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Knock-in of integrin β1D affects primary but not secondary myogenesis in mice

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SUMMARY

Integrins are extracellular matrix receptors composed of α and β subunits involved in cell adhesion, migration and signal transduction. The β1 subunit has two isoforms, β1A ubiquitously expressed and β1D restricted to striated muscle. They are not functionally equivalent. Replacement of β1A by β1D (β1D knock-in) in the mouse leads to midgestation lethality on a 50% Ola/50% FVB background [Baudoin, C., Goumans, M. J., Mummery, C. and Sonnenberg, A. (1998). *Genes Dev.* 12, 1202-1216]. We crossed the β1D knock-in line into a less penetrant genetic background. This led to an attenuation of the midgestation lethality and revealed a second period of lethality around birth. Midgestation death was apparently not caused by failure in cell migration, but rather by abnormal placentation. The β1D knock-in embryos that survived midgestation developed until birth, but exhibited severely reduced skeletal muscle mass. Quantification of myotube numbers showed that substitution of β1A with β1D impairs primary myogenesis with no direct effect on secondary myogenesis. Furthermore, long-term primary myotube survival was affected in β1D knock-in embryos. Finally, overexpression of β1D in C2C12 cells impaired myotube formation while overexpression of β1A primarily affected myotube maturation. Together these results demonstrate for the first time distinct roles for β1 integrins in primary versus secondary myogenesis and that the β1A and β1D variants are not functionally equivalent in this process.

Key words: β1 integrins, Knock-in, Myogenesis, Muscle mass, Cell migration, Placentation, Mouse

INTRODUCTION

Cell-extracellular matrix (ECM) interactions play a crucial role during embryonic development (DeSimone, 1994; Darribère et al., 2000). Cells interact with the ECM via a variety of receptors, the integrins being the most common (Hynes, 1992; Hynes, 1999). Integrins consist of heterodimer complexes of α and β subunits (van der Flier and Sonnenberg, 2001). The β1 subunit associates with at least 12 different α subunits and forms the largest and most abundantly expressed subfamily. In the mouse, it occurs as two highly homologous isoforms: β1A and β1D (van der Flier et al., 1995; Zhidkova et al., 1995). These are not functionally redundant; we have shown previously that replacement of β1A with β1D is lethal in embryos homozygous for the knock-in allele (*β1D ki/ki*) (Baudoin et al., 1998). Moreover, β1A and β1D have different binding affinities for the cytoskeletal proteins talin, filamin, α-actinin (Belkin et al., 1997) and the integrin linked kinase (ILK) (Hannigan et al., 1996). The stronger binding of β1D to talin and the observation that fibroblasts isolated from *β1D ki/ki* embryos show impaired migration in vitro (Baudoin et al., 1998), raises the possibility that cell migration might be affected when only β1D is expressed.

During mouse development, β1A and β1D expression is mainly non-overlapping: β1A is expressed ubiquitously, while β1D is detectable in the heart from E11.0, increasing sharply around birth, and in skeletal muscle from E17.5 (van der Flier et al., 1997; Brancaccio et al., 1998). In both cases β1D completely displaces β1A perinatally, suggesting distinct roles for each in striated muscle in vivo.

In vertebrates, myogenesis occurs sequentially, starting when proliferating myoblasts induce myogenic regulatory factors, such as Myf5, MyoD and myogenin, followed by an irreversible exit from the cell cycle, phenotypic differentiation and fusion of myoblasts to form multinucleated elongated myotubes (Buckingham, 2001). During mouse development,

there are two waves of myogenesis involving three distinct populations of myoblasts. Between E11.5 and E15.5 the formation of primary myotubes results from the fusion of primary (embryonic) myoblasts, while secondary (foetal) myoblasts remain proliferative. From E15.5, secondary myoblasts progressively enter the muscle differentiation programme. Finally, a third population of myoblasts (adult) develops and contributes to secondary myogenesis until well after birth and also gives rise to the quiescent satellite cells of adult muscle (Wigmore and Dunglison, 1998).

Antibody perturbation experiments have suggested a role for β1 integrins during several steps of myogenesis, including migration, differentiation and fusion (McDonald et al., 1995; Gullberg et al., 1998). However, in wild type/β1-null chimeric mice, β1-deficient myoblasts migrate, differentiate and fuse with wild-type myoblasts/myotubes (Fässler and Meyer, 1995). Thus the role of β1 integrins in myogenesis in vivo is presently not clear. In vitro studies show that exogenous β1D inhibits cell cycle progression in cultured C2C12 cells (Belkin and Retta, 1998), while exogenous β1A maintains proliferation in quail myoblasts when paired with a permissive α chain (Sastry et al., 1999). Together, these in vitro results suggest not only that integrin β1 may play a role in myogenesis, but that β1A and β1D might have different functions.

We studied in detail the role of β1A and β1D during myogenesis in vivo in the β1D knock-in mice, which were crossed into a less penetrant genetic background than used previously. This revealed a second period of lethality. Our results show that the replacement of β1A with β1D supports normal cell migration during development, but placentation is abnormal and the most likely cause of midgestational death. Furthermore, we show for the first time that β1 plays an important role during myogenesis in vivo, β1D being incapable of replacing β1A functionally during primary myogenesis.

MATERIALS AND METHODS

Animals and embryos

Generation of β1D knock-in mice on a mixed background (50% 129Ola/50% FVB) has been described previously (Baudoin et al., 1998). Here, heterozygous individuals were backcrossed four times onto a FVB background. Homozygous *β1D ki/ki* embryos were obtained from heterozygous crossings. The day of the vaginal plug was designated embryonic day (E) 0.5. Following cervical dislocation, embryos were collected from E8.5-18.5 or pregnancies were allowed to reach term. DNA isolated from tail biopsies or visceral yolk sacs was used for genotyping as described previously (Baudoin et al., 1998). For embryos sectioned in paraffin or paraplast, DNA was extracted from the embedded material using the TaKaRa DEXPAT™ kit.

Primordial germ cells

E10.5 embryos were fixed in 4% paraformaldehyde (PFA) for 2.5 hours at 4°C, embedded in Paraplast and stained for alkaline phosphatase (AP) activity (Lawson et al., 1999). Primordial germ cells (PGCs) in each embryo (n=8 for wild-type and n=6 for *β1D ki/ki* embryos) section were counted on the basis of the strong cytoplasmic AP activity and morphology (Chiquoine, 1954).

PGCs were isolated by flow cytometry as described by Abe et al. (Abe et al., 1996) and cDNA isolated by standard procedures. RT-PCR was performed on 2 ng cDNA and PCR conditions were 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 54°C for 30 seconds and 72°C for 30 seconds. Primers for integrin β1A (282 bp) and β1D (363 bp) were 5′-GGCAACAATGAAGCTATCGT-3′ and 5′-CCCT-CATACTTCGGATTGAC-3′; for Oct4, 5′-GGAGAGGTGAAAC-CGTCCCTAGG-3′ and 5′-AGAGGAGGTTCCCTCTGAGTTGC-3′ (Anderson et al., 1999); and for HPRT, 5′-GCTGGTGAAAAGGAC-CTCT-3′ and 5′-CACAGGACTAGAACACCTGC-3′ (Johansson and Wiles, 1995).

In situ hybridisation

Digoxigenin-labelled RNA probes were prepared from linearised plasmids (Sambrook et al., 1989). Whole-mount in situ hybridisation was performed as described by Henrique et al. (1995). mRNA was visualised using BM Purple (Roche). The expression of *Snail* (n=8 for *β1D ki/ki* and wild type or heterozygous) and *pax3* (n=2 for *β1D ki/ki* and heterozygous) were analysed in toto; some embryos were embedded in Technovit 8100™ and sectioned.

Histology

E18.5 embryos were fixed for 10 days in 4% PFA, washed for 10 days in PBS, 5 days in 0.83% NaCl, 10 days in 1:1 mix of 0.83% NaCl and 100% ethanol and stored in 70% ethanol, all at 4ºC. After embedding in paraffin (Histowax), 7 μm sections were processed for Haematoxylin and Eosin staining.

Immunohistochemistry

E18.5 cleidomastoideus muscle was fixed in 2% PFA, dissected out and embedded in Tissue-Tek OCT compound. E14.5 placenta, E18.5 thoracic body wall, and E14.5, E16.5, E17.5 and E18.5 lower hindlimb muscles were embedded without fixation, but immediately frozen in liquid nitrogen-chilled isopentane. Transverse cryosections (5-10 μm) were fixed in 2% PFA and processed as described by Venters et al. (Venters et al., 1999). Some E14.5 placentae were fixed in 4% PFA and embedded in paraffin. Paraffin sections (7 μm) were processed as described previously (Zwijsen et al., 1999) and some were processed for Haematoxylin and Eosin staining.

Primary antibodies used were rabbit anti-EHS laminin (Sigma), rat anti-mouse endothelial glycoprotein (MECA32, DSHB), mouse anti-slow myosin heavy chain (slow MHC, NOQ7.1.1A) (Draeger et al., 1987), mouse anti-neonatal/fast MHC (MY-32, Sigma), rabbit anti-protein gene product 9.5 (PGP9.5) (Thompson et al., 1983), rat anti-muscle acetylcholine nicotinic receptor (mAb35; DSHB) and rabbit anti-phospho-histone H3 (UBI). Secondary antibodies were Alexa 488 (Molecular Probes) or TRITC-conjugated (Sigma) goat anti-rabbit IgG, FITC- or TRITC-conjugated goat anti-rat IgG (Sigma) and HRP-conjugated goat anti-mouse IgG (Southern Biotech). For the latter, diaminobenzidine was used as a substrate.

Myotube quantification

Primary and secondary myotubes were quantified by counting slow MHC-positive (primary) and slow MHC-negative (secondary) myotubes (Harris et al., 1989; Sheard and Duxson, 1996). Consecutive sections were labelled with the MY32 antibody, which stains all myotubes at E18.5.

In E18.5 body wall muscles, primary and secondary myotubes were counted per unit area of intercostal and serratus dorsalis muscles. The number of myotubes from the sternum to the vertebra in one section per individual (n=2 for wild type and n=3 for *β1D ki/ki*) was counted and the number of myotubes/mm^2 calculated.

In E18.5 cleidomastoideus and lower hindlimb muscles, primary and secondary myotubes were counted in 3-6 serial sections, from each individual (n=3 or 4 per genotype). The widest part of the hindlimbs and the red area of cleidomastoideus were used. Primary myotubes were counted in E14.5 lower hindlimb and the myotube diameter measured in extensor digitorum longus (EDL) muscle in at least 15 myotubes from each individual (n=3 per genotype). Primary

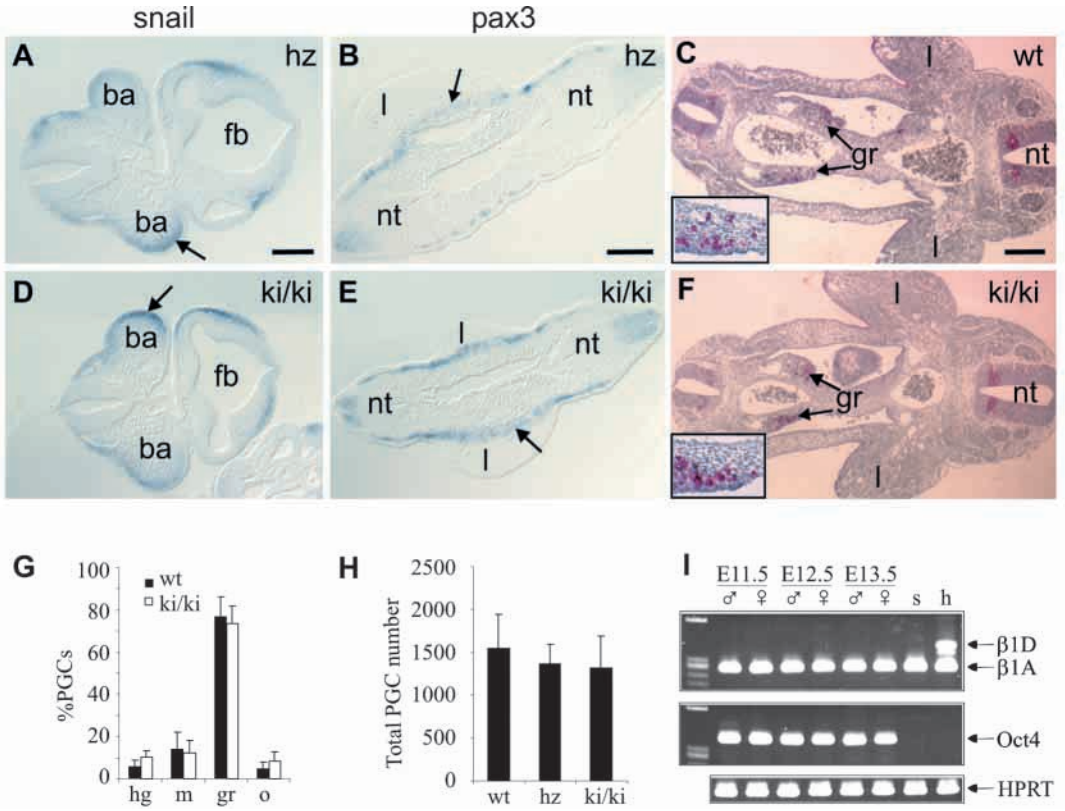


Fig. 1. Different migratory cell types behaved normally in *β1D ki/ki* embryos. (A-D) Transverse sections showing (A,D) *snail* expression in migratory neural crest cells (arrows) and (B,E) *pax3* expression in migratory limb muscle precursor cells (arrows) of E9.5 heterozygous (A,B) and *β1D ki/ki* (D,E embryos). (C,F) PGC distribution was analysed in transverse serial sections of E10.5 wild-type (C) and *β1D ki/ki* (F) embryos. An enlargement of the right gonadal ridge is shown. (G,H) At E10.5, PGCs were detected by AP staining in the hindgut (hg), mesenterium (m), gonadal ridges (gr) and ectopic regions (o) of *β1D ki/ki*, heterozygous and wild-type embryos in the quantities indicated. (I) RT-PCR detection of β1A and β1D in isolated PGCs from different stages. Oct4 is a PGC marker at these stages. HPRT is a loading control. ba, first branchial arch; fb, forebrain; gr, gonadal ridges; h, newborn heart; l, limb bud; nt, neural tube; s, E11.5 gonadal somatic tissue. Scale bars: 200 μm (A,B,D,E), 400 μm (C,F).

and secondary myotubes were also counted in E16.5 (n=1 per genotype) and E17.5 (n=2 per genotype) EDL muscle.

Differences in the numbers of myotubes were tested either by analysis of variance (body wall, E16.5 hindlimb) or nested analysis of variance. Differences in myotube diameter were tested by nested analysis of variance.

TUNEL assay

Apoptosis was analysed on cryosections of E14.5 placentae (n=2 or 3 per genotype), and E14.5, E16.5 and E17.5 (n=2, 1, 2 per genotype, respectively) hindlimb muscles using the Cell Death Detection Kit (Roche). TUNEL-positive nuclei were counted in the tibialis anterior, (TA), EDL and peroneus group (P) muscles using at least 5 serial sections per individual.

C2C12 differentiation

The mouse myoblast cell line C2C12 (ATTC CRL 1772) was grown in DMEM supplemented with 20% (v/v) foetal calf serum and high glucose (5 g/l). The C2C12/β1D and C2C12/β1A cells were generated by retroviral transduction with cDNA constructs for human β1A and β1D, as described previously (Gimond et al., 1999), but using 2×10^4 C2C12 cells and infection for 6 hours. Myogenic differentiation was induced as described by van der Flier et al. (van der Flier et al., 2002).

For immunofluorescence, cells were processed as described by van der Flier et al. (van der Flier et al., 2002), using mouse anti-sarcomeric MHC (MF20; DSHB) and FITC-conjugated goat anti-mouse IgG (Sigma). To-Pro 3 (Molecular Probes) was added to the last PBS wash. The number of MF20-positive cells (MHC^+, total myoblasts and myotubes) and respective nuclei were counted in three microscopic fields at 10× magnification and three to seven microscopic fields at 16× magnification. The fusion index (%) was calculated as the ratio of nuclei in myotubes (>3 nuclei) to the total number of nuclei in MHC^+ cells. Student's t-test was used to analyse the differences between wild-type and infected cells.

Western blotting was performed as in van der Flier et al. (van der Flier et al., 1997). Primary antibodies were mouse anti-p21 (UBI), rabbit anti-pRB (Santa Cruz), MF20, rabbit anti-connexin43 (Sigma), rabbit anti-β1Acyto (kind gift from U. Mayer) and mouse anti-β1Dcyto (2B1) (van der Flier et al., 1997). Secondary antibodies were HRP-conjugated sheep anti-mouse and donkey anti-rabbit (Amersham Pharmacia).

RESULTS

Two periods of lethality for *β1D ki/ki* mice

Embryos between E8.0 and E18.5 were collected and genotyped (Table 1). The genotypes showed a Mendelian

Table 1. Number of embryos of each genotype recovered from heterozygous crossings

Stage	+/+	+/*ki*	*ki/ki*
E8.0-E9.5	65^{1*} (26.4%)	105^{4*} (42.7%)	76^{1*} (30.9%)
E10.0-E11.5	93 (26.0%)	194^{4*} (54.0%)	70^{6*} (20.0%)
E12.0-E13.5	21 (20.0%)	59 (56.2%)	25^{1*}(23.8%)
E14.0-E16.5	26 (17.0%)	$107^{2*,2‡}$ (70.0%)	$20^{2†,6*,1‡}$ (13.0%)
E17.0-E18.5	44 (25.6%)	$112^{1†}$ (65.1%)	$16^{4†,1*,13‡}$ (9.3%)
P1-4	54 (39.1%)	79 (57.3%)	$5^{5‡}$ (3.6%)
>P20	51 (41.1%)	72 (58.1%)	$1^{1‡}$ (0.8%)

n^{*}, the number of embryos showing abnormalities, including open or kinked neural tube, reduced branchial arches or abnormal head shape and eccentric limbs.

Dead or reabsorbed embryos were not included in the table but are given as $n^{†}$. ‡Number of shorter/thinner embryos.

distribution until E13.5, after which the *β1D ki/ki* genotype frequency decreased from about 24% to 9% of the total embryos recovered. The embryos that survived this first period of lethality developed further and were alive at E18.5. This contrasts with our previous results on a mixed background (50% 129Ola/50% FVB), where only two of a total of 35 embryos, which were collected at E16.5 and genotyped as *β1D ki/ki*, were dead and highly abnormal (Baudoin et al., 1998). Since the gross morphology of the *β1D ki/ki* embryos at E18.5 on a mainly FVB genetic background was relatively normal, we allowed subsequent litters to develop to term. Of the 138 pups on P1, five were *β1D ki/ki*; three were dead and one survived for 1 day. The fifth (male) *β1D ki/ki* pup survived for 5 months and was fertile. Of his offspring (two litters), nine individuals were heterozygous and six *β1D ki/ki*. These *β1D ki/ki* pups died soon after birth. The relatively normal gross morphology of *β1D ki/ki* embryos at E18.5 was therefore only exceptionally reflected in viable offspring and survival to adulthood. Thus, the FVB background resulted in reduced penetration of the phenotype and revealed a second period of lethality, occurring at, or soon after, birth.

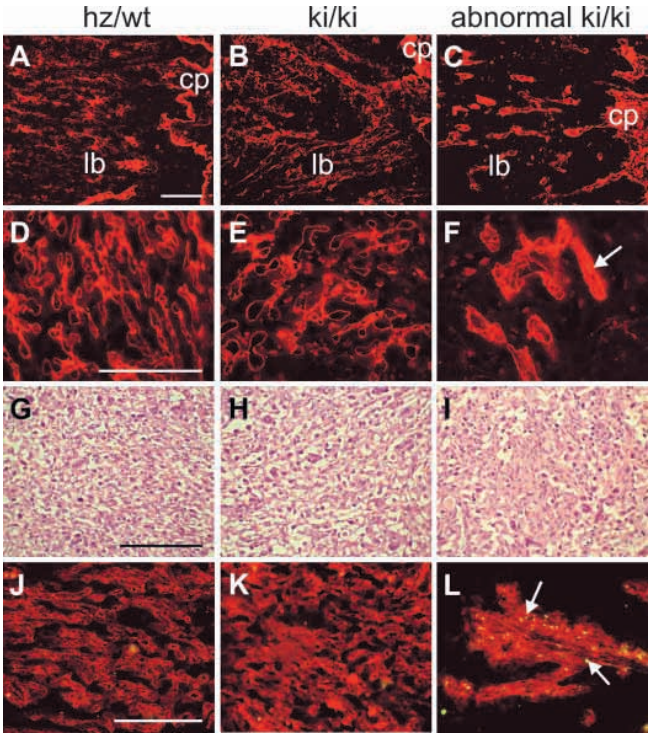


Fig. 2. At E14.5, abnormal *β1D ki/ki* embryos showed defective vascularisation and increased apoptosis of endothelial cells in the placental labyrinth. (A,D,G) Heterozygous, (J) wild-type (B,E,H,K) normal *β1D ki/ki* and (C,F,I,L) abnormal *β1D ki/ki* embryos. (A-C) Laminin immunostaining, in transverse sections of E14.5 placentae, showing the basal lamina of foetal blood vessels. (D-F) Higher magnification of the labyrinth in A-C showing reduced branching and obstructed blood vessels in abnormal *β1D ki/ki* embryos (arrow). (G-I) Haematoxylin and Eosin staining. (J-L) Transverse sections showing MECA32- (red) and TUNEL- (green) positive cells (arrows in L). cp, chorionic plate; lb, labyrinth. Scale bar: 200 μm.

Gross morphology of *β1D ki/ki* embryos

Between E8.0-E13.5, we observed similar external defects as described previously (Baudoin et al., 1998). These included abnormal head or branchial arches, open or kinked neural tube, and retarded or eccentric limbs (Table 1). However, the frequency was considerably lower (7.4% compared with 27.5% between E10.0 and E13.5), showing that on the present genetic background, replacement of β1A by β1D had less severe morphological consequences. Furthermore, *β1D ki/ki* embryos showed no signs of haemorrhage. Thus, from the external morphology alone, no defects severe enough to cause the previously observed early lethality were detected.

Migratory cells behave normally in *β1D ki/ki* embryos

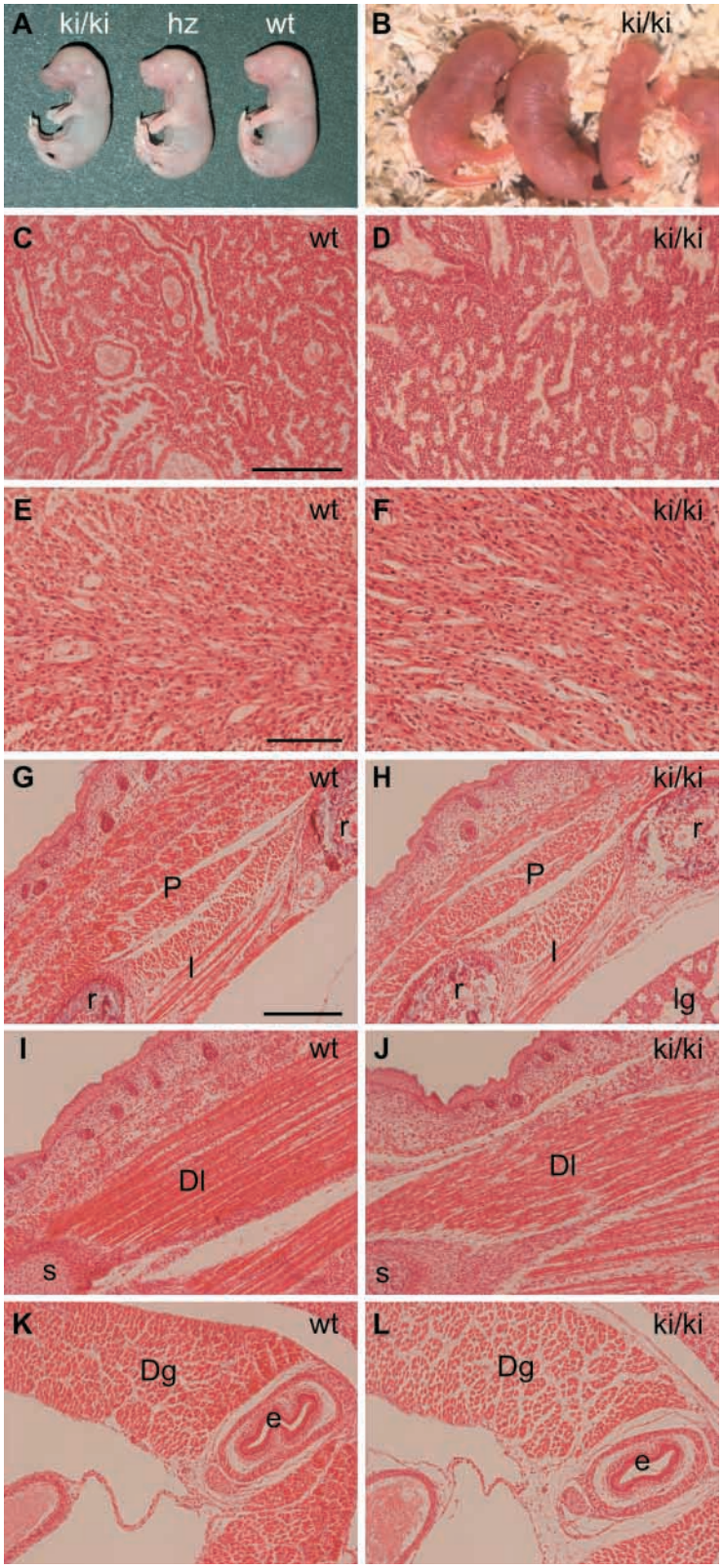
One of the most prominent defects in E11.5 *β1D ki/ki* embryos on a mixed background was a reduction in the size of the first branchial arch (Baudoin et al., 1998), raising the possibility that the migration of *β1D ki/ki* cranial neural crest cells into the branchial arches could be impaired. Although on a mainly FVB background, only 7.4% of the *β1D ki/ki* embryos had a phenotype suggesting problems with cell migration, we determined whether a general failure in cell migration contributed to the first period of lethality. The behaviour of three migratory cell populations (cranial neural crest cells, limb muscle precursor cells and primordial germ cells; PGCs) was analysed in *β1D ki/ki* embryos and their littermates.

In E9.5 embryos there were no differences between *β1D ki/ki* embryos and their littermates in the expression of *snail* (Fig. 1A,D), which encodes a transcription factor in cranial neural crest cells (Nieto et al., 1992) and *pax3* (Fig. 1B,E), a marker for muscle precursor cells (Goulding et al., 1994).

Since wild-type PGCs only express integrin β1A (Fig. 1I), any delay in the rate of PGC migration in *β1D ki/ki* embryos would be due to the exclusive expression of the β1D splice variant. Comparison of PGC distribution in both E10.5 *β1D ki/ki* and wild-type embryos revealed no significant differences (Fig. 1C,F,G). Furthermore, the absolute number of PGCs in each of the genotypes was similar (Fig. 1H).

Although we cannot exclude defective cell migration in the few *β1D ki/ki* embryos showing external defects, our data show that three cell populations that undergo long-range cell

migration behave normally in the *β1D ki/ki* embryos analysed. These results suggest that on a predominantly FVB genetic background, β1D is able to support cell migration in vivo as efficiently as β1A.



Abnormal vascularisation and increased apoptosis in the placental labyrinth causes early death of *β1D ki/ki* embryos

The *β1D ki/k*i embryos that ceased development between E13.5-E14.5 were exceptionally pale (not shown), raising the possibility of placental malfunction. We examined the distribution of laminin, a marker for foetal blood vessels (Harbers et al., 1996), in placentae of E14.5 embryos. The results showed that embryonic blood vessels present in the labyrinth of both heterozygous and morphologically normal, living *β1D ki/k*i embryos were uniform in diameter and regularly spaced (Fig. 2A,B,D,E,G,H). In contrast, the labyrinth of the pale *β1D ki/ki* embryos contained a reduced number of large calibre blood vessels, the lumen of the vessels was irregular and many were obstructed. Furthermore, there was less branching, although strong laminin immunoreactivity was maintained (Fig. 2C,F,I). TUNEL assay and immunostaining with MECA32 antibody (recognising endothelial cells) revealed increased apoptosis in labyrinthine endothelial cells in two of three pale *β1D ki/ki* placentae, compared to wild-type or morphologically normal *β1D ki/ki* placentae (Fig. 2J-L). However, phospho-histone H3 immunostaining showed no difference in the number of proliferating cells (not shown). Interestingly, the one *β1D ki/ki* placenta that did not show increased apoptosis, did show a marked increase in proliferation (not shown), suggesting placental recovery (Plum et al., 2001).

These results suggested that some *β1D ki/ki* embryos have placentae with a reduced network of foetal blood vessels, and that placental endothelial cells undergo apoptosis. This could compromise maternal-foetal nutrition and be the cause of early lethality. However, the fact that the 36% *β1D ki/ki* embryos, surviving this period, developed normally until birth indicates that this defect is of variable severity on the FVB genetic background.

Reduction in muscle mass in late gestation *β1D ki/ki* embryos

The *β1D ki/ki* embryos recovered alive between E14.0 and E18.5 were as advanced in development as their wild-type littermates. However, a large proportion was thinner and a few were also shorter than heterozygous and wild-type embryos (Fig. 3A). This was particularly evident between E17.0-E18.5 where 13 of 16 *β1D ki/ki* embryos exhibited this phenotype.

All *β1D ki/ki* pups alive on P1 were both thinner and shorter than their littermates (Fig. 3B). Two of the five

Fig. 3. Late gestation *β1D ki/ki* embryos and newborn pups are thinner/shorter than their littermates and exhibit a reduction in muscle mass. (A,B) Gross morphology of E18.5 embryos (A) and newborns (B). Note the curved posture of the E18.5 *β1D ki/ki* embryo. (C-L) Transverse Haematoxylin and Eosin-stained sections of E18.5 wild-type (C,E,G,I,K) and *β1D ki/ki* (D,F,H,J,L) embryos showing lung (C,D), heart (E,F), thoracic body wall (G,H), shoulder (I,J) and diaphragm (K,L) muscles. Dg, diaphragm; Dl, deltoideus; e, esophagus; I, intercostal; lg, lung; P, pectoral; r, rib; s, scapula. Scale bar: 200 μm.

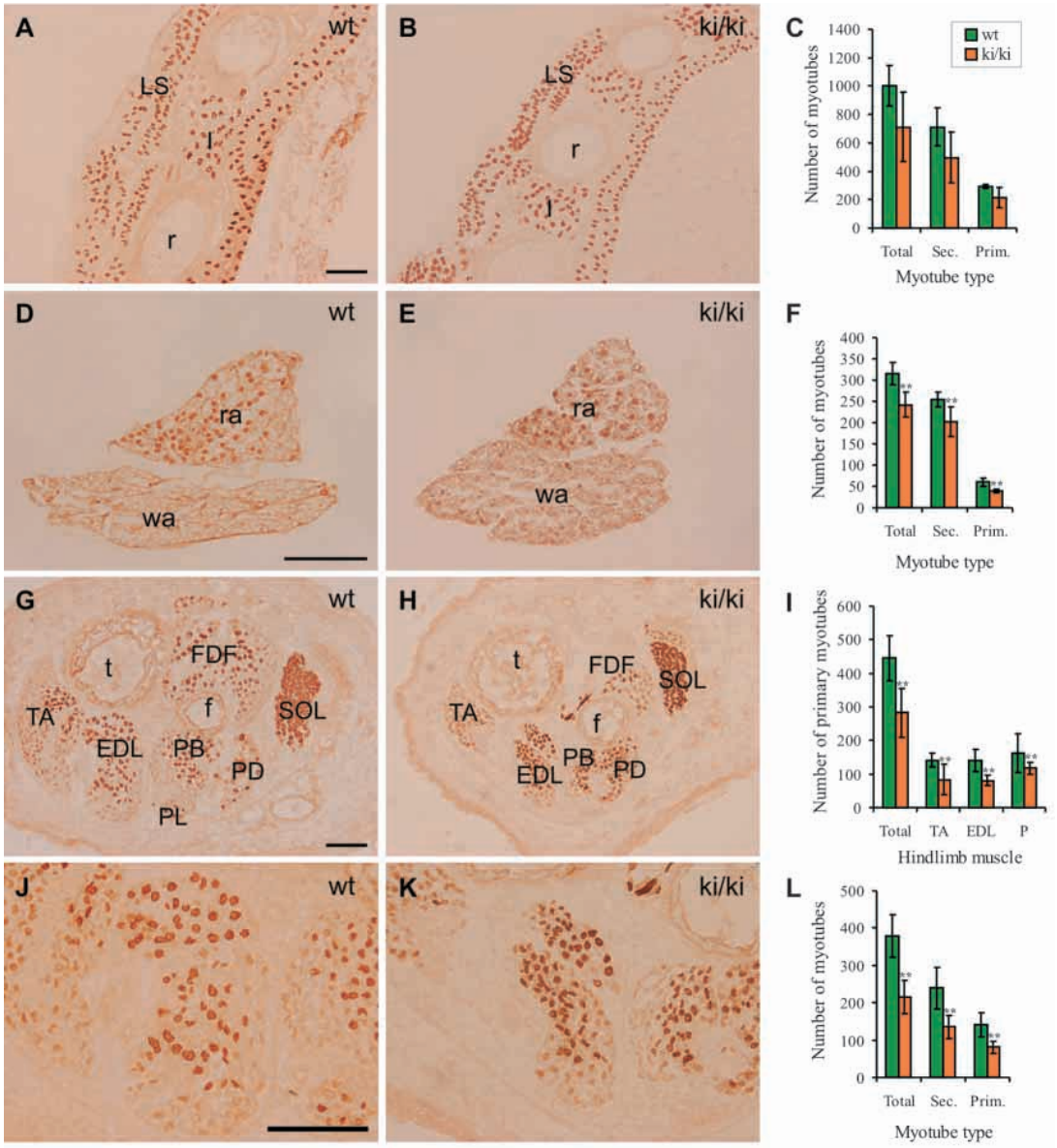


Fig. 4. Different muscle types of E18.5 embryos stained with the anti-slow MHC antibody show a reduction in primary and secondary myotubes in *β1D ki/ki* embryos (B,E,H,K) compared to wild-type littermates (A,D,G,J). Transverse sections of body wall (A,B), cleidomastoideus (D,E) and lower hindlimb (G,H) muscles. J and K are higher magnifications of EDL muscles in G and H. (C,F,I,L) Graphical representation of the differences in myotube number between wild-type and *β1D ki/ki* E18.5 muscles. (C) Total, secondary and primary myotube numbers/mm^2 in intercostal/serratus dorsalis muscles. (F) Total, secondary and primary myotube numbers in red area of cleidomastoideus muscle. (I) Total number of primary myotubes in lower hindlimb represents the sum of myotubes in TA, EDL, and P group. (L) Total, secondary and primary myotubes in EDL muscle. Bars represent means ± s.d.; $^{**}P \leq 0.01$. EDL, extensor digitorum longus; f, fibula; FDF, flexor digitorum fibularis; I, intercostal; LS, latissimus dorsi/serratus dorsalis; P, peroneus group (including: PB, peroneus brevis; PD, peroneus digiti; PL, peroneus longus); r, rib; ra, red area of cleidomastoideus; SOL, soleus; t, tibia; TA, tibialis anterior; wa, white area of cleidomastoideus. Scale bars: 200 μm.

pups alive on P1 were also purple (suggesting respiratory distress) and unable to feed. The *β1D ki/ki* male that survived to adulthood behaved normally, but remained smaller than all littermates (weight on P36 was 15 g compared with an average of 22 g for 4 male littermates).

Histological analysis of E18.5 *β1D ki/ki* embryos did not reveal obvious gross morphological abnormalities, and all major organs, e.g. lung (Fig. 3C,D), heart (Fig. 3E,F) and kidneys (not shown), appeared normal. However, there was a marked reduction in skeletal muscle mass (Fig. 3G-L), probably causing the overall size reduction. Furthermore, while wild-type and heterozygous embryos/neonates had a straight spine and upright head, the posture of *β1D ki/ki* embryos was characterised by pronounced spinal curvature and downward facing head (Fig. 3A). Interestingly, myogenin-deficient mice (Hasty et al., 1993; Nabeshima et al., 1993) displayed a similar posture and gross phenotype, and also died perinatally due to a severe reduction in skeletal muscle mass.

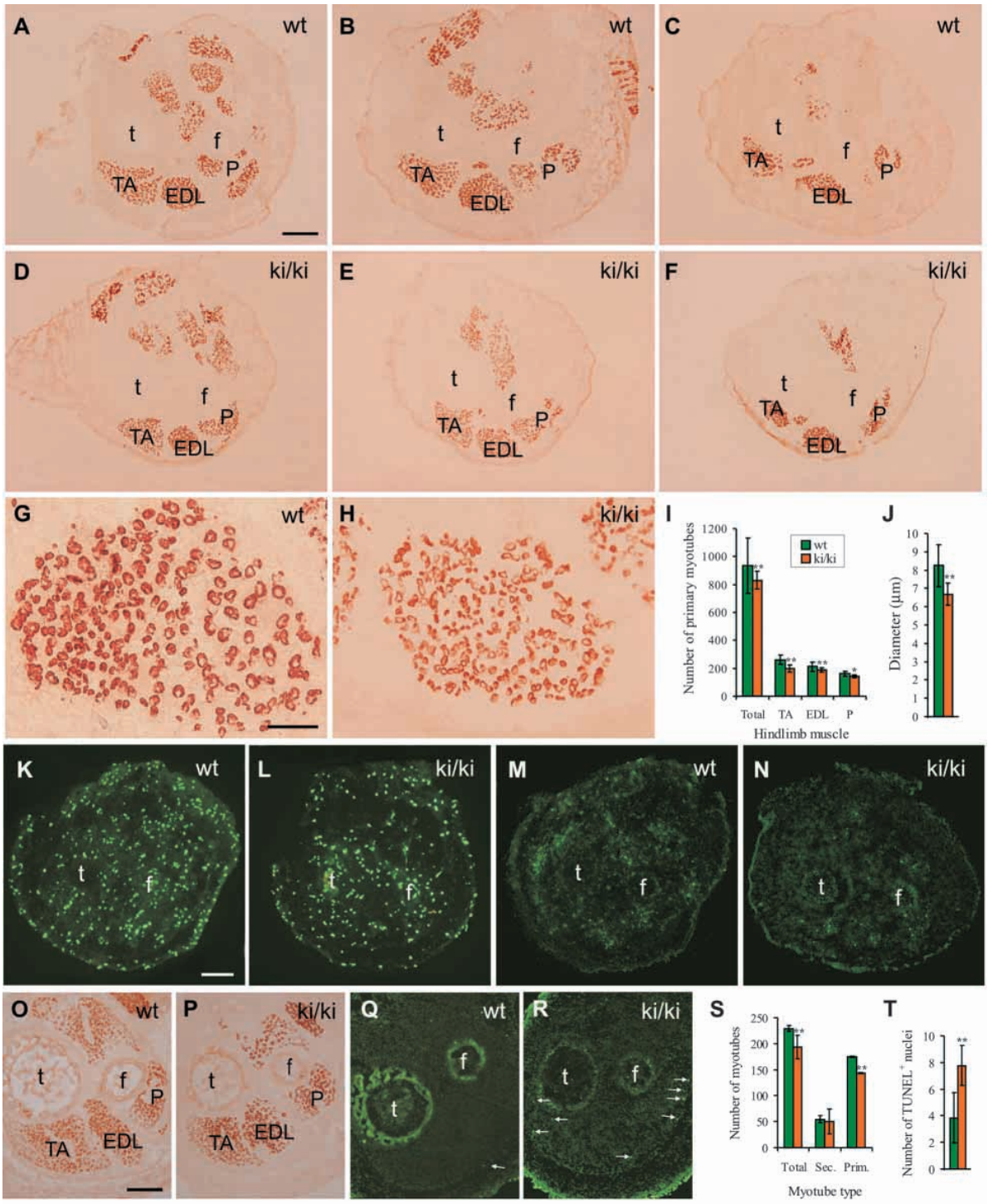


Fig. 5. Hindlimb muscles of *β1D ki/ki* E14.5 embryos (D,E,F,H) have reduced numbers of primary myotubes compared to wild-type embryos (A,B,C,G). Transverse sections of lower hindlimb: (A,D) upper (B,E) middle and (C,F) lower region, stained with anti-slow MHC, showing that muscle size is always reduced in *β1D ki/ki* embryos. (G,H) Higher magnification of EDL in (B,E) showing a clear difference in morphology of the primary myotubes. (I) Graphical representation of the differences in primary myotube numbers in TA, EDL, P muscles and their totals in E14.5 wild-type and *β1D ki/ki* embryos. (J) Primary myotube diameter in EDL, showing a difference in size between wild-type and *β1D ki/ki*. (K-N) Transverse sections of E14.5 hindlimbs (middle region), stained for phospho-histone-H3 (K,L) and subjected to TUNEL assay (M,N) show no differences in proliferation or apoptosis in muscle regions between wild-type (K,M) and *β1D ki/ki* (L,N) embryos. (O-R) Adjacent transverse sections of E17.5 lower hindlimbs stained for slow MHC (O,P) and exposed to the TUNEL assay (Q,R) show a reduction in primary myotubes and increased apoptosis (arrows) in *β1D ki/ki* (P,R) compared with wild type (O,Q). (S-T) Graphical representation of primary, secondary and total myotube numbers in E17.5 EDL (S) and TUNEL-positive nuclei in E17.5 TA, EDL and P per section, in wild type and *β1D ki/ki* (T). For abbreviations see Fig. 4. Legend. Bars represent means ± s.d. **P*≤0.05, ***P*≤0.01. Scale bars: 200 μm (A-F,K-R), 50 μm (G,H).

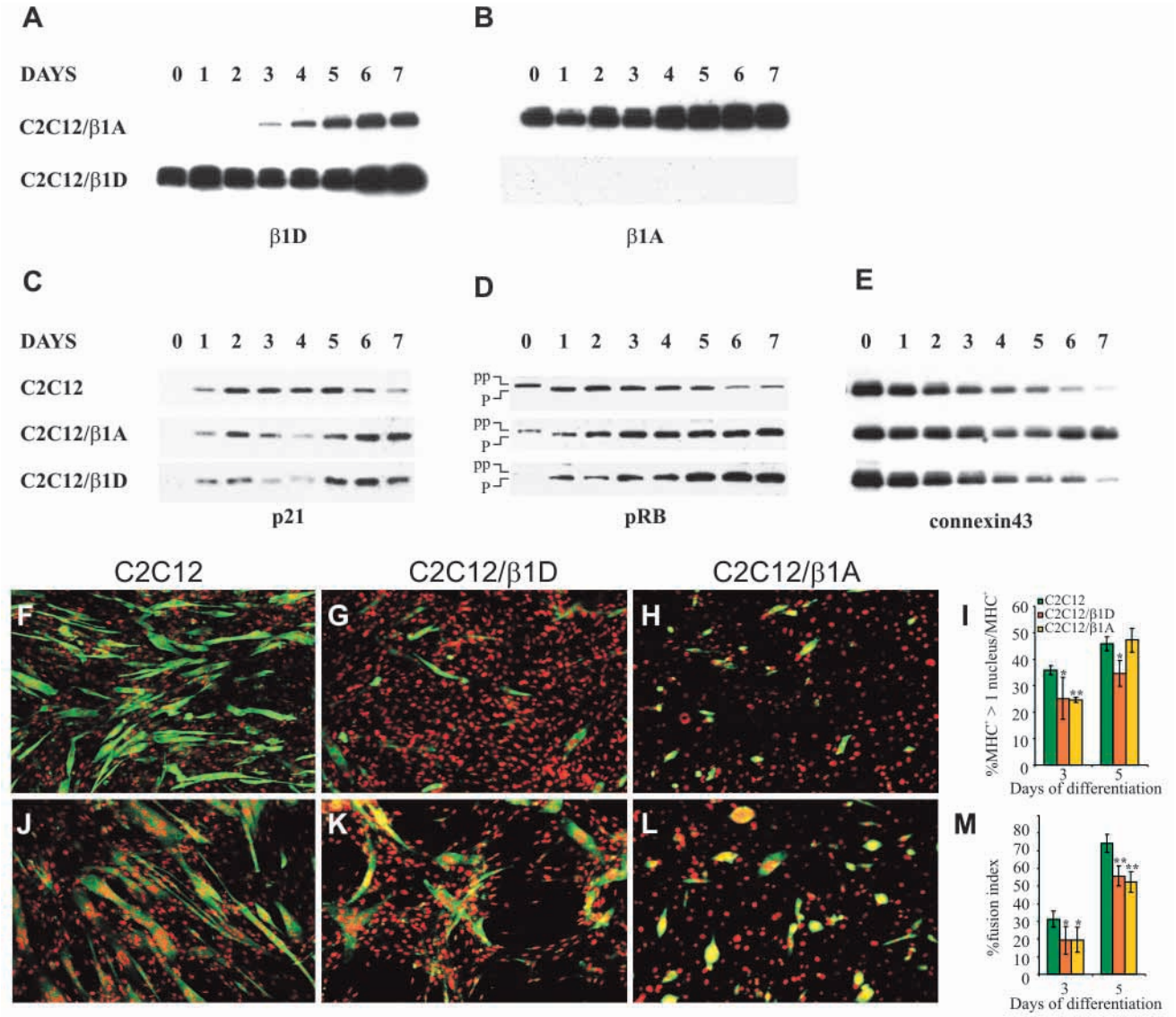


Fig. 6. Myogenic differentiation of C2C12/β1D and C2C12/β1A cells. (A,B) β1D and β1A expression during differentiation of C2C12/β1D and C2C12/β1A cells. (C-E) Expression of p21 (C), pRB (D) and connexin43 (E) analysed by western blotting. During differentiation, p21 is upregulated and pRB is dephosphorylated in all cell groups analysed. Connexin43 was downregulated in C2C12 and C2C12/β1D, but not in C2C12/β1A. (F-H,J-L) Immunostaining for MHC (green) and nuclear To-Pro3 staining (red). C2C12/β1D and C2C12/β1A had fewer MHC^+ cells than C2C12 at day 3 (F-H) and day 5 (J-L) of differentiation and C2C12/β1A myotube morphology was abnormal (H,L). (I) Percentage of MHC^+ cells with >1 nucleus. After 3 days of differentiation, the number of C2C12/β1D and C2C12/β1A myotubes was less than the C2C12 control. After 5 days, the number of C2C12/β1A myotubes is similar to the C2C12 control, in contrast to the number of C2C12/β1D myotubes, which was still lower. (M) Fusion index (percent), being the ratio of number of nuclei in myotubes (cells with >3 nuclei) to the total number of nuclei in MHC^+ cells. After 3 and 5 days of differentiation, both C2C12/β1D and C2C12/β1A myotubes have fewer nuclei than C2C12 myotubes. Bars represent means ± s.d. *$P \leq 0.05$; **$P \leq 0.01$.

Reduced primary and secondary myotube numbers in E18.5 *β1D ki/ki* embryos

The next question to be considered was whether the reduction in muscle mass resulted from selective loss of either primary or secondary myotubes, or whether these were affected equally. This is important since maternal malnutrition has been shown to affect the development of secondary, but not primary myotubes (Wilson et al., 1988). Thus, a selective reduction in secondary myotubes could indicate an indirect effect on skeletal muscle development due to placental insufficiency, rather than a direct effect of the *β1D ki/ki* genotype in skeletal muscle cells.

The number of primary and secondary myotubes were compared in three different muscle groups in E18.5 wild-type and *β1D ki/ki* embryos, namely cleidomastoideus (neck), intercostal/serratus dorsalis (trunk) and lower hindlimb muscles. A comparable reduction in the number of both primary (27%) and secondary (30%) myotubes per mm^2 in crosssections of trunk muscles of *β1D ki/ki* embryos was observed but this was not statistically significant (Fig. 4A-C).

However, there was a significant reduction in both primary (35%) and secondary (20%) myotube numbers within the red part of cleidomastoideus muscle (Fig. 4D-F). Interestingly, the ratio of secondary/primary myotubes was slightly higher in *β1D ki/ki* (5.20) than in wild-type (4.23) embryos.

Furthermore, *β1D ki/ki* lower hindlimb muscles showed a significant reduction (37%) in primary myotubes as did the individual muscles assayed (tibialis anterior, TA; extensor digitorum longus, EDL; and the peroneus group, P) (Fig. 4G-I). Secondary myotubes were counted only in EDL muscles (Fig. 4J-L); the results showed a significant decrease (44%) in *β1D ki/ki* embryos. The ratio of secondary/primary myotubes in EDL muscles was similar in wild-type (1.71) and *β1D ki/ki* (1.67) embryos at this developmental stage. These results demonstrate that the reduction in muscle mass observed in E18.5 *β1D ki/ki* embryos is due to a reduction in the number of both primary and secondary myotubes. However, since primary myotubes serve as a scaffold for the formation of secondary myotubes (Duxson et al., 1989), a reduction in primary myotubes will cause a proportional reduction in secondary myotubes, evident from E18.5 onwards (Ashby et al., 1993; Kegley et al., 2001). Together these results suggested that the reduction in muscle mass of the *β1D ki/ki* embryos was not due to placental insufficiency, but was rather a direct effect of defective primary myogenesis.

Primary myotube formation and survival are affected in *β1D ki/ki* embryos

The reduction in the number of primary myotubes in E18.5 *β1D ki/ki* embryos may result from the formation of fewer primary myoblasts, their reduced proliferation or subsequent loss by, for example, apoptosis. Primary myogenesis starts around E12.0 in the mouse, with the number of primary myotubes reaching a maximum at around E14.5 in hindlimb muscles, without secondary myotubes being present (Ashby et al., 1993; Kegley et al., 2001).

Serial sections of the lower hindlimb of E14.5 *β1D ki/ki* and wild-type embryos were stained with anti-slow MHC antibody. *β1D ki/ki* hindlimb muscles were smaller than wild type at all levels (Fig. 5A-F), and showed a significant average reduction in the number of primary myotubes (16%; Fig. 5I). Fewer serial sections of each muscle were obtained (not shown), suggesting that these muscles were also shorter. In addition, myotubes of *β1D ki/ki* embryos were smaller (Fig. 5G,H), EDL myotube diameter being 81% of those of wild type (Fig. 5J). This suggested that the exclusive presence of β1D resulted in a reduced capacity to form primary myotubes, which was reflected both in the lower number formed and their smaller size.

To determine whether this could be due to reduced proliferation of primary myoblasts, E14.5 hindlimbs sections were stained for phospho-histone H3, but no differences were observed between the number of proliferating cells per section of wild-type and *β1D ki/ki* embryos at this developmental stage (Fig. 5K,L).

At E14.5, apoptosis is a normal feature of developing muscle (Ashby et al., 1993). TUNEL assay carried out on sections of E14.5 hindlimbs revealed no differences in apoptosis between wild-type and *β1D ki/ki* embryos (Fig. 5M,N), suggesting that the presence of β1D does not lead to increased cell death in primary myoblasts or early myotubes.

It is, however, clear from the results above (see Fig. 4I and Fig. 5I) that the reduction in the number of primary myotubes in *β1D ki/ki* hindlimbs is much greater at E18.5 (37%) than at E14.5 (16%). Thus about 21% of *β1D ki/ki* primary myotubes are apparently lost during this period. To pinpoint the exact period of myotube loss, we exposed alternate cryosections of wild-type and *β1D ki/ki* E16.5 and E17.5 hindlimbs to anti-slow MHC antibody and TUNEL assay. Quantification of primary myotubes in E16.5 EDL showed a similar difference between wild type and *β1D ki/ki* as observed in E14.5 (not shown), suggesting that myotube loss had not yet started. However, at E17.5, primary myotube numbers in *β1D ki/ki* EDL (Fig. 5O,P) were reduced by 18% as compared to the wild type (Fig. 5S), an intermediate value between the reduction observed at E14.5 (11%) and E18.5 (40%) (Fig. 4I and Fig. 5I). The number of secondary myotubes was, however, identical both at E16.5 (not shown) and E17.5 (Fig. 5S) between genotypes, confirming that reduced number of secondary myotubes as a result of defective primary myogenesis becomes evident only by E18.5 (Ashby et al., 1993). The TUNEL assay revealed a twofold increase in apoptosis in *β1D ki/ki* hindlimb muscles compared with those of wild type both at E16.5 (not shown) and E17.5 (Fig. 5Q,R,T). Together these data show that there is an increase in apoptosis in *β1D ki/ki* muscles at E16.5, but myotube loss is only evident by E17.5.

Denervation has been shown to result in reduction of primary myotube numbers, although the myotube loss was observed before E15.5 (Condon et al., 1990; Ashby et al., 1993). Neurites differentiate and migrate normally on a permissive substrate in *β1D ki/ki* embryoid bodies (Gimond et al., 2000). No evidence for abnormal innervation was observed in E14.5 *β1D ki/ki* hindlimb sections stained for PGP9.5, a nerve marker (not shown). Clusters of acetylcholine receptors were also present in normal numbers in hindlimb muscles of E18.5 *β1D ki/ki* embryos (not shown), suggesting normal development of neuromuscular junctions.

Thus, these results show a significant impairment in primary myogenesis in E14.5 *β1D ki/ki* embryos, not caused by reduced myoblast proliferation, increased apoptosis or absence of nerves. In addition, our data indicate that long-term primary myotube survival is affected when β1A is replaced by β1D.

Differential effects of β1A and β1D overexpression during myogenic differentiation of C2C12 cells

To gain insight in the putative roles of β1A and β1D during myogenic differentiation, C2C12 cells were stably infected with either β1D or β1A constructs and differentiation and fusion parameters analysed. β1A is expressed by proliferating C2C12 cells and is downregulated during myogenic differentiation, while β1D becomes upregulated (Belkin et al., 1996). Infection of C2C12 cells with either splice variant caused constant and high expression of that splice variant (Fig. 6A,B) and infection with β1D totally inhibited the expression of β1A (Fig. 6B).

First, we analysed the effects of β1A and β1D on cell cycle parameters by western blotting for cyclin-dependent kinase inhibitor p21 and tumour suppressor retinoblastoma protein (pRB), markers for irreversible cell cycle arrest (Gu et al., 1993; Andrés and Walsh, 1996; Walsh and Perlman, 1997). No

differences were observed between wild-type and infected cells in the onset of p21 and dephosphorylation of pRB (Fig. 6C,D).

MHC, a marker for myoblast differentiation, was upregulated 2 days after induction of differentiation in C2C12 cells, as expected, and the levels of expression steadily increased until day 7 (Andrés and Walsh, 1996; Dedieu et al., 2002). Although both C2C12/β1A and C2C12/β1D cells upregulated MHC at a similar rate (not shown), immunostaining for MHC showed clear differences between infected and control cultures (Fig. 6F-H,J-L).

After 3 days of differentiation, both C2C12/β1A and C2C12/β1D cells showed a similar reduction in number of multinucleated MHC^+ myotubes/MHC^+ cells (30% reduction compared to C2C12 control) and in fusion index (Fig. 6L,M). After 5 days of differentiation, the number of multinucleated MHC^+ myotubes/MHC^+ cells formed by C2C12/β1A cells was similar to the C2C12 control, but the number of nuclei present per myotube was significantly lower. In contrast, both the number of multinucleated MHC^+ cells and the number of nuclei per myotube were reduced in C2C12/β1D cells (Fig. 6L,M).

During differentiation, C2C12/β1D myotubes elongated normally, but the elongation of C2C12/β1A myotubes was blocked and myotubes were rounded and had little cytoplasm (Fig. 6H,L). Finally, we analysed the expression of connexin43, a gap junction protein downregulated in C2C12 myotubes (Reinecke et al., 2000). C2C12/β1A cells did not downregulate this protein, in contrast to C2C12/β1D cells (Fig. 6E), despite a similar fusion index. Thus, we propose that the failure to downregulate connexin43 correlates with the failure in myotube elongation rather than fusion as such.

These data thus show that overexpression of β1A and β1D in C2C12 cells differentially affects myogenic differentiation.

DISCUSSION

β1D ki/ki embryos exhibit two periods of lethality on a FVB background

Phenotypes resulting from genetic modifications in mice are often highly dependent on the genetic background of the mice carrying the mutation. Here, we crossed *β1D ki/ki* mice generated on a mixed genetic background [50% 129Ola/50% FVB (Baudoin et al., 1998)], into a predominantly FVB background. We observed that the defects previously associated with the genotype became milder and less frequent and were unlikely to be responsible for the early lethality described.

We demonstrated that the great majority of *β1D ki/ki* embryos lost during midgestation, died not because of abnormal cell migration or haemorrhage as previously, but from defective placentation. Furthermore, the remaining *β1D ki/ki* embryos were lost at birth, most likely as a consequence of reduced muscle mass, which affected, among other muscles, the diaphragm and therefore their ability to breath.

By studying the phenotype of the β1D knock-in mice in a predominantly FVB background, we revealed the importance of β1 during placental labyrinth development and also demonstrated that precocious and exclusive expression of β1D in skeletal muscle leads to a reduction in muscle mass.

Replacement of β1A with β1D supported cell migration in vivo

Cell migration on a predominantly FVB background was not perturbed by the substitution of β1A by β1D in any of the migratory cells analysed in vivo (neural crest cells, limb muscle precursor cells and PGCs), meaning that their migratory behaviour was unaffected by the exclusive presence of β1D. Independent experiments have shown that myoblasts and neural crest cells derived from mES cells deficient in β1 were able to migrate normally in vivo in wild-type/β1-null chimeric embryos (Fässler and Meyer, 1995; Hirsch et al., 1998). This suggested that migration in general was not dependent on β1 integrins. An alternative explanation, however, could be the low percentage of chimerism used in those experiments. Thus β1-null cells could have adhered to wild-type cells and been passively transported to their destination. However, PGC colonisation of the gonads has been shown to be dependent on β1 integrins, by analysis of wild-type/β1-null chimeric mice (Anderson et al., 1999) and therefore β1 is necessary for normal PGC migration even in the presence of a large number of wild-type cells. Our results clearly show that replacement of β1A with β1D had no effect on either the total number of PGCs or their migration towards the gonads. This strongly suggests that β1D is able to support normal cell migration in vivo on a predominantly FVB background.

Placental labyrinth defects are responsible for early lethality in *β1D ki/ki* embryos

The placental labyrinth starts forming immediately after chorioallantoic fusion, when foetal blood vessels growing from the allantois contact the chorionic plate, the precursor of the labyrinthine trophoblast. The chorionic trophoblast cells then proliferate, differentiate and fuse into multinucleated syncytiotrophoblast, a prerequisite for labyrinthine trophoblast branching morphogenesis and subsequent embryonic vascular invasion (Anson-Cartwright et al., 2000; Rossant and Cross, 2001).

In the mouse, β1 integrin is strongly expressed in both the labyrinthine trophoblast and foetal blood vessels throughout their development (Bowen and Hunt, 1999). α4β1 in the chorionic plate plays an important role in chorioallantoic fusion by binding to VCAM1 expressed by the allantois (Yang et al., 1995; Kwee et al., 1995; Gurtner et al., 1995). This early event does not appear to be affected in *β1D ki/ki* embryos. However, labyrinthine branching defects were observed in the minority of VCAM1-null embryos that underwent chorioallantoic fusion (Gurtner et al., 1995), suggesting that VCAM1-α4β1 interactions are also important during later stages. αvβ1 and αvβ3 heterodimers are expressed in the labyrinth (Bowen and Hunt, 1999), which is poorly developed in 80% of αv-null embryos (Bader et al., 1998). Furthermore, α7β1 is strongly expressed in the chorionic plate, but is downregulated during labyrinthine branching (Klaffky et al., 2001). Although placental defects have not been described in α7-null embryos, about half of these embryos are lost at midgestation (Mayer et al., 1997), raising the question of whether α7 is important in trophoblast development as suggested by in vitro assays (Klaffky et al., 2001). Although α6β1 is present in labyrinthine blood vessels, α6-null embryos

develop to term (Georges-Labouesse et al., 1996), indicating normal placental development. Laminin receptors are likely to play a role in labyrinthine development since inactivation of the laminin α5 chain (present in laminin 10/11) causes a reduction in labyrinthine branching and leads to embryonic lethality between E13.5 and E16.5 (Miner et al., 1998).

β1A is the only β1 splice variant present in the early placenta and trophoblast cell lines (Klaffky et al., 2001). Based on the phenotype observed in two out of three *β1D ki/ki* embryos, we hypothesise that the replacement of β1A by β1D might impair chorionic trophoblast morphogenesis, leading to abnormal vascularisation of the labyrinth (Rossant and Cross, 2001). Alternatively, β1D expression on invading embryonic blood vessels might impair their morphogenesis directly.

The placenta was not examined in *β1D ki/ki* embryos on a mixed background (Baudoin et al., 1998). We cannot rule out placentation defects, at least in part, causing the observed embryonic lethality, since 5 of the 13 *β1D ki/ki* embryos analysed at E12.5 were severely anaemic. It was suggested that the anaemia was the result of the extravasation of red blood cells through weak-walled vessels throughout the bodies of *β1D ki/ki* embryos. However, in the predominantly FVB background described here, extravascular blood cells were not observed and we believe that the anaemia resulted largely or entirely from abnormal placentation.

Precocious expression of β1D inhibits primary myogenesis

From its expression pattern it is clear that β1D is not involved in primary myogenesis. β1D is detected on the surface of myotubes from E17.5 and then becomes enriched in myotendinous junctions and at costameres, where it might be important for the formation of strong adhesion sites (van der Flier et al., 1997).

We show here that precocious and exclusive expression of β1D in skeletal muscle affects primary myogenesis. Not only were there fewer primary myotubes at E14.5, but they were also smaller in diameter and shorter.

Fewer primary myotubes could be the result of: (1) a reduction in the number of primary myoblast precursors, (2) a reduction in the proliferation rate of primary myoblasts, (3) their increased apoptosis and (4) an inhibition in myoblast differentiation and subsequent fusion.

Our results suggest that myogenic precursor cells delaminate and migrate normally towards the limb buds in *β1D ki/ki* embryos and that proliferation and apoptosis is not altered at E14.5. However, we cannot exclude an earlier reduction in the numbers of myoblast precursors. The fact that primary myotubes are smaller in *β1D ki/ki* embryos does, however, strongly suggest a defect in myoblast differentiation and/or fusion. A significant reduction in primary myotubes was also found in embryos lacking the transcription factor NFATC3 (Kegley et al., 2001). However, there, the E15.0 EDL primary myotubes were normal in size and morphology, leading the authors to exclude a defect in differentiation and/or fusion and favour a reduction in the pool of primary myoblasts.

Several studies have suggested a role for β1 integrins in the differentiation and fusion of myoblasts in vitro (Sastry et al., 1996; Gullberg et al., 1998), but because β1-null cells participate in the formation of skeletal muscle in β1-null/wild type chimeras (Fässler and Meyer, 1995) the role of β1 in this process in vivo has been questioned. However, Hirsch et al. (Hirsch et al., 1998) have shown that while β1-null myoblasts isolated from E16.0 embryos (i.e. secondary myoblasts) fuse and form normal myotubes, myoblasts derived from β1-null mES cells (i.e. predominantly primary myoblasts) show a significant inhibition in myotube formation. This study together with our data strongly suggest that β1A plays an important role in the formation of primary myotubes and that substitution of β1A by β1D significantly impedes this process.

Long-term myotube survival is clearly affected in *β1D ki/ki* embryos. This could be due to inhibition of myotube growth caused by a progressive impairment in the addition of myoblast nuclei to the early myotubes (Zhang and McLennan, 1995). Alternatively, the precocious presence of β1D might interfere with the establishment of myotube connections to the surrounding basement membrane (Vachon et al., 1996) or the myotendinous junction (Miosge et al., 1999), compromising myotube survival by forming strong adhesion sites too early.

Overexpression of β1D and β1A produces different effects on C2C12 differentiation

C2C12 cells overexpressing β1A or β1D exhibit inhibition of myotube formation, suggesting that excessive amounts of either β1 splice variant interferes with this process. However, the more long-term effect of β1D overexpression shows that this splice variant causes a stronger inhibition. Strikingly, myotube morphology was severely affected in C2C12/β1A cells and downregulation of connexin43 did not occur, suggesting that β1A inhibits myotube maturation. This is probably due to overexpression since inhibition in myotube maturation is not observed in β1D knock-out mice, where β1A expression persists in mature muscle fibres (Baudoin et al., 1998). Overall, these results suggest that C2C12 myotube formation and maturation is dependent on a quantitative balance of different integrins, an idea supported by studies on the effect of integrin α-subunit ratios on β1A signalling during myoblast differentiation (Sastry et al., 1996; Sastry et al., 1999).

Secondary myogenesis is unaffected in *β1D ki/ki* embryos, suggesting different roles for β1 integrins in primary versus secondary myogenesis

At E16.5 and E17.5, the number of secondary myotubes was similar between *β1D ki/ki* and wild-type embryos, while at E18.5, it was significantly lower in *β1D ki/ki* embryos. The ratio of secondary to primary myotubes, however, was similar in the muscles studied at E18.5. Thus the number of secondary myotubes is only reduced when the reduced number of primary myotubes in *β1D ki/ki* embryos becomes a limiting factor for the formation of more secondary myotubes (Ashby et al., 1993). These results suggest that secondary myogenesis is unaffected in *β1D ki/ki* embryos. It is becoming evident that different regulatory pathways control these two waves of myogenesis in vivo. For example, in myogenin null embryos secondary myogenesis is much more affected than primary myogenesis (Venuti et al., 1995), while inactivation of NFATC3 causes a selective reduction in primary myotubes (Kegley et al., 2001). Our data show that primary myogenesis is more sensitive to the precocious presence of β1D than secondary myogenesis. This not only demonstrates different roles for β1A and β1D, but also strongly suggests that β1

integrins play distinct roles in primary versus secondary myogenesis in vivo.

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