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Targeted inactivation of myogenic factor genes reveals their role during mouse myogenesis: a review[1]

HANS-HENNING ARNOLD* and THOMAS BRAUN

Department of Cell and Molecular Biology, Technical University of Braunschweig, Braunschweig, Germany

ABSTRACT The role of the four myogenic regulating genes *Myf-5, myogenin, MyoD,* and *MRF4 (herculin, Myf-6)* during mouse embryogenesis has been investigated by targeted gene inactivation. Null mutations for the MyoD gene generate no skeletal muscle phenotype due to a compensatory activation of the *Myf-5* gene. Mice carrying a homozygous Myf-5 mutation exert considerably delayed myotome formation with unexpected consequences. While skeletal myogenesis in these mutant mice resumes normally at the onset of MyoD expression, a skeletal defect of the ribs persists. Apparently, Myf-5 and MyoD individually are not absolutely essential for skeletal muscle development, most likely because they have overlapping or redundant functions. In fact, double mutants lacking both, MyoD and Myf-5, fail to develop skeletal musculature and the muscle forming regions seem to be devoid of myoblasts. Homozygous inactivation of the myogenin gene leads to drastically reduced myofiber formation. These mice accumulate apparently normal numbers of myoblasts which are arrested in their terminal differentiation program. Myf-6 null mutant mice exhibit drastically reduced expression of Myf-5 for reasons presently unknown. The phenotype is very similar to Myf-5 mutants with an additional reduction of deep back muscles and minor alterations in sarcomeric protein isoforms. Based on the phenotypes obtained from these various gene "knock-out" mice, we now begin to understand the regulatory network and the homostatic relationship of genes which are critically involved in myogenesis of vertebrates.

KEY WORDS: *MyoD protein family, vertebrate myogenesis, determination and differentiation, knock-out mice*

Introduction

The existence of dominant-acting regulators in muscle cell differentiation has been predicted from early cell fusion experiments which demonstrated that muscle-specific genes can be transactivated in non-muscle nuclei of heterocaryons between differentiated skeletal muscle cells and various non-muscle cell types (Blau *et al.*, 1983; Wright, 1984). These observations have led to the concept that muscle nuclei may direct the synthesis of factors which specifically activate gene expression in heterologous nuclei within the same cell. The first factor resembling this property was described when MyoD was isolated and shown to activate the complete myogenic program in 10 T 1/2 fibroblast thereby converting these non-muscle cells to the skeletal muscle phenotype (Davis *et al.*, 1987). This remarkable capacity evoked the idea that MyoD may constitute the "master regulator" for myogenesis. However, subsequently to the isolation of *MyoD*, three additional genes have been identified which also exerted similar myogenic activity. The members of this family are *MyoD*, myogenin (Edmondson and Olson, 1989; Wright *et al.*, 1989), *Myf-5* (Braun *et al.*, 1989a), and *MRF4* (Rhodes and Konieczny, 1989), the latter independently isolated as *herculin* (Miner and Wold, 1990) and *Myf-6* (Braun *et al.*, 1990a). These proteins are related to each other by a highly conserved structural motif, referred to as basic helix-loop-helix (bHLH) domain. The conserved region is necessary for the myogenic function and mediates dimerization and sequence-specific DNA-binding. Little sequence conservation exists among the four proteins outside of the bHLH domain which suggests that their biological activity may depend primarily on this domain. The expression of these genes is strictly limited to skeletal muscle cells excluding cardiac and smooth muscle. They encode nuclear phosphoproteins which have been shown by many investigators to function as gene-specific transcription factors (for review see Weintraub *et al.*, 1991a; Olson, 1990; Arnold and Braun, 1993). Their potential target genes, such as genes encoding sarcomeric proteins, etc., frequently contain cis-acting DNA control elements, referred to as E-boxes. Upon heterodimerization with ubiquitously expressed bHLH proteins, for instance the products of the

Abbreviations used in this paper: bHLH, basic helix-loop-helix; ES cells, embryonic stem cells; PGK, phosphoglucokinase; neo, neomycin; MyHC, myosin heavy chain,; RT-PCR, reverse transcriptase-polymerase chain reaction.

***Address for reprints:** Department of Cell and Molecular Biology, Technical University of Braunschweig, 38106 Braunschweig, Germany. FAX: 531.3918178.

[1]This article is dedicated to Helga and Walter Kersten on the occasion of their official retirement.

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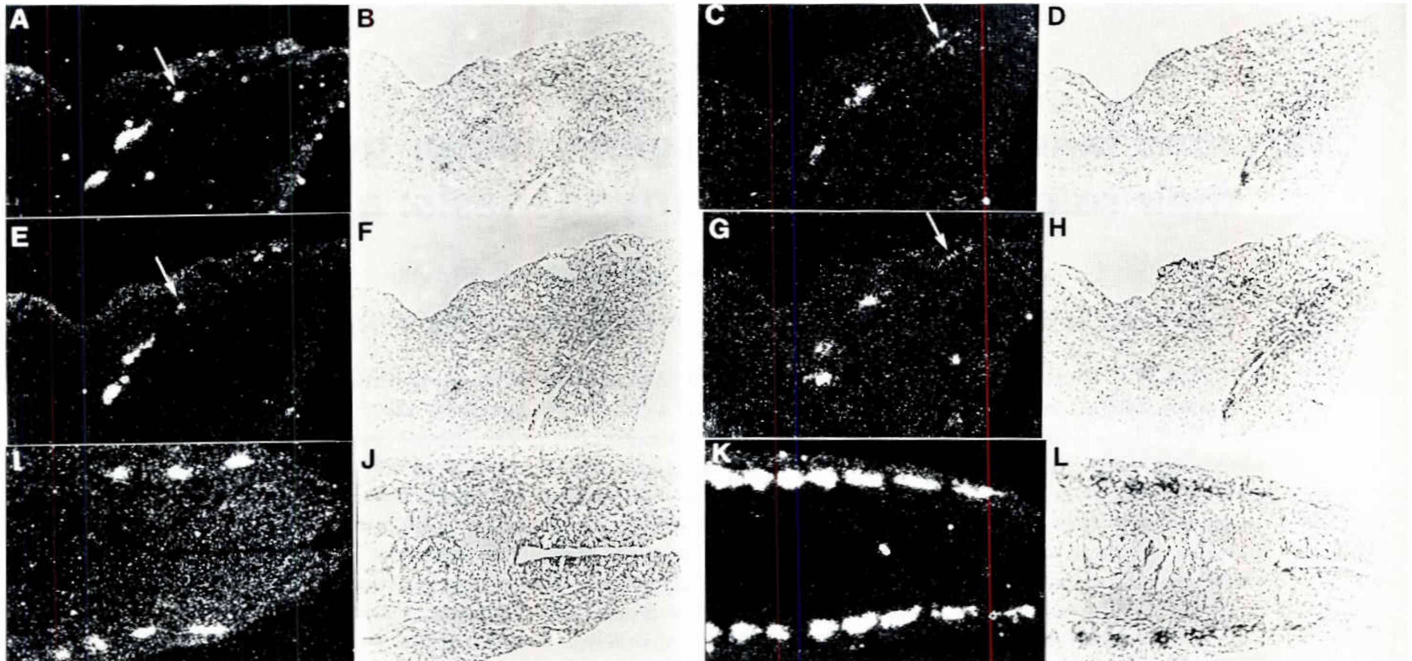

Fig. 1. Expression of MyoD and myogenin transcripts in the caudal somites of *Myf-5* mutant embryos at the 40 somite stage. *Adjacent sections of Myf-5 mutant* **(A-H)** *or wild-type* **(I-L)** *embryos were hybridized to MyoD* **(A,E,I)** *or myogenin* **(C,G,K)** *probes. In Myf-5 mutants myogenin transcripts were present only in MyoD expressing somites, whereas in wild-type embryos myogenin mRNA is already present in the most caudal somites prior to MyoD mRNA. The most caudal MyoD positive somite of the mutant embryo begins to accumulate myogenin transcripts (marked by the arrow). First and third panel: dark-field illumination, second and forth panel: bright-field illumination. Magnification, 250x.*

E2A gene, muscle-specific bHLH factors bind to these E-boxes with high affinity and lead to transcriptional activation of the targets. However transactivation requires not only the bHLH domain but also depends on non-conserved transactivation domains located in the NH_2- and / or C00H termini of the various myogenic factors (Braun *et al.,* 1990b; Yutzey *et al.,* 1990; Weintraub *et al.,* 1991b). Interestingly, conversion of non-muscle cells to the stable skeletal muscle phenotype by forced expression of one myogenic protein is frequently accompanied and may even require the activation of one or more of the endogenous myogenic bHLH genes (Braun *et al.,* 1989b; Thayer *et al.,* 1989). This auto- and crossactivation in most tissue culture cell lines has made it difficult to assess the individual roles of each individual factor and their functional interplay during the myogenic process.

In established muscle cell lines MyoD and/or Myf-5 are expressed in proliferating myoblasts prior to overt muscle differentiation but continue to be present at somewhat lower levels in differentiated myocytes. In contrast, myogenin transcripts and protein are not present in myoblasts but begin to accumulate at the onset of differentiation in all skeletal muscle cell lines studied to date. Expression of MRF-4 / herculin / Myf-6 does not prevail in most established myogenic cells until later stages of prolonged culturing (Braun *et al.,* 1990a; Miner and Wold, 1990; Montarras *et al.,* 1991). Taken together, these observations have led to the speculation that Myf-5 and MyoD may play a role in early events of myogenesis presumably during myogenic determination, whereas myogenin and / or MRF-4 may be part of the terminal differentiation program. This view implies that the biological functions of Myf-5 and/or MyoD in myoblasts would be different from those in myotubes and would also be distinct from myogenin and MRF-4. Alternatively but not mutually exclusive, the time of activation of the individual myogenic factor genes in cells at different developmental stages may also have important functional consequences. Unfortunately, the available tissue culture systems do not allow studies to distinguish between these various possibilities, mainly because of lack of suitable markers for myogenic stem cells. In this report, we review the current state of knowledge of *in vivo* functions associated with the four myogenic bHLH factors. Here, we concentrate particularly on their developmental expression patterns and on phenotypes observed in gene knock-out mice lacking one or several members of the MyoD family.

Temporal expression during mouse development is distinct for each gene of the myogenic bHLH family

Overexpression of MyoD, myogenin, Myf-5, and MRF-4 in fibroblasts generates virtually identical muscle cells *in vitro.* These observations have led to the proposal that the four transcription factors may more or less play the same role as "myogenic master genes" at a "nodal point" of myogenesis (Weintraub *et al.,* 1991a). Their different developmental expression patterns, however, challenged this view of completely overlapping or redundant functions and suggested that each gene may serve different aspects of myogenesis *in vivo.*

The spatio-temporal expression of the four genes has been determined by *in situ* hybridizations during mouse embryogenesis (Sassoon *et al.,* 1990; Bober *et al.,* 1991; Ott

et al., 1991). Investigations on serial sections revealed that each gene is activated at another developmental time in muscle cells or their progenitors in somites, visceral arches, and limb buds. Transcripts can be detected first in cranial somites and successively in all somites along the rostro-caudal axis. The same antero-posterior sequence is seen for their expression in muscle progenitors in visceral arches and limb buds.

Myf-5 transcripts appear as soon as segmentation of somites occurs in 8 day p.c. mouse embryos. The expression is initially restricted to dermomyotomal cells in the dorsal-medial quadrant of still epithelial somites and later spreads to the entire myotomes and all skeletal muscles of the body (Bober *et al.,* 1991; Hinterberger *et al.,* 1991; Ott *et al.,* 1991). The concentration of Myf-5 mRNA and protein reaches maximal levels between day 9.5 and 10.5 p.c. and then declines rapidly beginning in the rostral part of the embryo and proceeding in caudal direction. This transient activity of the *Myf-5* gene is in marked contrast to the continuous and persistent synthesis of myogenin and MyoD throughout prenatal development.

Accumulation of myogenin mRNA is first detectable in rostral somites of 8.5 day p.c. embryos, approximately 12 hours after Myf-5. At this time, myotomal muscle begins to form and muscle-specific marker genes, such as α-actin, start to be expressed. In limb buds and muscle progenitors of jaw muscles and tongue, myogenin expression also lags behind Myf-5 by approximately the same margin.

MRF4 (Myf-6) expression is first seen in somites about 12 h after myogenin. Like Myf-5, this gene is activated for a short transient period and transcripts can be detected by *in situ* hybridization for only about 2 days. In contrast to the other family members, Myf-6 mRNA is restricted to myotomes and can not be found by *in situ* hybridization in limb buds, visceral arches and axial skeletal muscles between day 8 and 15 of embryogenesis. In fetal mice, Myf-6 transcripts reappear from day 16 onwards in all skeletal muscles where they constitute the major transcripts for myogenic factors until and after birth. Thus, Myf-6 appears in a characteristic biphasic time pattern not seen for the other members of this family. The reappearance of Myf-6 mRNA in fetal skeletal muscle coincides approximately with the formation of secondary muscle fibers and the onset of innervation.

MyoD expression in mature somites begins about 24 h after Myf-6 at day 10.5 p.c. when MyoD transcripts accumulate in myotomes, limb buds, and facial muscles. Like myogenin, its expression persists throughout prenatal life. The late activation of MyoD expression *in vivo* is not in agreement with an obligatory function in muscle determination upstream of the other myogenic factor genes. In contrast, the early expression of Myf-5 suggests that it may play a role in activating one or several later expressed members of this gene family. It is also interesting to note that the auto- and crossactivation of muscle control genes frequently observed in tissue culture cells upon overexpression of any one of the factors may not be operating in this simple fashion *in vivo.*

Targeted inactivation of single genes in mice allows to study their individual physiological importance *in vivo*

A variety of *in vitro* assays had suggested largely similar properties and biological activities for all members of the MyoD family (for review see: Arnold and Braun, 1993; Emerson, 1993; Olson and Klein, 1994).

We wanted to know whether these genes are also functionally redundant *in vivo* or whether they play distinct roles in progenitor commitment, myoblast proliferation, terminal differentiation, and in the maturation and regeneration of skeletal muscle. In order to genetically dissect the roles of the different myogenic factors in myogenesis, we and others employed gene targeting in embryonic stem (ES) cells as a means to introduce specific

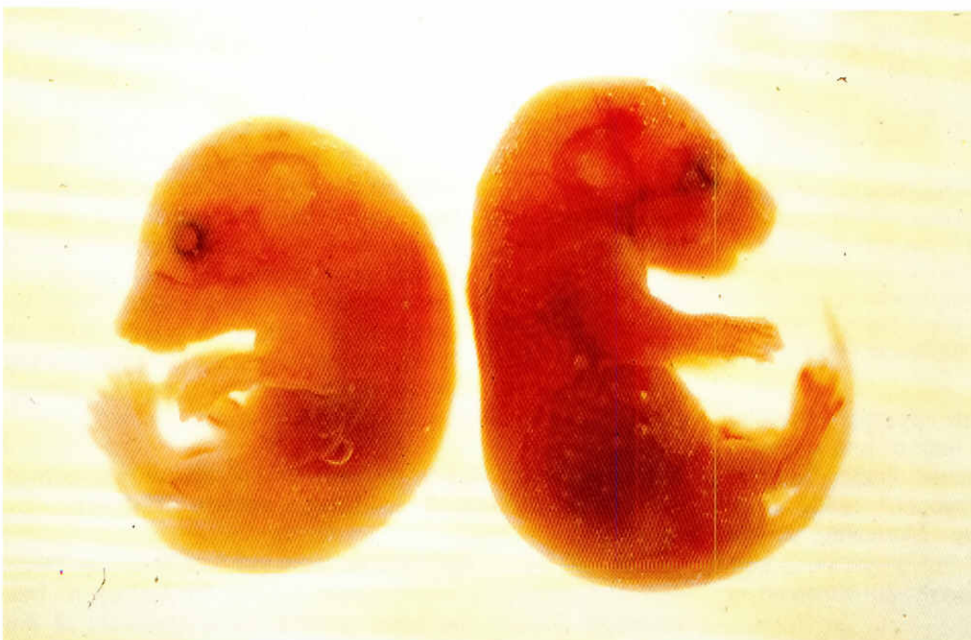

Fig. 2. Mice carrying homozygous null-mutations in the Myf-5 and MyoD genes lack skeletal muscle. *Left fetus: double homozygous Myf-5 (-/-), MyoD (-/-). Right fetus: heterozygous littermate Myf-5 (+/-) / MyoD (+/-). Animals were delivered by cesarian section on the 18th day of gestation. Newborn pups lacking MyoD and Myf-5 are born alive but are immobile and die shortly after birth. Mutant animals have a severely distorted posture (arched back) and appear entirely translucent due to the absence of all skeletal muscle. Lung and liver are clearly visible through the body wall.*

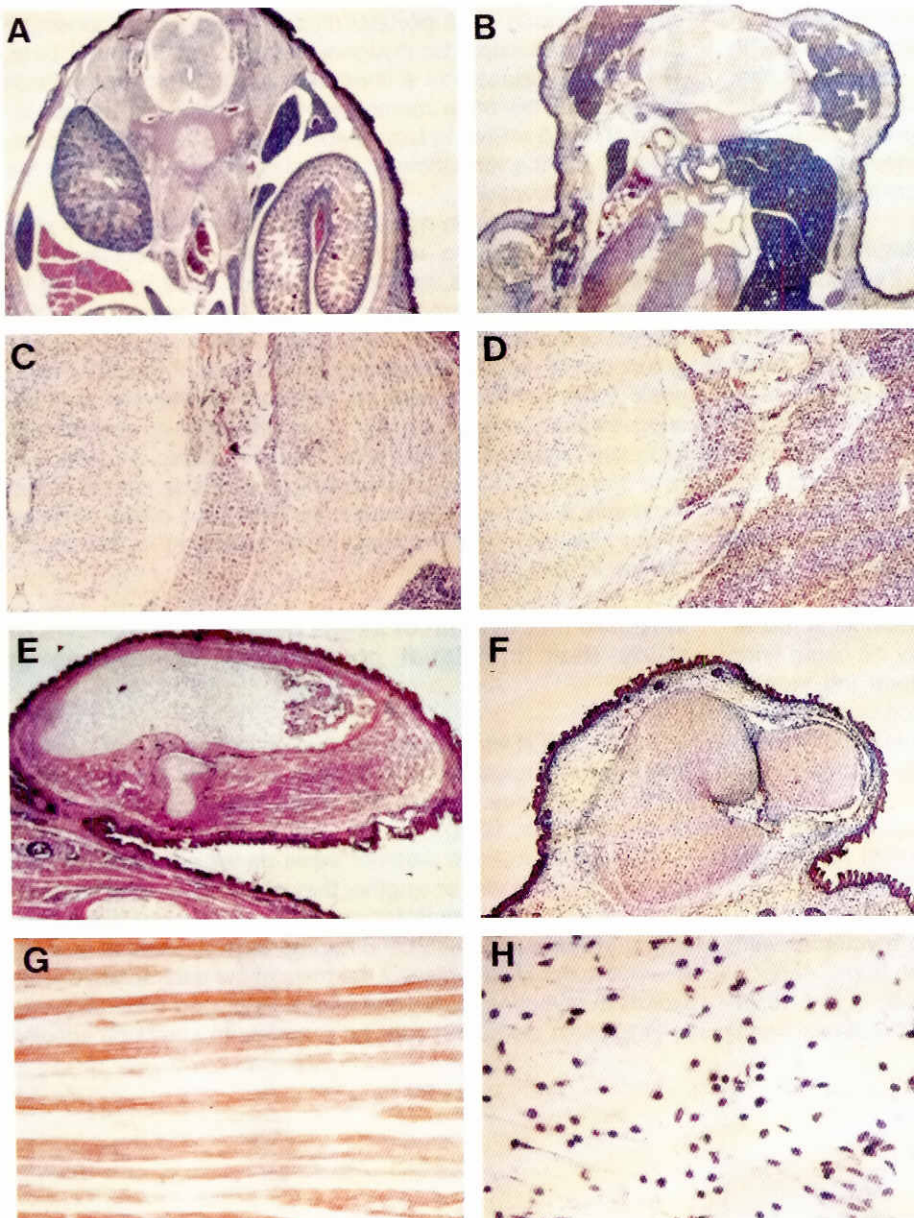

Fig. 3. Histology of mutant mice lacking Myf-5 and MyoD. *Hematoxylin- and eosin-stained transversal sections through wild-type* **(A,C,E,G)** *and double-homozygous mutants* **(B,D,F,H)** *reveal the lack of skeletal muscles in the trunk* **(B,D)** *and in the limbs* **(F)** *while heart muscle tissue appears normal* **(B)**. *Corresponding sections from a wild-type littermate are shown on the left panels for comparison. In double-mutants the areas normally covered by skeletal muscle is occupied by neighboring organs* **(B,D)** *or loose connective tissue which does not react with anti-myosin heavy chain antibody MF-20* **(H)** *while corresponding sections from wild-type embryos are stained* **(G)**.

mutations into the germ line of mice. This approach allows to study the phenotypic consequences of hetero- and homozygous mutations in otherwise isogenic mouse strains. To date, null-mutations for all four genes of the myogenic bHLH family have been obtained by this technique. Moreover, double mutants lacking certain combinations of two genes have also been studied. Here, we review the phenotypes observed in these mice.

Inactivation of the MyoD gene results in upregulation of Myf-5 expression and apparently normal muscle development

Rudnicki *et al.* (1992) have targeted the *MyoD* gene by a replacement construct in which the first exon and part of intron 1 were replaced by the selectable *PGK-neo* gene. Upon introduction of this null mutation into the germ line of mice, they generated heterozygous and homozygous animals which were viable and fertile and did not exhibit any apparent deficits in skeletal muscles. In particular, the histology of muscles from various body regions and the expression of numerous muscle-specific marker genes as well as the ultrastructure of sarcomeres appeared unaltered. The motility and evoked movements of homozygous animals were indistinguishable from heterozygous and wild-type littermates. These results indicate that MyoD is not essential for the initiation or progression of normal muscle development consistent with the idea that other members of the myogenic bHLH family may substitute for MyoD in myogenesis. Examination of the expression of the other myogenic bHLH genes in heterozygous and homozygous MyoD mutant mice revealed normal levels of myogenin and Myf-6 transcripts. However, Myf-5 mRNA which is normally down-regulated and not easily detectable on Northern blots of neonatal mice was clearly recognized in heterozygous mice and at even higher levels in homozygous MyoD mutants. These results suggest that Myf-5 expression is normally suppressed by MyoD in a dosage dependent manner and the elevated and sustained expression of Myf-5 in the absence of MyoD may actually be responsible for the lack of a muscle phenotype. However, it has been observed that in mixed litters of all possible genotypes the homozygous mutants survive weaning at a lower frequency than predicted. This phenomenon has not been seen in litters of purely homozygous mutants suggesting that under competitive conditions a fraction of these mutants may die in the postnatal period.

Homozygous Myf-5 mutant mice show abnormal somite development resulting a severe rib defect

The *Myf-5* gene has been targeted in ES cells with an insertion vector carrying the *PGK-neo* gene in the first exon. This construct disrupts the open reading frame upstream of the

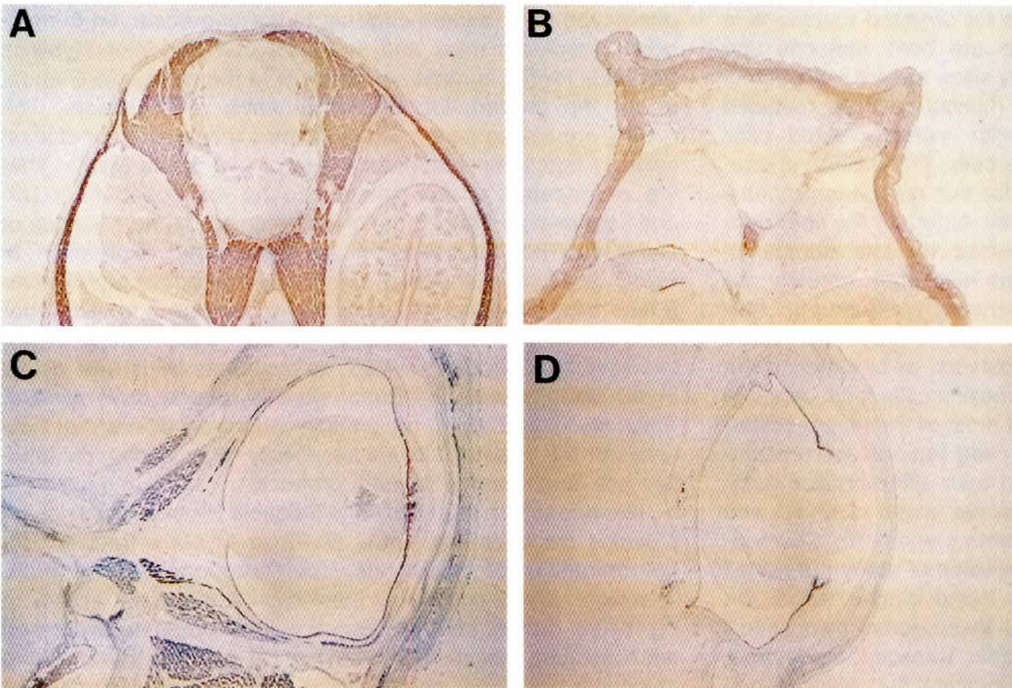

Fig. 4. Mice lacking both Myf-5 and MyoD are completely devoid of skeletal myocytes derived from somites and prechordal plate mesoderm. *Immunohistochemical detection of skeletal muscle cells with anti-myosin heavy chain antibody MY32 in wild-type* **(A,C)** *and double-homozygous mutants* **(B,D)**. *No myocytes are found in double homozygous mutants at the trunk level* **(B)** *and in the orbit* **(D)**. *Corresponding sections from wild-type littermates are shown in A and C. Note that muscles of the trunk and extraocular muscles of the orbit have different developmental origins.*

bHLH domain and yields no functional Myf-5 protein (Braun *et al.,* 1992a). The mutation in animals is recessive lethal in the perinatal period. The cause of death in homozygous Myf-5 mice is their inability to breathe owing to the absence of the major distal parts of the ribs and consequently the lack of a functional rib cage. Other skeletal abnormalities were not observed. Interestingly, skeletal muscles in newborn mutants revealed no morphological abnormalities and expressed the normal set of muscle-specific marker genes including the myogenic factors, MyoD, myogenin, and Myf-6. These unexpected results suggested that, while Myf-5 seems dispensable for the development of skeletal muscle perhaps because other members of the myogenic bHLH family substitute for Myf-5 activity, it plays a crucial role for the formation of complete ribs. It should be noted here that Myf-5 is not expressed outside of muscle cells and their precursors and is particularly absent from sclerotome which gives rise to the axial skeleton including the ribs. How then may this phenotype arise? A partial answer to this question came from developmental studies which showed that early somitogenesis is impaired in homozygous Myf-5 mutant embryos. While heterozygous and wild-type littermates developed myotomes between day 8.5 and 10 p.c., no myotomal cells were detectable in homozygous embryos until day 10.5 p.c. The expression of myogenin, normally activated at day 8.5 in cranial somites, was also conspicuously absent suggesting that it may require the action of Myf-5 either directly or indirectly. At day 10.5 p.c., when the *MyoD* gene starts to be expressed in normal mouse embryos, the first myotomal cells expressing MyoD and myogenin can also be detected in Myf-5 mutants (see Fig. 1). Subsequently, skeletal muscle develops quite normally until it is indistinguishable from wild-type animals at birth (Braun *et al.,* 1994). Taken together these results indicate that the *Myf-5* gene is essential to establish the early myotome, but MyoD can also generate myotomal cells independently of Myf-5. Thus, two independent entry-points to myogenesis one controlled by Myf-5 and another one controlled by MyoD seem to exist making a simple model of functional hierarchy unlikely (Braun and Arnold, 1994). Interestingly, the significant delay of myotome formation in Myf-5 mutants has no major impact on skeletal muscle development, however, it severely affects normal development of the sclerotome, particularly of its lateral part which is located adjacent to the myotome and contains the rib progenitor cells. This observation leads to the hypothesis that either inductive or permissive interactions of early myotomal cells with sclerotomal precursors may be required to allow complete rib development (Holtzer, 1968). It will be of utmost importance to define the molecules which mediate these interactions (Hall, 1977).

The myogenin gene is required for myocyte differentiation *in vivo*

The importance of myogenin for myocyte differentiation has been suggested by the strict correlation of its expression at the onset of differentiation in all skeletal muscle cell lines studied so far, whereas the other factors MyoD, Myf-5 or Myf-6 are variably expressed (Edmondson and Olson, 1989; Wright *et al.,* 1989). Further evidence for myogenin's essential role in differentiation came from muscle cells expressing adenovirus protein Ela which suppresses the activation of the myogenin gene and concommitantly blocks differentiation, although these cells continue to express Myf-5 or MyoD (Enkemann *et al.,* 1990; Braun *et al.,* 1992b). Furthermore, antisense oligonucleotides that inhibit myogenin expression prevent myoblast differentiation (Brunetti and Goldfine, 1990; Florini and Ewton, 1990).

In order to test the role of myogenin in embryonic muscle development directly, Hasty *et al.* (1993) and Nabeshima *et al.*

(1993) generated mice homozygous for targeted mutations in the *myogenin* gene. These animals are born immobile and decease immediately after birth. They show severe reduction of skeletal muscle mass. Histology of muscle sections revealed drastically reduced fiber density with mononucleated cells replacing most of the mature muscle cells. This severe phenotype includes the diaphragm which for this reason is probably non-functional and causes the lethality. Although the fiber density in muscles was severely diminished, the total number of nuclei in the muscle forming regions was approximately the same in homozygous myogenin mutants compared to heterozygous and wild-type littermates. This suggests that these areas were populated either by myoblasts, or by unfused but differentiated myocytes, or with non-muscle cells for instance of mesenchymal origin. It turns out that most of the mononucleated cells observed in the muscle forming regions are presumably myoblasts which are arrested in their differentiation pathway, since they express virtually normal levels of MyoD and lack myosin heavy chain and actin, both markers for differentiated muscle cells. Significantly, expression of many but not all muscle-specific marker genes was found diminished in the absence of myogenin indicating that these genes have differential requirements for the myogenic transcription factors. Taken together these observations indicate that myogenin is not essential for commitment of cells to the myogenic lineage, but is important for terminal differentiation. Moreover, the normal levels of MyoD in homozygous myogenin mutants show that the MyoD gene is unable to overcome the severe muscle deficiency caused by the lack of myogenin. Interestingly, the absolute requirement of myogenin for differentiation seems to be contingent on the *in vivo* situation, as myoblasts obtained from myogenin null mutants differentiate in culture quite readily (Nabeshima *et al.,* 1993).

Double mutant mice lacking MyoD and Myf-5 fail to form skeletal muscle

Mice carrying null mutations in the myogenic regulatory genes *Myf-5* or *MyoD* have apparently normal skeletal muscle, indicating that both genes individually are not required for myogenesis. The expression patterns of the remaining myogenic bHLH factors in these mutants suggested possible functional redundancy, particularly for Myf-5 and MyoD. To address whether these two factors actually substitute for one another in myogenesis, Myf-5 and MyoD mutants were intercrossed to generate double homozygous mutant animals (Rudnicki *et al.,* 1993). Null mutants for both genes were born alive but were immobile and died soon after birth. The phenotype of newborn pups is shown in Figure 2. Expression analysis indicated that no skeletal muscle-specific mRNAs including all four myogenic bHLH transcripts were made in the double mutants. Histological examination revealed the complete absence of skeletal muscle fibers and desmin-expressing myoblasts. The muscle forming regions were partly devoid of cells or were populated by loose mesenchymal tissue or taken up by other structures, such as the internal organs (Fig. 3). This lack of myocytes is not restricted to muscles originating in somites but also involves the extraoccular muscles which are derived from prechordal plate mesoderm (Fig. 4). This phenotype is in marked contrast to the one observed in myogenin knock-out mice and suggests that Myf-5 or MyoD is essential for the commitment of cells to the myogenic lineage, or for the propagation or maintenance of myoblasts. The results are also in agreement with the expectations for at least partially functional redundant roles of Myf-5 and MyoD. That the redundancy is incomplete can be concluded from two observations: 1) The rib deficiency caused by the absence of Myf-5 can not be overcome by the normal expression of MyoD; 2) MyoD null mutants with only one active Myf-5 allele (these mice produce only 50% Myf-5 compared to wild type) have severely reduced skeletal muscle masses. Interestingly, the reverse genotype (Myf-5 null mutants with one active MyoD allele) results in normal skeletal muscle development. Thus, Myf-5 and MyoD have either inherently different activity levels or the threshold levels necessary for full biological function are not the same for both proteins.

Taken together, the MyoD/Myf-5 double knock-out phenotype provides evidence that either Myf-5 or MyoD is required for determination of myoblasts, their propagation, or both. Therefore, Myf-5 and MyoD must have a functional activity in myoblasts distinct from the transactivation of genes expressed after myotube fusion. This activity must also be different from myogenin which is not essential for the establishment of myoblasts. The identification of target genes for Myf-5 and MyoD in myoblasts or their progenitors will be important to understand how these cells are specified.

Targeted disruption of the forth myogenic factor, Myf-6

The role of Myf-6 in the network of molecules controlling myogenesis has so far attracted minor attention. This is in part due to its restricted expression in established cell lines severely limiting the experimental access to study Myf-6 functions. *In vivo* analysis of Myf-6 in mice offers a much better approach to assess the individual role of Myf-6, its interaction with other myogenic factors, and its possible impact on terminal muscle differentiation. We have inactivated the *Myf-6* gene in ES cells and generated mouse strains thereby completing the targeted inactivation experiments for all four myogenic factors in mice (Braun and Arnold, 1995).

Inactivation of the *Myf-6* gene was achieved with a targeting construct containing a deletion between nucleotides -5 to +207 which were replaced by the PGK-neo cassette in sense orientation. This deletion removes the initiation codon of *Myf-6* and therefore disrupts the gene. Myf-6 mRNA of normal size was readily detected on Northern blots from wild-type and heterozygous newborn mice but not from homozygous mutants. At birth all homozygous mutant mice were capable of spontaneous movements or reacted to mechanical stimulation, but died postnatally within a few minutes. Autopsy revealed that the rib cage was severely malformed with only the proximal parts of the ribs next to the vertebral column developed normally. The observed rib phenotype in Myf-6 minus mice was almost indistinguishable from that in Myf-5 mutant mice. However, the rib stumps in general were longer than in the Myf-5 mutants which consistently display only short rib rudiments.

Immunohistochemistry with anti-myosin heavy chain (MyHC) and anti-myogenin antibodies revealed the lack of primary

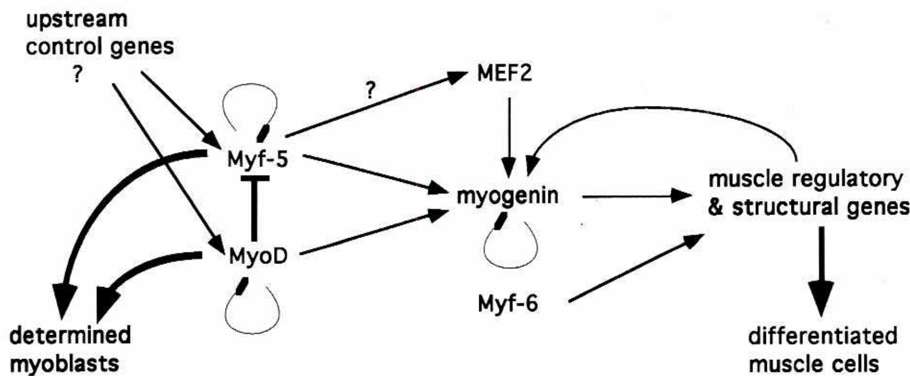

Fig. 5. Genetic relationship of myogenic control genes. *Gene activations and feedback loops are indicated by thin arrows. Cellular consequences of gene activities are pointed out by thick arrows.*

myocytes in somites of homozygous mutants at E10.5, an observation very similar to Myf-5 mutants. To distinguish whether the failure of myogenic differentiation in the absence of Myf-6 was due to a block of differentiation of myoblasts or whether the development of myoblast precursors was inhibited, we examined the presence of myoblasts using Myf-5 as marker and myogenin for comparison. Myf-5 and myogenin mRNAs were undetectable in somites of homozygous mutant Myf-6 embryos by *in situ* hybridization at E9.5 and E10.5. Thus, disruption of the *Myf-6* gene appears to interfere with the development of Myf-5 positive myoblasts in early somites. At birth normal levels of MyoD and myogenin were observed, whereas Myf-5 expression was still significantly reduced and only detectable by RNAse protection.

The Myf-5 phenocopy generated by the Myf6 mutation may be related to the fact that both genes are closely linked on mouse chromosome 10, approximately 8 kb apart.

Formally Myf-5 down-regulation in Myf-6 mutant mice may be explained in two ways: first, the *Myf-6 gene* mutation may affect Myf-5 expression in cis. A simple mutation of a *Myf-5* upstream regulatory element responsible for gene regulation seems unlikely, because only exon sequences of the *Myf-6* gene were deleted and mutations within the *Myf-5* gene were virtually excluded. However, it is possible that the insertion of the *PGK-neo* gene interferes with a remote Myf-5 activating element which is located outside of both genes. In fact, a promoter/enhancer competition model has been proposed for a mutation of the locus control region in the *globin* cluster (Kim *et al.,* 1992; Fiering *et al.,* 1993).

Second, Myf-6 may be required to switch on *Myf-5* gene expression in the dermomyotome or maintain its activity in somites. This mechanism, however, would not be compatible with the expression pattern of Myf-6 which seems to follow Myf-5 expression as previously defined by *in situ* hybridization (Bober *et al.,* 1991; Hinterberger, *et al.,* 1991). Based on RT-PCR, however, Myf-6 transcripts can be detected in immature somites and even in unsegmented mesoderm indicating that low level Myf-6 transcription occurs much earlier than previously noticed. Whether this level of expression is sufficient to account for the biological activity, however, can not be assessed in this way. Recent analysis of Myf6/Myf-5 compound heterozygous mice which carry the Myf6 mutation on one chromosome and the Myf-5 mutation on the other revealed the same rib defects and down-regulation of Myf-5 expression as our Myf6 homozygous mutants. This indicates by a formal genetic approach that the mutated Myf6 allele affects Myf-5 activity in cis (Braun and Arnold, unpublished results).

Data on Myf-6 expression *in vivo* and in cell culture have led to the hypothesis that Myf-6 may be primarily responsible for the maturation of myocytes. Histological and immunohistochemical investigations in homozygous Myf-6 mutants do not support this hypothesis. Mature secondary myotubes with regular arrays of sarcomeres were observed in mutants without significant differences to wild-type controls. However, expression of some muscle-specific genes examined by Northern blot and RNase-protection was different in Myf-6 mutants and control littermates. Certain muscle markers, in particular those expressed in fast fibers, showed slightly reduced expression. The most drastic decrease was observed for embryonic MyHC. This observation may reflect the various dependence of structural muscle genes on different transcription factors. We also noted a reduction of muscle fibers in deep axial muscles which are thought to originate from myotomal cells of the somite while hypaxial muscles originating from the dermomyotome appeared normal. Interestingly, Nabeshima and co-workers reported the reverse distribution in the myogenin knock-out mice, e.g. stronger reduction of differentiated muscle cells in limbs than in axial muscles (Nabeshima *et al.,* 1993). However, it should be pointed out that the muscle phenotype of Myf-6 mutant mice is rather mild compared to myogenin. In the light of the results obtained in Myf-5 and MyoD mutants and in mutants simultaneously carrying both inactivated genes, it appears likely that compensatory mechanisms might also be operative between Myf-6 and myogenin. Crosses between Myf-6 and myogenin deficient mice will shed light on this question and will reveal whether the absence of both factors results in a complete block of differentiation.

In summary, Myf-6 null mutant mice which also have drastically reduced levels of Myf-5 (in essence Myf-6/Myf-5 double knock-outs) form fairly normal skeletal muscle with some alterations in the expression of sarcomeric protein isoforms. An essential function of Myf-6 during maturation of myotubes as previously proposed on the basis of its expression pattern and on tissue culture experiments, appears rather unlikely considering the knock-out phenotype. However, this result may be explained by myogenin compensating for the role of Myf-6.

Conclusions and Perspectives

Despite the remarkable muscle-forming activities of Myf-5 and MyoD in constitutively expressing tissue culture cells, mice

with homozygous gene-targeted mutations in either Myf-5 or MyoD produce essentially normal skeletal muscle (Braun *et al.*, 1992; Rudnicki *et al.*, 1992). The reason for this apparent paradox has been resolved in double homozygous mutants lacking both Myf-5 and MyoD. These mutant animals are incapable of forming muscle and express no muscle markers and no myogenin (Rudnicki *et al.*, 1993) indicating that Myf-5 and MyoD share redundant functions required for establishing or maintaining myoblasts in embryos and activating the myogenin gene *in vivo*. Since Myf-5 and MyoD are activated at different times during development and no evidence has been presented so far that both proteins are present in the same myoblast, an extreme view could be that one myogenic lineage could be established dependent on Myf-5 and a second dependent on MyoD.

Mice with a homozygous targeted mutation of myogenin produce myoblasts with normal levels of MyoD, but these myoblasts fail to differentiate to functional myofibers indicating that myogenin is required for the terminal differentiation program. Thus, while Myf-5 and MyoD direct the formation and control the identity of the correct number of myoblasts in the appropriate positions of the body, myogenin exerts its unique function in myoblasts but apparently does not contribute to their generation. This simple epistatic relationship of myogenic control genes is illustrated in Figure 5.

It is presently unclear how Myf-5 and MyoD actually establish the determined myogenic state in cells, since reliable markers for committed myoblasts are limited to the myogenic control genes themselves. Therefore, the identification of myoblast-specific target genes for Myf-5 and MyoD will be important to allow a better understanding of this determining function. One of the potential target genes for MyoD and Myf-5 is myogenin whose activation in myoblasts also seems to require another muscle transcription factor MEF-2 (Edmondson *et al.*, 1992; Buchberger *et al.*, 1994). Whether *MEF-2* gene activation also depends on Myf-5 or MyoD is currently unknown, although in tissue culture cells MEF-2 expression can be induced by these bHLH proteins and also by myogenin.

The unique function of myogenin in activating downstream muscle structural genes is indicated in myogenin-deficient mice which express normal levels of MyoD and presumably Myf-5, yet fail to activate most of the muscle markers. Surprisingly, when these myoblasts are cultured *in vitro*, they readily differentiate, most likely under the direction of MyoD or Myf-5 (Nabeshima *et al.*, 1993). This observation suggests that MyoD and Myf-5 in the embryo are subject to negative control either by external signals or by cell contacts which are removed by the culture conditions. In fact, several growth factors, such as bFGF and TGF-β, and oncogene products, such as fos, jun, and myc, are known to inhibit the activity and expression of myogenic bHLH proteins. Whether or not the same genes are mediating control in the embryo remains to be seen.

The role of Myf-6 in mouse myogenesis is still not quite clear. Particulary, the early expression in somites is enigmatic, since in its absence mice develop fairly normal skeletal muscle. Unfortunately, the only available Myf-6 knock-out has a recessive lethal phenotype, probably due to the down-regulation of Myf-5. Thus, postnatal consequences of *Myf-6* gene inactivation cannot be assessed in this model. Nevertheless, the mild muscle phenotype in homozygous Myf-6 mutants which also lack most of Myf-5 suggests that a functional overlap with other bHLH factors, most likely with myogenin may also exist for this gene.

Post Scriptum: During the preparation of this manuscript two additional Myf6 mutant alleles have been published with somewhat different phenotypes. While the Myf6 mutation generated in E. Olson's group yields viable animals with normal Myf-5 expression and less severe rib defects, a second allele made in B. Wolds' laboratory results in a lethal phenotype with intermediate severity of the rib distortions (Zhang *et al.*, 1995; Patapoutian *et al.*, 1995).

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