Biochemical and Biophysical Research Communications **272,** 138–143 (2000)
doi:10.1006/bbrc.2000.2703, available online at http://www.idealibrary.com on IDEAL®

Skeletal Muscle Regeneration Is Not Impaired in *Fgf6* −/− Mutant Mice

Frederic Fiore,* Alain Sébille,† and Daniel Birnbaum*
**U.119 INSERM, 27 Bd. Leï Roure, IFR57, Université de la Méditerranéee, 13009 Marseille, France; and*
†U.523 INSERM, Faculté de Médecine Saint-Antoine, 75012 Paris, France

Received April 10, 2000

FGF6 is a member of the fibroblast growth factor family. The *Fgf6* gene is almost exclusively expressed in adult and developing skeletal muscle. We have obtained mice deficient in FGF6 by targeting the *Fgf6* gene by homologous recombination. We studied regeneration of adult skeletal muscle in *Fgf6* −/− mice derived on a standard inbred background. Muscle degeneration was induced by notexin drug or crush injury. The defect in FGF6 did not modify the kinetics of muscle regeneration. We bred *Fgf6* −/− mice with *mdx* dystrophin deficient mice; *Fgf6* −/−:*mdx* and *mdx* muscles were similar. Our study suggests that FGF6 does not play a role in muscle regeneration, i.e., in satellite cell proliferation and fusion, or that this role is strictly compensated by other factors, possibly other FGFs.
© 2000 Academic Press

Key Words: **FGF; FGF6; growth factors; *mdx* mouse; muscle regeneration.**

Skeletal muscle growth and regeneration are vital processes to breathing and movements and are sustained by adult muscle precursor cells (also called satellite cells). The *in vivo* proliferation, and differentiation and fusion into muscle fibers, of these cells are controlled by a network of growth factors, signaling molecules and transcription regulators (1–3). The control of muscle mass, i.e., the number and cross-sectional areas of the muscle fibers, during exercise, stress and aging is also an important process. Like for developmental myogenesis, the controls of these processes begin to be unraveled but only few of the molecular pathways have been precisely defined so far. Adult skeletal muscle function is also highly dependent on the integrity of muscle fibers. Membrane cytoskeletal components such as dystrophins and dystrophin-associated proteins are major elements of this integrity (4). Factors and mechanisms that play a role at this stage are not precisely known.

The FGF (fibroblast growth factor) family is a large family of more than twenty growth factors (5, 6). They play important roles in cellular communication processes during development and adult life (5). Several FGFs are active in myogenesis (7). One of these, FGF6, has a tissue expression essentially restricted to developing and adult skeletal muscle (8–13). During development, it may be an important component of the signaling events associated with the somite. In the adult, its expression in adult skeletal muscle fibers, which express FGF receptors, suggests that it may participate in the control of muscle maintenance and/or regeneration.

To better understand the importance of FGF6 for skeletal muscle physiology, mice with a homozygous disruption of the *Fgf6* gene have been generated in two laboratories (14, 15). In both cases, *Fgf6* −/− mice are viable, develop normally, can reproduce, and have morphologically normal muscle masses. Skeletal muscle regeneration has been studied in these *Fgf6* −/− mice. In one case, a regeneration defect accompanied by fibrosis was associated with the absence of functional *Fgf6* gene (15).

In this work, we studied skeletal muscle regeneration in *Fgf6* −/− mice of pure C57BL6 background and by using different models of muscle degeneration.

MATERIAL AND METHODS

Animals

Fgf6 mutant. *Fgf6* −/− deficient mice were generated by targeted mutagenesis (14) and initially maintained on a C57BL6/129Ola hybrid genetic background. We subsequently obtained *Fgf6* −/− mutants on a C57BL/6J background by backcross with C57BL/6J mice until F6 generation. All muscle injury experiments shown here were done with this genetic background.

Fgf6:mdx mutants. The *mdx* mice are homozygous mutants with dystrophin deficiency. The C57BL/10 *mdx:mdx* mice were bred in the Paris lab. Mice deficient in both *Fgf6* and dystrophin (*Fgf6* −/−:*mdx* double mutants) were generated by cross breeding the single mutants. The genotypes of the mutant mice were determined by Southern blot analysis for *Fgf6* deficiency and allelic-specific oligonucleo-

0006-291X/00 $35.00
Copyright © 2000 by Academic Press
All rights of reproduction in any form reserved.

tide PCR for dystrophin (16). Females *mdx* (homozygous dystrophin deficiency) were bred with male *Fgf6* −/−. Resultant F1 offspring was as follows: all animals were *Fgf6* −/−, males were *mdx* and *Fgf6* +/−, females were +/*mdx* and *Fgf6* +/−. This F1 generation was used to produce the double mutant mice. According to Mendelian ratio, F1 male (*mdx* and *Fgf6* +/−) and female (+/*mdx* and *Fgf6* +/−) crossing gave 12.5% of F2 *Fgf6* −/−:*mdx*: double mutants.

Fgf6:Fgf4 mutants. Because the homozygous *Fgf4* mutation is embryonic lethal, only *Fgf6* −−:*Fgf6* +/− mutant were obtained by crossing *Fgf6* −/− (outbred mutant) with *Fgf4* +/− (17).

Histological Analysis

Because 4%-paraformaldehyde fixation muscle and paraffin embedding process can induce artefacts in analysis of muscle (i.e., fiber damage accompanying imperfect dehydration and rehydration), we chose to freeze muscles in liquid nitrogen-cooled isopentane. Blocks of tissue were oriented so that transverse or longitudinal sections could be obtained. 10 μm sections were cut on a cryotome and dried before hematoxylin/eosin staining.

Muscle Injury

Crush injury. All animals (*Fgf6* −/− mutants or C57BL/6 controls) were males aged 8 weeks (25–28 g). They were anaesthetized with a mix of Ketamin/Xylasin by intraperitoneal injection (0.1 ml to 1 mg/ml per 10 g weight). The *tibialis anterior* (TA) muscle of each mouse was exposed and carefully dissected of its overlying fascia. Crush injury was made transversely across the middle region of TA with a pair of forceps and during 10 s. This produced an injury of approximately 2 × 4 mm. The skin was then sutured. Muscles were analyzed at different stages of regeneration (day 4, 7 and 10 after injury).

Myonecrotic injection. Male mice aged 8 weeks were anaesthetized with a mix of Ketamin/Xylasin by intraperitoneal injection. TA muscle of each mouse was exposed and injected (Hamilton polylabo No. 7110 N) with 10 μl per TA of snake venom (Notexin 50 μg/ml, Sigma No. N6271). The Notexin was adjusted to 50 μg/ml by phosphate saline buffered. Muscles were analyzed at different stages of regeneration (days 4, 7, 10 and 14 after myonecrotic injury).

Molecular Biology

Reverse transcribed (RT) RNAs from wild type and mutant adult (tibialis anterior) muscle were amplified by polymerase chain reaction (PCR) using the following pairs of oligonucleotide primers for *Fgf6*: sense primer 5′ (ATTGGGAAAGCGGCTATTTGG) 3′, located at the end of exon 1 and antisense primer 5′ (TTGCATTCGTCCTGGAAGCT) 3′, located in exon 3.

Primers for murine β2 microglobulin are described in (12).

The RT-PCR products were run on 2% agarose gel and analyzed by BET staining and Southern blot hybridization for *Fgf6* specific amplification. The membrane was hybridized with ^{32}P radiolabeled O4 probe, located in exon 2 (8).

RESULTS

As a preliminary analysis, we verified the absence of *Fgf6* RNA in *Fgf6* −/− mice in C57BL/6 background by Southern blot hybridization of PCR products of reverse transcribed RNA (Fig. 1). No *Fgf6* transcript was seen in these mice by this technique (lanes 1 and 3).

Muscle Regeneration Is Not Impaired in Fgf6 −/− Mice

Muscle regeneration was studied using two different tests. First, we induced muscle degeneration by injecting the drug Notexin in the *tibialis anterior* muscle. This test induces an injury that is dose-dependent, reproducible and easy to control. Sections of muscle at different stages of regeneration showed that both *Fgf6* +/+ and *Fgf6* −/− mice behave similarly. In the two types of mice regeneration progressed at the same pace and was completed by day fourteen (Fig. 2). At this time, centronucleated fibers—a signature of a regenerated muscle—were visible in the mice.

Second, we induced muscle degeneration by mechanical injury (crush method), which is more experimenter-dependent. As in the previous test, *Fgf6* +/+ as well as *Fgf6* −/− showed identical kinetics of regeneration with all types of muscle tested (Fig. 3).

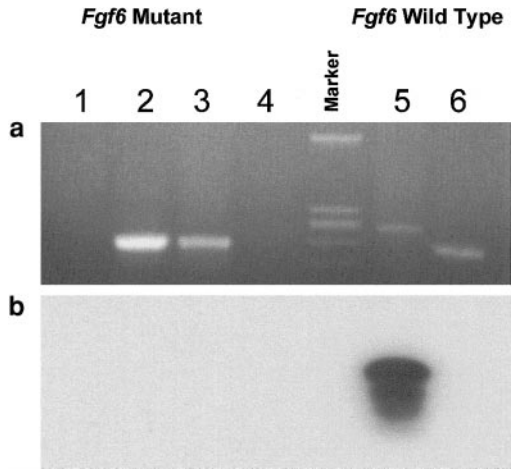

FIG. 1. Absence of *Fgf6* transcript in skeletal muscle of *Fgf6* −/− mutant mice. Reverse transcribed RNAs from *Fgf6* −/− mutant or wild type adult mouse muscle were amplified by PCR using oligonucleotide primers for *Fgf6* or β2 microglobulin and visualized by ethidium bromide staining (a) or hybridization with *Fgf6* exon 2 specific ^{32}P probe (b). Lanes are as follows: *Fgf6* −/− (lanes 1, 2, 3), *Fgf6* +/+ (lanes 5, 6) and control without reverse transcribed RNA (lane 4). Marker is *Hae*III fragments of phage ϕX174. *Fgf6* primers (lanes 1, 5), β2 microglobulin primers (lanes 2, 5). *Fgf6* and β2 microglobulin primers (lanes 3, 4).

Muscle Regeneration Is Not Impaired in Fgf6 −/−: mdx Mice

To further investigate a potential effect of Fgf6 in muscle regeneration, we derived *Fgf6* −/−:*mdx* mice and compared their respective muscles. *Mdx* mice show continuous degeneration/regeneration cycles and represent a model of spontaneous muscle degeneration. Artefacts that can result from the experimental induction of degeneration/regeneration are not present. Centronucleated fibers are a hallmark of all adult mdx muscles.

Several muscles were analyzed (Fig. 4). They all had a similar appearance. No differences in the number or appearance of fibers was seen.

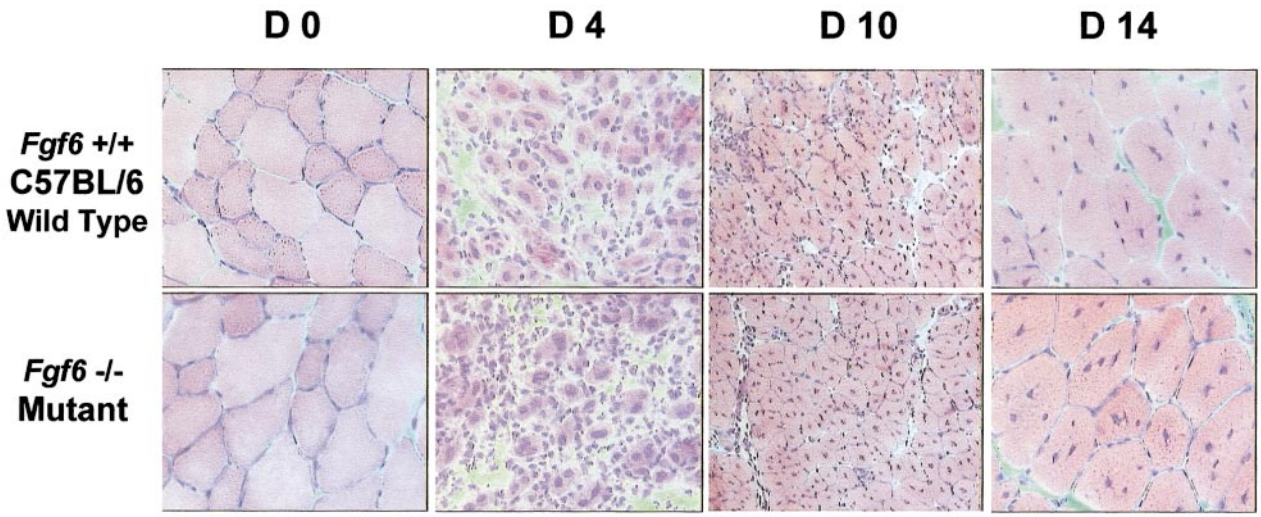

FIG. 2. Absence of differences in the kinetics of muscle regeneration in *Fgf6* −/− mice. Transverse sections from tibialis anterior (TA) muscle of 8-week-old *Fgf6* −/− inbred mouse mutant or C57BL/6 wild type controls. TA muscle were injected by notexin drug and studied at different stages of regeneration (days 4, 10 and 14). No difference in regeneration is observed. Hematoxylin–eosin staining, magnification ×200.

DISCUSSION

Absence of Defect in Muscle Regeneration in Fgf6 −/− Mice

The *Fgf6* gene has a predominant expression in muscle. We have studied muscle regeneration in *Fgf6* −/− mutant mice. No apparent defect could be evidenced (14; this work). This is in contrast to the study reported by Floss and co-workers (15) in which *Fgf6* −/− mice showed a regeneration defect with fibrosis and myotube degeneration. At least three phenomena could explain this difference.

First, discrepancies can be related to the use of different targeting constructs. This is not the case since in both studies the targeting strategy is similar.

Second, the type of degeneration test could introduce a possible bias (18). Floss *et al.* (15) used the freeze-crush injury method. We studied degeneration/

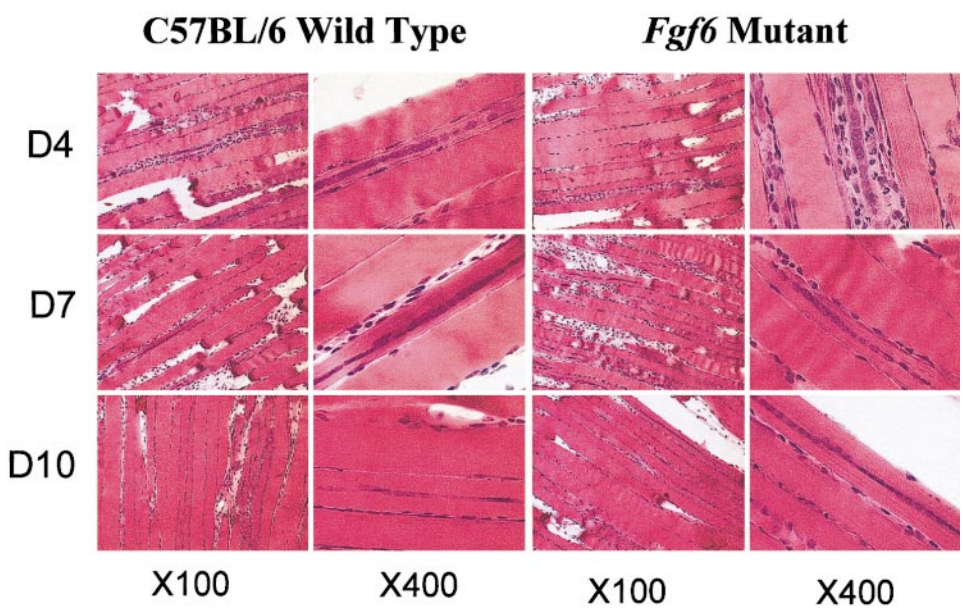

FIG. 3. Absence of differences in the kinetics of muscle regeneration in *Fgf6* −/− mice. Longitudinal sections from tibialis anterior (TA) muscle from 8-week-old *Fgf6* −/− inbred mouse mutant or C57BL/6 wild type controls. TA muscle was crush-injured and studied at different stages of regeneration (days 4, 7 and 10). No difference in regeneration is observed. Hematoxylin–eosin staining, magnification ×100, ×400.

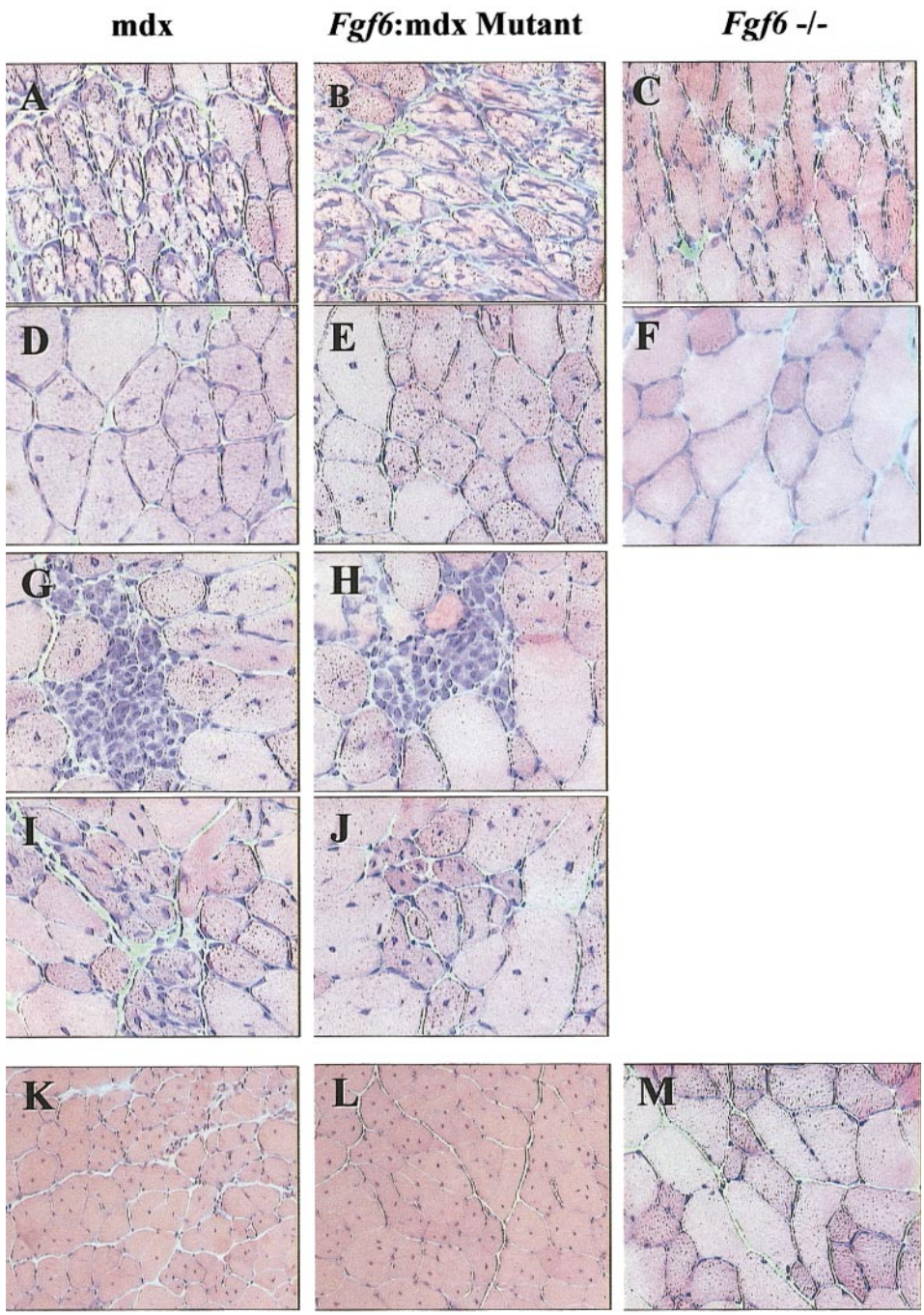

FIG. 4. Comparative analysis of skeletal muscle of *mdx*, *Fgf6* −/−:*mdx* and *Fgf* −/− mouse. Transverse sections from 12-week-old (A–J), 14-month-old (K, L) and 24-month-old animals (M). Hematoxylin–eosin stained sections of: diaphragm (A–C), tibialis anterior (D, E, K–M), TA necrosis foci (F, G); musculus erector tronci (H, I). No variation in fiber size, amount of necrosis and fibrosis is seen in the double mutant (B, E, G, I). No difference is observed in 14-month-old double mutant muscle (L). Note the presence of centronucleation in *mdx* and *Fgf6* −/−:*mdx* mice, and the absence of fibrosis in old *Fgf6* −/:*mdx* and *Fgf6* −/− mice.

regeneration by using two different tests: injection of the notexin drug and crush injury. The results were identical in both assays and there was no delay in the kinetics of regeneration of *Fgf6* −/− injured muscle.

Third, to eliminate a possible bias due to variations in the kinetics of regeneration of different mouse genetic background, we derived *Fgf6* −/− mutant mice on a standard inbred C57BL/6 background. Indeed, differences in the gravity of phenotypes due to the influence of genetic background have been observed in several instances of gene targeting. For example, it has been well established that the genetic background in-

fluences the phenotype of *Egfr* −/− (19, 20) and *p130* −/− mice (21). Since muscle regeneration is also influenced by the genetic background (22, 23), it was important to proceed to a rigorous comparison of *Fgf6* −/− mutants and wild type mice in the same genetic C57BL/6 background. To date, we cannot explain the differences between our study and the one conducted by Floss and coworkers (15).

To extend our observations, additional experiments were carried out.

First, degeneration tests were done on different muscles, including the maxillary muscle; in rodents, this muscle is in prolonged activity and its function should be more sensitive to genetic defects and stress. No delay in muscle regeneration was observed with this muscle. Second, we bred *Fgf6* −/− with *mdx* mice. The *mdx* mice have a defect in dystrophin; their muscles show continuous degeneration/regeneration cycles and the myofibers are all centronucleated as early as a few weeks after birth. The *mdx* mouse is therefore a spontaneously occurring degeneration model, in which chronic muscle degeneration is independent of exterior manipulations. We did not observe any aggravation of the *mdx* phenotype in the *Fgf6* −/−:*mdx* mutants. Finally, the observation of *Fgf6* −/− mice at old age did not show any particular defect (see Fig. 4M).

Thus, the observation of muscle and muscle regeneration in *Fgf6* −/− mice did not give us a clue to the function of FGF6.

What Could Be the Role of FGF6?

At least two potential explanations may account for our failure to observe any defect in muscle regeneration of *Fgf6* −/− mice.

First, FGF6 may play a role in muscle regeneration but in our assay its absence is compensated by other factors. The absence of a detectable abnormal phenotype in a mouse mutant generated by gene targeting is often attributed to redundancy. FGF4 is potentially redundant to FGF6; it is the closest relative of FGF6 (70% identity over the FGF "core" region) (6), it binds to the same forms of FGFRs (24), and is coexpressed with FGF6 in embryonic muscle (25). We have bred *Fgf6* −/− with *Fgf4* +/− mice and we have not observed any variation in the kinetics of regeneration of *Fgf6* −/−:*Fgf4* +/− mice, as compared to *Fgf6* −/−. Thus, either FGF4 does not compensate for FGF6, or a single wild-type FGF4 allele is sufficient for compensation (embryonic lethality precluded testing *Fgf6* −/−:*Fgf4* −/− animals). Several other FGFs are expressed in muscle and may play a role in compensation (7). It shall be important to breed *Fgf6* −/− mice with mice deficient in other *Fgf* genes as they become available for analysis.

Second, in contrast to some other FGFs that stimulate satellite cell proliferation (7), including in *mdx* mice (30), FGF6 may not be directly involved in muscle regeneration, but in other processes associated with muscle development and function. Several such processes may require the activation of a tyrosine kinase receptor via the binding of FGF6 (31). For example, the assembly and function of the gigantic plasmalemnal-spanning dystrophin-glycoprotein complex, crucial for muscle integrity and function (4), may be regulated by phosphorylation. It shall be important in the future, to determine if this macromolecular assembly is regulated by FGFs and FGFRs. Potential muscle-based gene therapy (32) could benefit from this information.

ACKNOWLEDGMENTS

We are grateful to M. Goldfarb (NYU) for the gift of the *Fgf4* mutant mice, and F. Coulier (U.119), O. deLapeyrière (U.382), and C. Mawas (U119) for their advice and encouragement. This work was supported by Inserm and Institut Paoli-calmettes.

REFERENCES

1. Allbrook, D. (1981) Skeletal muscle regeneration. *Muscle Nerve* **4,** 234–245.
2. Chambers, R. L., and McDermott, J. C. (1996) Molecular basis of skeletal muscle regeneration. *Can. J. Appl. Physiol.* **21,** 155–184.
3. Anderson, J. (1998) Studies of the dynamics of skeletal muscle regeneration: The mouse came back. *Biochem. Cell Biol.* **76,** 13–26.
4. Culligan, K. G., Mackey, A. J., Finn, D. M., Maguire, P. B., and Ohlendieck, K. (1998) Role of dystrophin isoforms and associated proteins in muscular dystrophy. *Int. J. Mol. Med.* **2,** 639–648.
5. Goldfarb, M. (1996) Functions of Fibroblast Growth Factors in vertebrate development. *Cytokine Growth Factor Rev.* **7,** 311–325.
6. Coulier, F., Pontarotti, P., Roubin, R., Hartung, H., Goldfarb, M., and Birnbaum, D. (1997) Of worms and men: An evolutionary perspective on the fibroblast growth factor (FGF) and FGF receptor families. *J. Mol. Evol.* **44,** 43–56.
7. Olwin, B., Hannon, K., and Kudla, A. (1994) Are fibroblast growth factors regulators of myogenesis *in vivo*? *Prog. Growth Factor Res.* **5,** 145–158.
8. deLapeyrière, O., Rosnet, O., Benharroch, D., Raybaud, F., Marchetto, S., Planche, J., Galland, F., Mattei, M. G., Copeland, N., Jenkins, N., Coulier, F., and Birnbaum, D. (1990) Structure, chromosome mapping and expression of the murine *Fgf6* gene. *Oncogene* **5,** 823–831.
9. deLapeyrière, O., Ollendorff, V., Planche, J., Ott, M. O., Pizette, S., Coulier, F., and Birnbaum, D. (1993) Expression of the FGF6 gene is restricted to developing skeletal muscle in the mouse embryo. *Development* **118,** 601–611.
10. Han, J. K., and Martin, G. R. (1993) Embryonic expression of *Fgf-6* is restricted to the skeletal muscle lineage. *Dev. Biol.* **158,** 549–554.
11. Coulier, F., Pizette, S., Ollendorff, V., deLapeyrière, O., and Birnbaum, D. (1994) The human and mouse fibroblast growth factor 6 (FGF6) genes and their products: Possible implication in muscle development. *Prog. Growth Factor Res.* **5,** 1–14.
12. Pizette, S., Coulier, F., Birnbaum, D., and deLapeyrière, O. (1996) FGF6 modulates the expression of fibroblast growth factor receptors and myogenic genes in muscle cells. *Exp. Cell Res.* **224,** 143–151.

13. Rescan, P. Y. (1998) Identification of a fibroblast growth factor 6 (FGF6) gene in a non-mammalian vertebrate: Continuous expression of FGF6 accompanies muscle fiber hyperplasia. *Biochim. Biophys. Acta* **1443,** 305–314.
14. Fiore, F., Planche, J., Gibier, P., Sebille, A., deLapeyrière, O., and Birnbaum, D. (1997) Apparent normal phenotype of *Fgf6* −/− mice. *Int. J. Dev. Biol.* **41,** 639–642.
15. Floss, T., Arnold, H. H., and Braun, T. (1997) A role for FGF-6 in skeletal muscle regeneration. *Genes Dev.* **11,** 2040–2051.
16. Amalfitano, A., and Chamberlain, J. S. (1996) The mdx-amplification-resistant mutation system assay, a simple and rapid polymerase chain reaction-based detection of the mdx allele. *Muscle Nerve* **19,** 1549–1553.
17. Feldmann, B., Pouymeirou, W., Papaioannou, V. E., DeChiara, T., and Goldfarb, M. (1995) Requirement of FGF-4 for post-implantation mouse development. *Science* **267,** 246–249.
18. Lefaucheur, J. P., and Sébille, A. (1995) The cellular events of injured muscle regeneration depend on the nature of the injury. *Neuromusc. Disord.* **6,** 501–509.
19. Sibilia, M., and Wagner, E. F. (1995) Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science* **269,** 234–238.
20. Threadgill, D. W., Dlugosz, A. A., Hansen, L. A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R. C., Barnard, J. A., Yuspa, S. H., Coffey, R. J., and Magnuson, T. (1995) Targeted disruption of mouse EGF-receptor: Effect of genetic background on mutant mice. *Science* **125,** 230–234.
21. LeCouter, J. E., Kablar, B., Whyte, P. F. M., Ying, C., and Rudnicki, M. A. (1998) Strain-dependent embryonic lethality in mice lacking the retinoblastoma-related p130 gene. *Development* **125,** 4669–4679.
22. Grounds, M. (1987) Phagocytosis of necrotic muscle in muscle isografts is influenced by the strain, age, and sex of host mice. *J. Pathol.* **153,** 71–82.
23. Mitchell, C. A., McGeachie, J. K., and Grounds, M. D. (1992) Cellular differences in the regeneration of murine skeletal muscle: A quantitative histological study in SJL/J and BALB/c mice. *Cell Tissue Res.* **269,** 159–160.
24. Ornitz, D. M., Xu, J., Colvin, J. S., MxEwen, D. G., MacArthur, C. A., Coulier, F., Gao, G., and Goldfarb, M. (1996) Receptor specificity of the fibroblast growth factor family. *Proc. Natl. Acad. Sci. USA* **271,** 15292–15297.
25. Drucker, B., and Goldfarb, M. (1993) Murine FGF-4 gene expression is spatially restricted within embryonic skeletal muscle and other tissues. *Mech. Dev.* **40,** 155–163.
26. Guthridge, M., Wilson, M., Cowling, J., Bertolini, J., and Hearn, M. (1992) The role of basic fibroblast growth factor in skeletal muscle regeneration. *Growth Factors* **6,** 53–63.
27. Templeton, T., and Hauschka, S. (1992) FGF-mediated aspects of skeletal muscle growth and differentiation are controlled by a high affinity receptor, FGFR1. *Dev. Biol.* **154,** 169–181.
28. Johnson, S., and Allen, R. (1995) Activation of skeletal muscle satellite cells and the role of fibroblast growth factor receptors. *Exp. Cell Res.* **219,** 449–453.
29. Yablonka-Reuveni, Z., Seger, R., and Rivera, A. J. (1999) Fibroblast growth factor promotes recruitment of skeletal muscle satellite cells in young and old rats. *J. Histochem. Cytochem.* **47,** 23–42.
30. DiMario, J., and Strohman, R. C. (1988) Satellite cells from dystrophic (mdx) mouse muscle are stimulated by fibroblast growth factor in vitro. *Differentiation* **39,** 42–49.
31. McNeil, P. L. (1993) Cellular and molecular adaptations to injurious mechanical stress. *Trends Cell Biol.* **3,** 302–307.
32. Svensson, E., Tripathy, S., and Leiden, J. (1996) Muscle-based gene therapy: Realistic possibilities for the future. *Mol. Med. Today* **2,** 166–172.