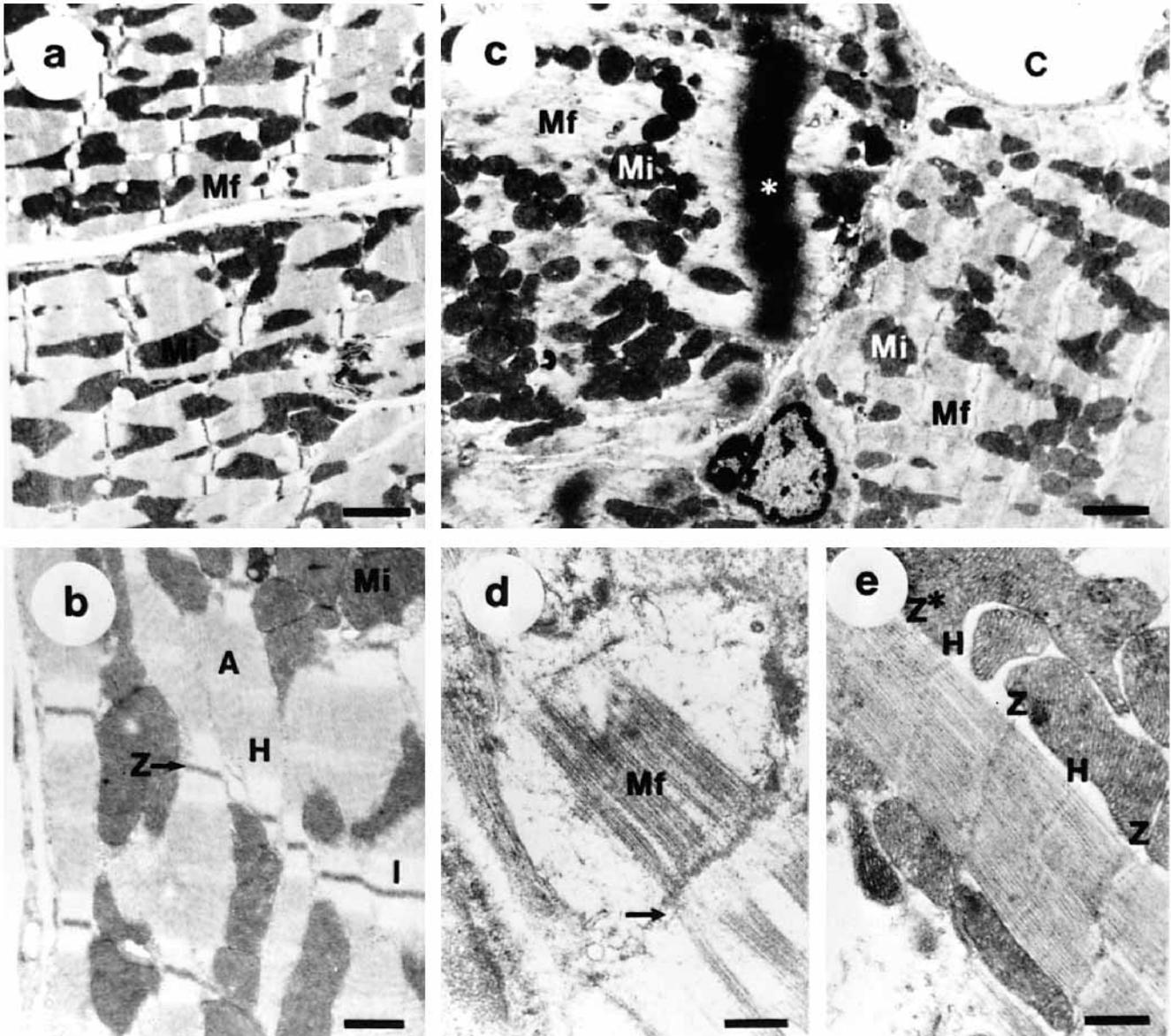


Fig. 7. Transmission electron microscopy of left papillary muscle cells of a β_1 integrin heterozygous (a,b) and a chimaeric mouse containing β_1 -null cells (c-e). (a) Longitudinal section of a β_1 integrin heterozygous cardiomyocyte with regular sarcomeric myofilament structures (Mf). (b) At higher magnification, regular sarcomeric structures including A-band (A), I-band (I) and M-line (H) of a β_1 integrin heterozygous cardiomyocyte are recognizable. (c) A longitudinal section of a β_1 integrin-knockout chimaeric heart with a regular structured cardiomyocyte (right) in direct neighborhood of a β_1 null cardiomyocyte (left) revealing intact mitochondria (Mi), but an irregular myofilament structure (Mf) and areas of electron-dense material (*). Sarcomeres are disarranged and Z-lines are missing. Myofilament bundles consisting mainly of myosin spread across the whole cell; c, capillary. (d) Myofilaments are incompletely anchored on the Z-line (arrow) and no regular I-band is developed. (e) Dense myofilament bundles are interrupted by Z-lines (Z) and partly incompletely developed (Z*). An I-band consisting mainly of actin is not developed. Myosin filaments are stabilized by a M-line (H). Bars: 1,600 nm (c); 1,400 nm (a); 600 nm (b,e); 500 nm (d).

tricular-specific *MLC-2V* mRNAs. Finally, the terminal stage was characterized by the presence of only pacemaker-like cells with unusual high frequency and/or arrhythmia.

The fate of the more specialized atrial- and ventricular-like cells, which appeared only transiently, is not clear. One possibility is that β_1 integrins confer survival to these cells. Apoptosis is a likely mechanism to eliminate such cells. Involvement of integrins in cellular signalling processes controlling apoptosis is a well-established phenomenon and has

been shown for a variety of other cell types (Meredith et al., 1993; Frisch and Francis, 1994; Boudreau et al., 1995).

While postnatal cardiac tissue of chimaeric mice showed a remarkable decrease in number and size of LacZ-positive areas, embryonic hearts of chimaeric mice exhibit large amounts of LacZ-positive areas. The small areas of postnatal LacZ-positive cardiomyocytes were surrounded by areas of necrotic tissue, suggesting that a loss of β_1 integrin leads to a deterioration of β_1 -null cells in this chimaeric cardiac tissue.

This could be due either to processes of degeneration or apoptosis, which leads to this reduction of β_1 -null cells in chimaeric cardiac tissue but not to degeneration of the LacZ-negative cardiac tissue.

The typical alterations in cardiac gene expression and in the appearance of action potentials were reflected in the morphology of β_1 -null cardiomyocytes. Although the sarcomeric components α -sarcomeric actin, sarcomeric MHC and α -actinin are detectable by immunohistochemistry in β_1 -null cardiomyocytes, the arrangement of myofilaments to myofibrils with appropriate sarcomeric structure is significantly altered, in particular in the cardiac specialization stage. These changes in the cytoarchitecture might also be responsible for the changes in cell morphology. Whereas normal cardiac muscle cells are triangular or spindle shaped, β_1 -null cells are more round and much smaller. This phenomenon has also been described with antibody perturbation experiments, which show that normal spreading and myofibrillogenesis of neonatal rat cardiomyocytes are altered in the presence of anti- β_1 integrin antibodies (Hilenski et al., 1992).

The ultrastructural observations confirm the immunohistochemical staining of contractile proteins in cardiomyocytes and demonstrate the absence of a regular arrangement of sarcomeric structure and irregularities in the Z-line as well as in the coupling of actin and myosin filaments with the Z-line. A regular configuration of myofibrils is not attained, which obviously leads to a fibrillation rather than to a regular contraction. This all leads to the suggestion that the function of β_1 integrin is related to the organization or maintenance of intact sarcomeric myofibrils. Similarly, β_1 -null cells in the chimaeric mice detected by staining for LacZ activity showed a disarrangement of the filaments to the Z-line and Z-line abnormalities.

These findings directly relate to reports showing that β_1 integrin is located in areas of myofibril-sarcolemma junctions in striated muscle (Bozyczko et al., 1989; Borg et al., 1990) and that integrins interact via α -actinin and talin (Horwitz et al., 1986; Otey et al., 1990) with the actin cytoskeleton. In the *Drosophila* mutant *mysospheroid*, which lacks the homologue of the mammalian β_1 integrin (Volk et al., 1990), abnormal sarcomeres are observed. In *Caenorhabditis elegans*, it was shown that integrin-mediated interactions in muscle cells with both cytoskeletal elements and constituents of the basement membranes are crucial for a normal sarcomeric organization. Lack of either vinculin (Barstead and Waterstone, 1991) thought to bridge the cytoplasmic tail of β_1 integrin via α -actinin to actin filaments, or of perlecan (Rogalski et al., 1993, 1995), a constituent of the basement membrane and shown to bind to the extracellular domain of β_1 integrins, results in abnormal sarcomeres. However, it is currently impossible to distinguish whether integrins take a direct role in the formation of the sarcomere or are important for maintaining the stability of the sarcomeric arrangement by attaching myofilaments at the costamere. The instability of the myofibrillar apparatus may lead to a degradation of the sarcomeric components and consequently of the specialized cardiac cells.

In conclusion, applying methods of cell biology, patch clamp technique, immuno-histochemistry and ultrastructural analysis, it is possible for the first time to show that loss of β_1 integrin leads to a delayed and unstable cardiac differentiation pattern. Therefore, cell-matrix involvement via integrins is

important for the maintenance of myofibrillar organization and a specialized cardiac phenotype.

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