Intermingling Versus Clonal Coherence During Skeletal Muscle Development: Mosaicism in eGFP/nLacZ-Labeled Mouse Chimeras

Daniel Eberhard and Harald Jockusch

To study the relative contributions of clonal coherence vs. myoblast intermingling to the formation of mammalian skeletal muscles, enhanced green fluorescent protein (eGFP) and nLacZ labels were used to analyze neonatal and adult mouse embryo aggregation chimeras. The eGFP marker allowed us to estimate absolute levels of the eGFP transgenic parental contributions as well as variances of levels within a chimeric individual; nLacZ served as a counter-label in neonatal chimeras. In mature chimeric muscle, free intracellular diffusion of eGFP led to an averaging of the eGFP level in individual myofibers. Chimerism, i.e., differing eGFP levels, was evident between corresponding right and left muscles, between adjacent back, body wall, limb (shank, foot), and extraocular muscles and among myofibers within these muscles. Inhomogeneities in muscles far from their somitic origin indicated unbalanced sampling from small founder pools. The results obtained with adult eGFP→0 chimeras were corroborated by neonatal chimeras with complementary eGFP→nLacZ labels. Developmental Dynamics 230:69–78, 2004.

© 2004 Wiley-Liss, Inc.

Key words: cell marking; eGFP; limb muscles; nLacZ; muscle development; mouse chimera; myoblast fusion; oligoclonal patches; toe muscles

Received 15 August 2003; Revised 11 December 2003; Accepted 11 December 2003

INTRODUCTION

In vertebrates, the bulk of skeletal muscles derives from precursor cells located in the dermomyotome of somites giving rise to longitudinally oriented muscles along the spine (epaxial muscles), and to thoracoabdominal and limb muscles (hypaxial muscles); muscles of the head derive from prechordal somitomeres and occipital somites (Tam, 1986; Christ and Ordahl, 1995; Dietrich, 1999). Precursors of epaxial muscles are nonmigratory, whereas myogenic hypaxial precursors invade their target sites either as an expanding epithelial sheet or by migration as individual mesenchymal cells as is the case of occipital, cervical, and limb muscles (Dietrich, 1999; Christ and Brand-Saberi, 2002). Limb muscles originate from the lateral dermomyotome, from where myogenic precursor cells migrate into the somatopleural mesoderm of the limb rudiments, proliferate, and condense into premuscular masses that give rise to individual muscles (Christ and Brand-Saberi, 2002).

The myofiber orientation in muscles is established by a scaffold of primary fibers with subsequent accretion of secondary fibers and hypertrophic growth (Duxson and Sheard, 1995; Patel et al., 2002). Except during regeneration by activation of satellite cells, postnatal growth of muscle is mainly due to hypertrophy (Grounds et al., 2002).

Although there is increasing knowledge on molecular processes governing the commitment and differentiation of myogenic cells in mammals (Buckingham et al., 2003), information regarding pattern formation processes during myogen-
esis is mostly based on descriptive studies (Kardon, 1998), the analysis of chick/quail chimeras (Le Douarin and McLaren, 1984; Christ and Or Dahl, 1995), or retroviral labeling in the chick (Rees et al., 2003). In the mouse, the anterior-posterior and mediolateral myotome formation has been analyzed by LacZ/LacZ mosaics (Eloy-Trinquet et al., 2000; Eloy-Trinquet and Nicolas, 2002), a technique based on the detection of β-galactosidase in clones that have undergone a rare somatic recombination.

Hereditary cell autonomous markers have been used in mouse aggregation chimeras to obtain information on the clonal origins of tissues. In favorable cases, one usually observes patches or cells with like phenotype, the spatial distribution and sizes of which can provide information on underlying developmental processes (Jannaccone, 1987; West, 1999). However, in mature chimeric skeletal muscle, retrospective analysis of clonal distribution is complicated due to intermingling of myogenic precursors and subsequent myoblast fusion (Mintz, 1970), processes that tend to abolish clonal borders. Yet, on the other hand, information on just these processes may be obtained by an appropriate marker. Allelic variants of glucose phosphate-isomerase (GPI) were used to prove the polyclonal character of muscle fibers; the low eGFP signal was verified by staining for eGFP with an eGFP antibody. In epaxial muscles, labeled by staining for eGFP with an eGFP antibody. In epaxial muscles, left/right differences regarding the parental proportions of nLacZ and eGFP were not observable. Nevertheless, chimerism was detected in hypaxial-derived psos muscle in one specimen, where one side was eGFP negative. In thin body wall muscles of the neonatal chimeras, chimerism was detected, e.g., between adjacent abdominal muscles (Fig. 1C-E), where eGFP levels differed and the proportion of nLacZ-positive nuclei was lower in the high eGFP muscle.

**RESULTS**

**Validation of the eGFP/nLacZ-Labeling Method for Skeletal Muscle**

In the eGFP transgenic mouse strain C57BL/6-eGFP, eGFP is highly expressed in mature skeletal muscle (Okabe et al., 1997; Jockusch and Voigt, 2003). In muscles with mixed fiber type composition (e.g., tibialis anterior), the oxidative, small diameter fibers contained slightly higher eGFP levels than large diameter, glycolytic fibers (Jockusch et al., 2003). There were no indications for cytotoxic effects of eGFP; transgenic precursors and subsequent cell marker. By this method, we corroborate some previous notions of the origin of different muscle groups but also find unexpected heterogeneities in parental contributions. It is important to note that the maximal information that can be extracted from chimeric experiments is based, not on the average values, but on maximal differences within individual chimeras.

**Mosaicism in Skeletal Muscle of Neonatal and Adult Chimeras**

Based on their external appearance (McLaren, 1976), i.e., coat in adult and skin in neonatal mice, chimeras were classified as balanced (eGFP parental contribution \( P = 0.4-0.6 \)) or unbalanced (\( P < 0.4 \)). Two unbalanced neonatal C57BL/6-eGFP→129-DES-KO/nLacZ chimeras (\( P \approx 0.1 \)) and one unbalanced chimera with a low (\( P \approx 0.1 \)) eGFP contribution (aged 8 weeks) were analyzed in detail. Depending on the parental contribution to a chimera, the developmental origin (epaxial, hypaxial myotome, prechordal mesoderm), location (trunk, extremities, head muscles) and the sizes of muscles, eGFP levels and their variances differed widely. In the following, chimeric patterns in trunk, limb, and head muscles are evaluated.

**Back and Body Wall Muscles**

The backs of two neonatal C57BL/6-eGFP→129-DES-KO/nLacZ chimeras showed green fluorescent stripes due to skin chimerism (cf. McLaren, 1976). From their external appearance, there was no distinct chimerism in dorsal and body wall muscles. In sections, epaxial dorsal muscles contained eGFP- and nLacZ-positive fibers; the low eGFP signal was verified by staining for eGFP with an eGFP antibody. In epaxial muscles, left/right differences regarding the parental proportions of nLacZ and eGFP were not observable. Nevertheless, chimerism was detected in hypaxially derived psos muscle in one specimen, where one side was eGFP negative. In thin body wall muscles of the neonatal chimeras, chimerism was detected, e.g., between adjacent abdominal muscles (Fig. 1C-E), where eGFP levels differed and the proportion of nLacZ-positive nuclei was lower in the high eGFP muscle.

The external surfaces of dorsal muscles of adult balanced eGFP→0 chimeras appeared symmetrically and homogeneously fluorescent (Fig. 2A). In the unbalanced chimera (Fig. 2B) an asymmetric distribution of eGFP was evident and this asymmetry could be verified in tissue sections of the spine erector muscles (Fig. 2C-E).
Fig. 1. Validation of enhanced green fluorescent protein (eGFP) and nLacZ labeling in chimeras. A,B: eGFP is homogeneously distributed along muscle fibers of a low adult eGFP chimera (A) and in a neonatal chimera (B). A: eGFP fluorescence combined with Hoechst stain to visualize nuclei: l (low) h (high) eGFP level. B: Overlay of eGFP fluorescence, eGFP antibody (Cy3, red), and Hoechst stain. eGFP and Cy3 colocalize resulting in orange; g, gap between muscle fibers. C,D,E,E′: Chimerism in abdominal muscles (oea, obliquus externus abdominis; oia, obliquus internus abdominis; ta, transversus abdominis) in a low eGFP neonatal chimera. C: Skeletal β-actin staining (Cy3, red). D: Section adjacent to C, stained for β-galactosidase (nLacZ) combined with eosin. E: eGFP fluorescence. E′: Immunostaining for eGFP with Cy3 antibody. High eGFP levels correlate with low nLacZ and vice versa. Scale bars = 50 µm in A, 10 µm in B, 100 µm in C (applies to C–E′).

Fig. 2. Chimerism in trunk muscles. A,B: External view of dorsal muscles under ultraviolet light of a balanced (A) and an unbalanced, low enhanced green fluorescent protein (eGFP) chimera (B, montage of two frames). Note asymmetric distribution of eGFP (arrows) in B as evidence for chimerism. C: Spine erector muscles from the unbalanced chimera, at the level indicated by red line in B, showing a drastic left/right difference of eGFP levels. D,D′: Transverse sections of dorsal muscles of the unbalanced chimera seen in B with a sharp border between muscle segments with high and low (but not zero) eGFP levels (D′, enlargement of framed area in D). E: Corresponding section from balanced chimera with homogenously distributed eGFP levels. F–H: Chimeric intercostalis muscles in the unbalanced (F,G) and balanced (H) chimeras. D: eGFP fluorescence combined with Hoechst stain in C, D–H to visualize nuclei. r, rib. Fluorescence intensity plots in gray insets were taken along red dashed lines. Ordinates (short axes) are fluorescence intensities in relative units; abscissas (long axes) are distances at the same scale as the corresponding micrograph. Yellow diamonds and yellow bar indicate gaps in section or nonmuscle tissue that does not express eGFP. Scale bars = 500 µm in C,H, 200 µm in D,F, 100 µm in D′ (applies to D′–E).
2C). More caudally, heterogeneity was observable in the epaxially derived back muscles of the unbalanced chimera (Fig. 2D–D’), where separate asymmetric areas with high and very low (but not zero) eGFP fluorescence were present. In balanced chimeras, eGFP was homogeneously distributed along all levels of the epaxial back muscles observed (Fig. 2E).

In intercostal muscles, chimerism was observed in the adult unbalanced chimera, where eGFP levels differed between adjacent muscles, indicating clonal borders (Fig. 2F) and between individual myofibers appearing in fascial clusters of eGFP-positive myofibers (Fig. 2G). In contrast, eGFP was homogeneously distributed in all analyzed thoracic muscles of a balanced chimera (Fig. 2H), i.e., there were no borders separating areas with significantly differing eGFP levels.

**Limb Muscles**

All analyzed limb muscles of neonatal C57BL/6-eGFP→129-DES-KO/nLacZ contained β-galactosidase-positive nuclei and in most cases showed at least a low level of eGFP, as verified by an eGFP antibody (Fig. 3A). Distinct chimerism was apparent in hindlimb muscles of one specimen, where the eGFP levels differed significantly between muscles (Fig. 3A). In some muscles, fibers with differing eGFP levels were present (Fig. 3A’). Unexpectedly, the proportion of nLacZ nuclei was not drastically lower in muscles with a relatively high eGFP concentration (Fig. 3B), indicating that the two signals, eGFP fluorescence and fraction of blue stained nuclei, may not be proportional to the respective fraction of the genotypes (see Discussion section).

In hindlimb muscles of adult eGFP→0 mouse chimeras, distinct chimerism was observed in one balanced (Fig. 3C) and in the unbalanced chimera (Fig. 3D, D’), whereas all analyzed muscles of the other balanced chimeras were relatively homogeneous. In the hindlimb of the balanced chimera (Fig. 3C), the eGFP levels of the anterior (tibialis anterior complex) were drastically lower than the eGFP levels of the posterior compartment (gastrocnemius, soleus complex). In the tibialis anterior muscle (Fig. 3C; arrows), clusters of eGFP-positive myofibers were apparent at tendons, whereas muscles of the posterior compartment of the left leg were relatively homogeneous and contained only a few regions with lower eGFP levels. Distinct chimerism was apparent in all analyzed hindlimb muscles of the adult unbalanced chimera as indicated by varying eGFP levels between adjacent muscles, e.g., as observed between the soleus and lateral gastrocnemius in the left hindlimb (Fig. 3D); the latter muscle showed low (but not zero) eGFP levels. The posterior muscles gastrocnemius, plantaris, and soleus typically contained fascial clusters with 5 to 15 myofibers with similar eGFP levels (Fig. 3D’).

In neonatal chimeras, small foot muscles were all nLacZ-positive with very low or zero eGFP levels; only in one specimen, chimerism, i.e., differing eGFP levels between adjacent muscles, was clearly observable (not shown). In both hindlimbs of an adult, balanced chimera eGFP levels differed considerably between adjacent small foot muscles (interossei, as shown by overlays of eGFP fluorescence with skeletal muscle α-actin staining (Fig. 4). Thus, despite mingling of migratory myogenic precursors, the most distal limb muscles showed chimeric patterns of the parental label.

**Head Muscles**

Distinct chimerism was apparent in extraocular muscles (EOM) of neonatal chimeras where eGFP levels of muscles differed remarkably, e.g., the eGFP level in rectus superior was approximately twofold higher than in rectus lateralis (Fig. 5A, A’ and enlargements). Unexpectedly, the proportion of nLacZ nuclei was not drastically lower in EOM with a relatively high eGFP concentration (Fig. 5B and enlargement). Nevertheless, all EOM contained nLacZ-labeled myofibers (Fig. 5B and enlargement).

Apart from a few exceptions (e.g., rectus lateralis in one chimera, Fig. 5C, C’), the extraocular muscles of the adult balanced chimeras appeared homogeneously eGFP-positive, whereas eGFP mosa...
icism was detectable in the eye muscles of the unbalanced chimera, e.g., in the retractor bulbi (Fig. 5D). However, there was no fascicle in which all fibers were eGFP negative as in toe muscles.

In tongue muscles (oral part) of all three adult chimeras analyzed (not shown), eGFP levels were relatively homogeneous. There were no eGFP-negative myofibers detectable. The eGFP level in the tongue muscle of the unbalanced chimera was much lower (Fig. 6), but again no eGFP-negative fibers were observed.

Quantitative Comparison of eGFP Levels in Different Chimeric Muscles

Chimeric myofibers contain different eGFP levels reflecting the variable proportions of contributing eGFP transgenic myoblasts. Therefore, absolute eGFP levels in limb, head, and trunk muscles of one adult balanced chimera and the unbalanced were estimated by densitometry and compared with eGFP levels in muscles of nonchimeric transgenic controls (Fig. 6).

In transgenic adult controls, we have never observed a difference
that would distinguish homozygous transgenic from heterozygous transgenic specimens. Furthermore, the values of balanced chimeras are very close to those of the transgenic control (100% by definition). This finding indicates a signal saturation effect in approximately half of the nuclei carrying the eGFP transgene. On the other hand, values in a low eGFP chimera were clearly lower (67.2% ± 9; mean, ± SD; but 53.8 ± 11.6 when high eGFP epaxial muscles were excluded) than those in a balanced chimera, indicating that we are in the sensitive range of eGFP fluorescence that allows semiquantitative comparisons. The absolute variability in the unbalanced chimera was more than tenfold higher than in the balanced specimen. Significant left/right differences were not apparent in the balanced chimeras with exception of the tibialis anterior. In the unbalanced chimera, left/right eGFP levels were different in most muscle groups. eGFP levels also differed remarkably between adjacent muscles in the unbalanced chimera, e.g., between medial and lateral gastrocnemius muscle.

DISCUSSION

Technical Aspects of the eGFP and nLacZ Labels

Due to its high diffusibility (Arri-Dupont et al., 2000), eGFP enables to semiquantitatively evaluate the contribution of one aggregation partner to myofibers and is not restricted to the analysis of all-or-none signals. Prerequisite for this analysis is a homogeneous distribution of eGFP along single fibers, which was observed in transgenic controls, as well as in neonatal and adult chimeras. Similarly, in transplantation chimeras, cofusion of eGFP-positive with eGFP-negative myogenic cells led to a homogeneous distribution of eGFP along myofibers (Jockusch and Voigt, 2003).

Because eGFP levels in immature skeletal muscles are low (Jockusch and Voigt, 2003) and eGFP is further diluted in the chimeric fibers, neonatal chimeras with low eGFP contributions were more difficult to analyze than adult specimens but contributed valuable information in conjunction with the nLacZ counter-label. Low eGFP signals could be enhanced and discriminated from autofluorescence by immunostaining.

As eGFP diffuses along the whole muscle fiber, we cannot exclude that, even in the nonchimeric transgenic mouse, a few nuclei fail to express the eGFP gene. This kind of variegation or pseudochimerism, as has been described for the expression of other transgenes in mice (Dobie et al., 1997), would remain cryptic as long as all muscles are affected to the same degree. However, inhomogeneity between whole muscles has never been observed in the transgenic mouse, and a low level pseudochimerism within fibers would not affect the observation of authentic chimerism. In the tissue most closely related to skeletal muscle, myocardia, in which there is no cell fusion, we found absolutely no evidence for variegation in the transgenic mouse with respect to eGFP expression (Eberhard and Jockusch, manuscript in preparation).

Cytotoxic effects have been reported in the myocardia of eGFP transgenic mice (Huang et al., 2000), but in our stock, there were no indications for eGFP toxicity in either cardiac (Eberhard and Jockusch, manuscript in preparation) or skeletal muscle. In addition, pre- and perinatal eGFP expression is low and, thus, probably irrelevant for myoblast expansion and differentiation. For these reasons, it is unlikely that the distribution of eGFP transgenic myogenic cells is biased by the presence of this label. Presumably, background strain differences are of much greater influence on intra-individual competition between myoblasts of different parental origin than the eGFP transgenic label. Thus, the faster growth of the 129 strain-derived contribution (Petersen, 1979) has led to a preponderance of nLacZ-positive cells in muscles of neonatal C57BL/6-eGFP→129-nLacZ chimeras.

Dependence of eGFP Mosaicism on Parental Contributions

In adult eGFP→0 chimeras, there was a rough correlation between the parental contributions as judged by coat color chimerism (McLaren, 1976) and the overall levels of GFP: many muscles of a chimera with a low coat color contribution from the eGFP transgenic strain had a significant...
cantly lower eGFP content than a balanced chimera analyzed for comparison. On the other hand, the eGFP fluorescence level of the non-chimeric transgenic mouse was only slightly higher than that of a balanced chimera. Thus, the eGFP label behaves roughly proportional to the gene dosage (averaged over a given muscle fiber and a whole muscle) with low contributions, but approaches a saturation level at approximately 50% contribution. Conversely, in high-level eGFP muscles of neonatal chimeras, the proportion of β-galactosidase–positive nuclei was never as low as it should have been. Presumably, this excess of β-galactosidase–positive nuclei results from the diffusion of β-galactosidase mRNA and protein from the transgenic nucleus to neighboring nuclei in the muscle fiber.

**Patterns of Mosaicism in Skeletal Muscle**

Apart from the eGFP level differences due to different parental contributions, overall left/right asymmetry was particularly evident in the back muscles of the unbalanced chimera. It is generally accepted that the two halves of the vertebrate body develop largely independently and that cell migration from the midline, with the exception of tongue muscles (Huang et al., 1999), is confined to the ipsilateral side.

Clusters of eGFP-positive myofibers ("myofiber patches") were seen in some muscles, especially in the adult unbalanced chimera, but are rarely observable in neonates presumably due to a very low eGFP level. Such clusters of eGFP-positive myofibers may arise during establishment of myofiber orientation. During this process, secondary fetal fiber precursors, which are likely to be clonally related, accumulate on a scaffold of primary embryonic fibers (Duxson and Sheard, 1995; Patel et al., 2002; Buckingham et al., 2003). Similar patterns, i.e., clusters of labeled primary and secondary myotubes, were observed in the chick hindlimb after retroviral labeling of somitic cells (Rees et al., 2003).

Muscles derived from migratory myogenic precursor cells, e.g., limb and head muscles (but not tongue), were surprisingly inhomogeneous. Apparently, the influence of intermingling of myogenic precursors and subsequent myoblast fusion is counteracted by clonal isolation in the periphery resulting from unequal sampling of eGFP-labeled and unlabeled myoblasts from small pools (see below; Fig. 7).

The results obtained with adult eGFP→0 chimeras were corroborated by neonatal chimeras with complementary eGFP→nLacZ labels, indicating that there is no extensive myoblast migration or stem cell immigration after birth that would further level out regional differences. Wherever migration of potentially myogenic cells has been observed in experiments on adult mice, either cultured cells or traumatic conditions in the respective muscles were involved (Hughes and Blau, 1990; Ferrari et al., 1998; Jockusch and Voigt, 2003).

**Mechanisms Influencing Clonal Distribution During Muscle Development**

The initial distribution and sizes of patches in the premuscular masses depend on previous coherent vs. dispersed clonal growth (Fig. 7). Thus, premuscular masses may consist of a large number of many small patches ("salt and pepper" pattern, Fig. 7A) or, in case of previous coherent growth, contain few large patches ("coherence," Fig. 7B). In the chick, retroviral labeling of somitic cells showed that the labeled myogenic precursors can contribute to many muscles, i.e., they may intermingle with cells of adjacent somites before segmentation of the premuscular masses (Rees et al., 2003). Independently of the migratory history of a premuscu-
lar mass, an imbalance of parental proportions in individual muscles may arise, if the number of cells sampled from the premuscular masses is either low (and secondarily expands by proliferation; “founder effect,” Fig. 7Aa/Ba) or if the sample originates from a region with large coherent clones (Fig. 7B). In case of a fine grained “salt and pepper”-like founder pool and not too small sample size the initial parental proportion is approximately retained (Fig. 7Ab).

Thus, due to unequal sampling of myogenic cells, mosaicism should be more evident in low eGFP chimeras than in balanced chimeras, which was indeed observed. In balanced chimeras, such differences between clonal contributions tend to be abolished by random coalescence of patches of like parental type (West, 1975) and additional “infectious expansion” of eGFP-positive domains by myoblast cofusion at the borders (Jockusch and Voigt, 2003).

The described sampling and founder effects suggest that adjacent muscles that derive from different premuscular masses may contain different parental proportions, as evidenced by eGFP levels. This finding was indeed observed, e.g., in one adult balanced chimera where the gastrocnemius/soleus complex, which originates from the ventral premuscular mass (in chick; cf. Kardon, 1998), showed higher eGFP levels than the neighboring tibialis anterior complex deriving from the dorsal premuscular mass. Conversely, in retroviral-labeling experiments in chick, hindlimb muscles originating from the same premuscular masses were conjointly marked (Rees et al., 2003). Because abdominal wall muscles are reported to arise from a single premuscular mass at a given segment (Christ et al., 1983), the eGFP differences between layers as seen in unbalanced chimeras may be the result of unequal sampling or a founder effect; the same explanation may hold for the mosaicism observed in intercostal muscles.

Mosaicism in balanced chimeras suggests an oligoclonal origin, even of larger muscles and a relative coherent clonal expansion during establishment of the premuscular masses. On the other hand, in no case, not even in extraocular muscles, do our results support a strictly monoclonal origin of any muscle: both labels, eGFP and nLacZ, were found in almost every muscle investigated. Preliminary observations on LaacZ/LacZ mosaics have led to a similar conclusion (Buckingham et al., 2003).

The myocardium, a cross-striped muscle, in which there is no complication by cofusion, may serve as a negative control for migration and cofusion effects observed in skeletal muscle. It turns out that in the myocardial histogenesis clonal coherence is of much higher, and intermingling of much lower, importance than in skeletal muscle (Eberhard and Jockusch, manuscript in preparation).

As to biomedical applications of these findings, one has to take into account the several thousand-fold higher mass of human muscles that is achieved by additional rounds of myoblast proliferation with the possibilities of both, further intermingling and expansion of local clones. The reported patterns in eGFP-/-0 chimeric muscles might be relevant for the analysis of muscle diseases such as Duchenne muscular dystrophy (DMD; Pearson et al., 1963). In muscles of oligoclonal origin, X-inactivation of the only functional allele should lead to nearly dystrophin-free muscles and dysfunction. This was reported for extraocular muscles in human females hemizygous for the DMD mutation (Gearhart and Mintz, 1972). According to our results, regions of dystrophin deficiency due to X-inactivation might be even more conspicuous in toe muscles of carrier females.

In summary, we have shown that mosaicism can be observed in both neonatal and adult skeletal muscles of chimeric mice, despite the blurring effects of migration, intermingling and cofusion. The sensitivity of the eGFP marker allowed us to refute the existence of monoclonally derived muscles. The similarity between neonatal and adult mosaicism argues against any significant dispersal and integration into preexisting muscle fibers of myogenic cells, except during traumatic and regenerative processes.

**EXPERIMENTAL PROCEDURES**

**Transgenic Mice**

A breeding stock of C57BL/6 eGFP mice transgenic for the gene for enhanced green fluorescent protein (“green mice,” C57BL/6 Cr Slc TgN-(act-EGFP)OsbC15-001-FJ001), (Okabe et al., 1997) was obtained from Dr. Masaru Okabe through Dr. Melitta Schachner, ZMNH, Hamburg, 129-DES-KO/nLacZ mice (Li et al., 1996) were from Dr. Denise Paulin (Université Paris 7). The eGFP transgene, controlled by a chicken β-actin promoter and a CMV enhancer (Okabe et al., 1997), is expressed in several cell types, including smooth, skeletal, and cardiac muscle (Jockusch et al., 2003); nLacZ, driven by the endogenous desmin promoter, is exclusively expressed in the three types of muscle (Li et al., 1996). Due to random aggregation of Des+/-, Des+/+, Des+/+ embryos, one third of the β-galactosidase-positive neonatal chimeras contained desmin-deficient (Des−/−) cells; but this finding should not have any influence on the results of this study, because no abnormalities have been reported for neonatal homozygous desmin-deficient mice (Li et al., 1996, 1997).

**Production of Aggregation Chimeras**

Eight-cell morulae were collected from the oviduct-uterine junction from 2.5 days post coitum (dpc) superovulated C57BL/6 eGFP, and 129-DES-KO/nLacZ or CD-1 females by flushing with M2 medium (Sigma, M-7167). The zona pellucida was lysed with pronase (Sigma, P-5147); for aggregation, pairs of embryos were incubated overnight in medium M16 (Sigma, M-7292) at 37°C, 5% CO₂. On the following day, chimeric morulae or early blastocysts were transplanted into one uterine horn of a 2.5 dpc pseudopregnant CD1 female recipient mice (Hogan...
Preparation of Tissues and Immunohistochemistry

Organs of adult chimeras (killed by cervical dislocation at age 3–8 weeks) and decapitated neonatal chimeric animals were injected with 4% formaldehyde (FA, freshly prepared from paraformaldehyde) in calcium- and magnesium-free phosphate buffered saline (CMF-PBS) and post-fixed at 4°C for 24 hr in FA CMF-PBS (eGFP–0 chimeras in 4% FA; eGFP–LacZ chimeras in 2% FA), followed by 24 hr in CMF-PBS. They were subsequently shock-frozen in liquefied propane (−190°C) followed by liquid nitrogen.

Frozen sections (8–10 μm) were stained with Hoechst 33258 (Sigma B 2261) to visualize nuclei and embedded in Elvanol (10g Mowiol 4-88 + 40 ml PBS + 20 ml Glycerol). Immunostaining of skeletal muscle was performed with a polyclonal anti-skeletal muscle α-actin antibody (Clement et al., 1999) and an anti-rabbit Cy3-conjugated secondary antibody (Dianova, 211-160-003). eGFP was stained by using a polyclonal anti-GFP antibody (MBL, 598). Staining of sections for β-galactosidase was performed overnight in a solution of 4% (v/v) 5-bromo-chloro-indolyl–β-D-galactopyranoside (X-Gal, B 4252 Sigma), 1 mM MgCl₂, 150 mM sodium chloride; 3.3 mM potassium ferrocyanide (K₃Fe(CN)₆) 3H₂O, 3.3 mM potassium ferricyanide (K₃Fe(CN)₆) in 10 mM phosphate buffer (pH 7.0) at 37°C. Subsequently, sections were washed in 50 mM phosphate buffer (pH 7.0) and embedded in Elvanol (10 g Mowiol 4-88 + 40 ml PBS + 20 ml glycerol).

Microscopy, Documentation, Image Processing, and Densitometry

Sections were documented using a Nikon Coolpix 990 camera mounted on a Zeiss Axiosophot microscope. Images were assembled and processed with Photoshop 5.5 (Adobe Systems Incorporated). For figures, it was necessary to individually adapt exposure times when photographing muscles with low eGFP levels. Thus, although eGFP intensities may appear similar in two photomicrographs, the eGFP levels in the specimens may differ.

ImageJ (version 1.26; http://rsb.info.nih.gov/ij/) was used for densitometric evaluation. For this purpose, transverse muscle sections were photographed under equal illumination conditions but with different exposure times to produce an exposure series. The primary 16-bit color images (RGB format, red green blue) were converted to 8-bit grayscale images, in which eGFP intensity correspond to green fluorescence in the samples and are coded by a value between 0 and 255 (0 = black, no fluorescence; 255 = white, maximum fluorescence). Of each image, the mean gray value and standard deviation was determined. The gray values were plotted against the exposure time to identify the log phase and a line was fitted through the log phase using Sigma Plot 8.0 (Jandel Scientific). A base line was defined, and the intersections of base line and regression lines were calculated. All values were normalized to nonchimeric transgenic control tissue (gastrocnemius) and plotted. Densitometric profile plots were generated by specifying a region of interest (ROI) in the image and by using ImageJ’s plot profile feature, which calculates a two-dimensional graph of the intensities of each pixel along a specified line.

ACKNOWLEDGMENTS

We thank Professor Masaru Okabe (Osaka University) for providing the eGFP transgenic mouse strain, Dr. Volker Schmidt (now Physiological Institute, Medical Faculty, Würzburg University) and Sandra Heising for help with embryological techniques, Dr. Peter Heimann for critically reading the manuscript, and Dr. Sophie Clément and Professor Giulio Gabbiani (University of Geneva) for providing us with anti-actin antibodies.

REFERENCES


