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Short Contribution

Apparent normal phenotype of *Fgf6-/-* mice

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ABSTRACT To study the role of the sixth member of the FGF (fibroblast growth factor) family whose expression is restricted to skeletal muscle, we have derived mouse mutants with a homozygous disruption of the *Fgf6* gene. The animals are viable, fertile and apparently normal, indicating that FGF6 is not required for vital functions in the laboratory mouse.

KEY WORDS: *development, fibroblast growth factor, muscle, homologous recombination*

In mammals, the FGF (fibroblast growth factor) family comprises more than a dozen peptide regulatory factors (Smallwood *et al.*, 1996; Coulier *et al.*, 1997; Verdier *et al.*, 1997). They are involved in various biological processes during development and adult life, including formation of mesoderm during gastrulation, integration of growth and patterning during early post-implantation, and formation of tissues and organs, such as brain, ear, limb, hair and skeletal system. Their role, still not completely understood, is being established through the study of patterns of expression, gene invalidations, physiological experiments using application of beads soaked with recombinant proteins, and analysis of human hereditary skeletal disorders in which mutations in three of their four high affinity receptors have been found (Wilkie *et al.*, 1995; Yamaguchi and Rossant, 1995; Goldfarb, 1996).

The expression of FGF6 in tissue is restricted to developing and adult skeletal muscle (deLapeyrière *et al.*, 1993; Han and Martin, 1993; Coulier *et al.*, 1994). It may be an important component of signaling events associated with the somite (Grass *et al.*, 1996). Its expression in adult skeletal muscle fibers, which also express FGF receptors, suggests that it may also participate in muscle maintenance and regeneration (deLapeyrière *et al.*, 1991; Pizette *et al.*, 1996). To better understand the importance of FGF6 for skeletal muscle physiology, we generated mice with a homozygous disruption of the *Fgf6* gene.

Like most mammalian *Fgf* genes, *Fgf6* is composed of three exons. A replacement vector was constructed (Fig. 1a) resulting in the disruption of the *Fgf6* gene and in the inactivation of the FGF6 protein, as the reading frame is interrupted in the first exon. The *lacZ* gene was inserted downstream of the *Fgf6* promoter region to allow for visualization of *Fgf6* gene expression.

The linearized vector was introduced in ES cells and a double drug selection in the presence of G418 and ganciclovir drugs was applied. Double resistant clones were obtained and their DNA was analyzed by Southern blot hybridization in search for the recombination event using appropriate *Fgf6* (Fig. 1b) and *LacZ* (not shown) probes. Three clones (3, 4 and 26) were selected for further experiments, and injected into 3.5-day-old blastocysts. Injected blastocysts were transferred to pseudopregnant foster mothers. Chimeric animals were obtained with clone 26, and the males were bred with C57BL/6 females. As checked by Southern blot hybridization of genomic DNA from 3-week-old mice, *Fgf6+/-* heterozygous mice were obtained (Fig. 1c). They had an apparent normal phenotype.

Heterozygous males and females were mated. The genotype of 42 F2 mice was analyzed by Southern blot hybridization (Fig. 1c) and found to follow a Mendelian distribution. Reverse-transcribed RNA from embryos was analyzed after polymerase chain reaction with *Fgf6* specific oligonucleotide primers. No expression of *Fgf6* could be found in *Fgf6* -/- embryos (Fig. 1d) The targeted allele was transmitted through the germline with no variations in the expected Mendelian frequency. However, in some litters, an unexplained bias toward males was observed. The shape, size and body weight of males and females *Fgf6* -/- did not differ statistically from those of the *Fgf6* +/+ littermates. Hematoxylin-eosin stained sections of adult skeletal muscles showed no obvious histological abnormality. No difference was found in the skeletons of newborn *Fgf6* -/- and wild-type mice stained with Alizarin red and Alcian blue.

Fgf6 -/- embryos developed normally. X-gal specific staining of embryos (a total of ten *Fgf6-/-* and five *Fgf6+/-* litters) showed a muscle specific pattern of *Fgf6* gene expression. On day 10.5 (E10.5) post-conceptus, it was found in the myotomal compartment of the somites (Fig. 2a) but not in the limb buds. The same pattern was observed at E11.5 and E12.5. At E12.5, *Fgf6* expression was also detected in the myotomes that expand ventrolaterally at the level of the trunk (Fig. 2a,b). By E13.5, the signal became more extensive and appeared in the developing muscle of the limb

Abbreviations used in this paper: ES cells, embryonic stem cells; FGF, fibroblast growth factor; PBS, phosphate buffer saline.

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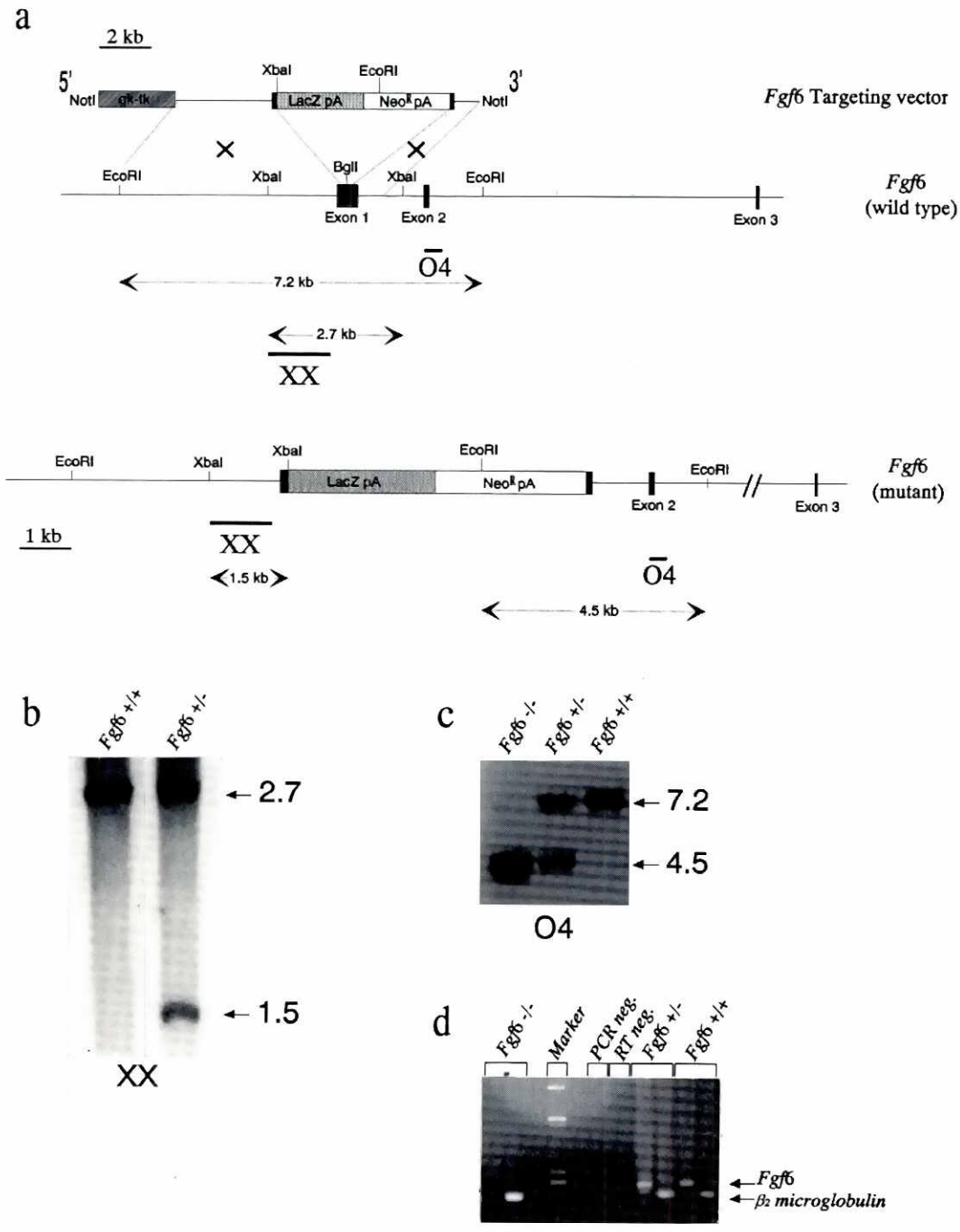

Fig. 1. Generation of FGF6-deficient mice by homologous recombination. (a) *Schematic organization of the mouse Fgf6 gene in between the targeting vector and the targeted allele. The targeting construct was generated by inserting a LacZ-Neo cassette into the first exon of Fgf6 (pA, polyadenylation signal). EcoRI and XbaI sites, the localization of the O4 and XX probes used to detect the rearrangement by Southern blot hybridization, and the size of the expected genomic fragments, are indicated.* **(b)** *Southern blot hybridization of XbaI-digested DNA from ES cells (clone 26) using the XX probe.* **(c)** *Southern blot analysis of EcoRI-digested DNA from wild-type (+/+), heterozygote (+/-) and mutant (-/-) mice using the O4 probe. DNA extracted from cells or tails was digested, electrophoresed in agarose gels, blotted onto nitrocellulose membranes and hybridized with ^{32}P radiolabeled XX or O4 genomic probes. The size of the positive bands (with correspondence in panel a) is indicated on the right.* **(d)** *Reverse-transcribed RNA from wild-type (+/+), heterozygote (+/-) and mutant (-/-) E14.5 embryos was amplified by PCR using oligonucleotide primers for Fgf6 and for β2 microglobulin. Marker is HaeIII fragments of phage φX174.*

(not shown). At later stages, *Fgf6* was widely expressed in the muscle masses (Fig. 2d). Heterozygous and homozygous mutants exhibited the same patterns of expression (not shown), suggesting that inactivation of the *Fgf6* gene did not have any severe effect upon muscle cells expressing this gene. In E11.5 myotomes, *Fgf6* signal, as described for other *Fgf*, was confined to longitudinally oriented myotomal cells (Fig. 2e), and was not present in the population of rounded cells. X-gal staining was found later on in the myotubes, with a stronger signal at the periphery of the muscle masses (Fig. 2f).

This exquisite pattern strengthened our belief that FGF6 may play an important role in muscle physiology. We thus studied the regeneration of *Fgf6* -/- muscles following a toxic injury. Five- to six-week-old wild-type or *Fgf6* -/- mice were injected with PBS or notexin in the hind limb muscle *tibialis anterior*. Notexin induces a rapid myofiber degeneration with conservation of capillaries and satellite cells. The course of the regeneration in normal mice is well known: the first myotubes appear at day 3 after injection, and regeneration is completed by day 10. Regenerated myofibers are characterized by their centronuclearization. In four experiments,

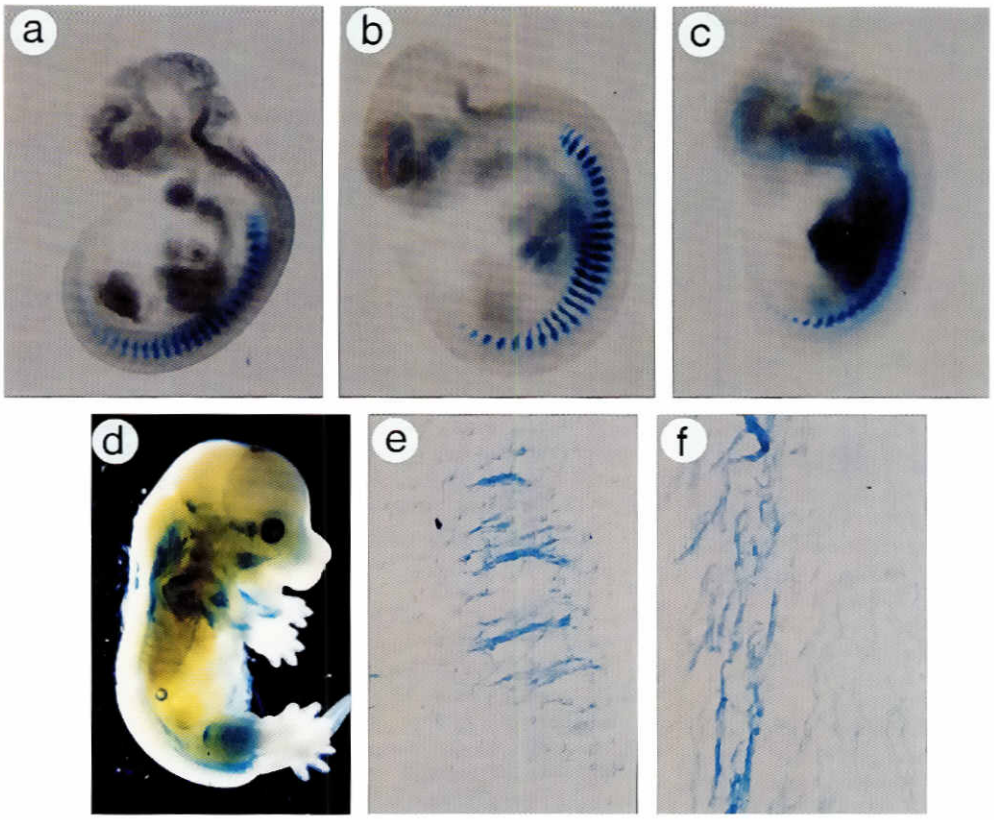

Fig. 2. Expression of *Fgf6* in *Fgf6* -/- embryos as followed by expression of *LacZ* gene and X-gal staining. *Fgf6 -/- embryos at day 10.5* **(a)**, *11.5* **(b)**, *12.5* **(c)** *and 15.5* **(d)** *post conceptus show Fgf6- specific X-gal staining in somites and developing muscles. Details of Fgf6 expression in myotome at E11.5 and dorsal muscle at E15.5 are shown in* **(e)** *and* **(f)**, *respectively.*

these characteristics were observed in all the muscles injected with notexin (not shown): either from controls (36 muscles), heterozygotes (15 muscles), or homozygotes (36 muscles). Differences in the kinetics of regeneration were observed between different strains of mice (Swiss, C3H, Balb/c, 129 and C57Bl6), but none could be attributed to *Fgf6* mutation. Thus, by using this type of conditions to induce muscle degeneration, no role of FGF6 could be demonstrated in muscle regeneration.

In conclusion, *Fgf6 -/-* mice are viable, develop normally, can reproduce and have morphologically normal muscle masses. FGF6 is not required for vital functions. Furthermore, there is apparently no gross abnormalities in the physiology of adult muscles. Therefore, a complete functional redundancy with other molecules, most likely other FGF, exists, both in the developing embryo and in the adult. This may not be surprising since several FGFs are thought to be active in skeletal muscle biology (Olwin *et al.*, 1994; Chambers and McDermott, 1996).

So far, gene invalidations of *Fgf* genes have not provided extensive information about their role in development. Aside from the knock-out of *Fgf4* that is embryonic lethal (Feldman *et al.*, 1995), they have led to animals with overall mild phenotypes (Mansour *et al.*, 1993; Hebert *et al.*, 1994; Guo *et al.*, 1996). Even in the case of FGF2, which is ubiquitous and has a large spectrum of activities, the null mutant is not severely affected (R. Zeller, personal communication). Thus, in the mammalian FGF family, functional redundancy is developed, and the absence of macroscopic phenotype of *Fgf6 -/-* mice is therefore not surprising. The availability of the *Fgf6 -/-* mice and the other *Fgf* mutants will allow interbreeding and will result in a wealth of information about the relative functional redundancy of distinct FGFs to provide a first complete picture of the interrelationships between members of a family of growth factors.

Experimental Procedures

Construction of the gene targeting vector

The mouse *Fgf6* sequence was isolated by screening a mouse cosmid library constructed by inserting *Eco*RI-digested, 35-45 kb-sized DNA from the 129/Sv mouse strain into the pHC79 vector (Boehringer Mannheim). A positive cosmid (CosES16) containing the whole *Fgf6* locus was isolated and used for the construction after restriction enzyme mapping. To disrupt *Fgf6*, 4 kb DNA from the 5' end and 1.2 kb from the 3' end were inserted into the targeting vector. The vector was pGNA (P. Brûlet, Institut Pasteur, Paris). Exon 1 of the *Fgf6* gene was disrupted by a *lacZ* reporter gene and a neomycin resistance cassette (neo^R) driven by the SV40 promoter. To interrupt FGF6 translation, the sequence corresponding to ATG codons and signal peptide was deleted. A viral thymidine kinase (tk) gene driven by the phosphoglycerate kinase promoter (gk) is present at the 5' end of the construct. The targeting vector was linearized by *Not*I enzyme, and electroporated in ES cells

Electroporation and selection of ES cells

E.14.1 ES cells (R. Murray, DNAX, CA) derived from mouse strain 129/ola were maintained on a monolayer of mitomycin C-inactivated, neomycin-resistant, fibroblast feeder cells. Twenty micrograms of *Not*I-linearized targeting vector were electroporated into a suspension of trypsinized cells (10^6) in cold phosphate-buffered saline (PBS) using a Bio-Rad gene pulser (240 V and 960 μF). Cells were then incubated at room temperature for 5 min, plated, and allowed to recover for 24 h before selection in medium containing G418 (300 μg/ml) and ganciclovir (2 μM). Cells were fed daily and, after 8 days, the resulting double-resistant ES clones were individually picked and transferred to 96-well plates with feeders. On the following day, each clone was trypsinized and divided in half. One half was frozen (-80°C) whereas the other half was plated into a 24-well plate without feeder cells and used to prepare DNA.

Analysis of targeted ES cells and animals

Cells in 24-well plates were washed in PBS, lysed in 350 μl lysis buffer containing 10 mM Tris-Hcl (pH 7.5), 10 mM NaCl, 10 mM EDTA, 0.5% Sarcosyl and 1 mg/ml proteinase K. The plates were incubated for 8 h at 60°C in a wet atmosphere. An equal volume of isopropanol was then added, and DNA was allowed to precipitate by gentle agitation. The supernatant was discarded and the DNA washed with 70% ethanol, air-dried, and the pellet resuspended in distilled water. DNA (10 μg) was digested in 50 μl of a restriction enzyme mixture (1x restriction buffer, 50 μg/ml of RNase-A,

and 20 units of *Eco*RI) for 5 h at 37°C, run on agarose gels and analyzed by Southern blot hybridization.

Reverse-transcribed RNA from wild-type, heterozygote and mutant E14.5 embryos were amplified by 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. For β2 microglobulin primers are described in Pizette *et al.* (1996).

Generation of chimeras

Targeted ES cells (clones 3, 4 and 26) were injected in C57BL/6 blastocysts. All injected blastocysts were transferred to pseudopregnant Swiss recipients. Chimeric males were crossed with C57BL/6 females, and tail biopsies from Agouti pups were genotyped by Southern blot analysis for transmission of the targeted allele.

Analysis of β-galactosidase gene expression

For detection of reporter gene expression in whole-mount preparations, embryos were removed from uteri of pregnant females, dissected free of maternal decidual tissue, rinsed twice with PBS and fixed for 20 min at 4°C in PBS containing 1% formaldehyde. After several washes in PBS, the embryos were incubated in PBS containing 5 mM $K_4Fe(CN)_6$ $3H_2O$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 0.02% Nodinet-P40, 0.01% sodium deoxycholate and 1 mg/ml 4-chloro-5-bromo-3-indolyl-β-galactoside at 30°C until color developed. Embryos were photographed on a Leica MZ6 microscope.

Muscle degeneration and histology

Six-week-old FGF6 deficient males, and control Swiss males, all weighing approximately 20 g at the time of muscle injury, were used for this study. Injury was induced by toxic muscle necrosis with notexin (Lefaucheur and Sebille, 1995). Toxicity of this drug, isolated from the venom of the Australian tiger snake, is based on phospholipase 2 activity. For this purpose, we exposed the *tibialis anterior* through a small incision and injected 10 μl of notexin (50 μg/ml), after which the wound was sutured. For this procedure we used a 10 μl microsyringe (Hamilton) and a $30G^{1/2}$ needle. Samples from the notexin-induced muscle necrosis were collected for morphology at 2, 4, 7, 10 and 22 days after injection of the toxin; wild-type control mice were processed similarly. The whole *tibialis anterior* was removed and fixed overnight in 4% paraformaldehyde/PBS buffer. For morphology, the fixed muscle samples were processed for paraffin embedding. Transverse sections were cut at 7 μm, dewaxed in xylene, rehydrated through an ethanol series, and stained with hematoxylin and eosin for structural analysis.

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