Expression of the Fgf6 gene is restricted to developing skeletal muscle in the mouse embryo

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SUMMARY

Fgf6, a member of the Fibroblast Growth Factor (FGF) family, is developmentally regulated and its expression is highly restricted in the adult. To gain further insight into the role of Fgf6, we studied its expression during embryogenesis using RNA in situ hybridization. Fgf6 expression is restricted to developing skeletal muscle. Fgf6 transcripts are first detected in the somites at 9.5 days post-conceptus, and expression continues in developing skeletal muscles up to at least 16.5 days post-conceptus. Fgfr4 is a putative receptor for FGF6. Its pattern of expression during myogenesis overlaps that of Fgf6, but both genes are not expressed in exactly the same population of cells. In addition, recombinant FGF6 protein is able to repress the terminal differentiation of myoblasts in culture, providing additional support to the concept that FGF6 plays an important role in myogenesis.

Key words: fibroblast growth factor, Fgf6, Fgfr4, mouse embryogenesis, myogenesis, skeletal muscle

INTRODUCTION

Intercellular communication plays a crucial role in embryonic development. Among a variety of signaling molecules are the fibroblast growth factors (FGF). They represent a family of structurally related mitogenic proteins (reviewed by Benharroch and Birnbaum, 1990; Goldfarb, 1990). In mammals, seven members of the FGF family are known at present, including aFGF/FGF1, bFGF/FGF2, INT2/FGF3, HST/K-FGF/FGF4, FGF5, FGF6 and KGF/FGF7. FGFs play important roles in various processes including development. They are involved in mesoderm induction and early embryonic pattern formation (Kimelman and Kirschner, 1987; Slack et al., 1987), as well as in later stages of embryonic development. FGF1 and FGF2 are broadly distributed in the embryo (Gonzalez et al., 1990; Fu et al., 1991) while the Fgβ3, Fgβ4 and Fgβ5 genes are expressed during gastrulation and later in embryogenesis (Wilkinson et al., 1988, 1989; Haub and Goldfarb, 1991; Hébert et al., 1991; Niswander and Martin, 1992).

FGFs may be particularly important in some specific developmental processes such as myoblast differentiation. In agreement with this function, FGF1, FGF2, FGF4 and FGF5 have been shown to influence the differentiation of cultured myoblasts (Lathrop et al., 1985; Clegg et al., 1987; Seed and Hauschka, 1988; Olwin and Rapraeger, 1992; Goldfarb, personal communication). In addition, the corresponding genes are expressed during myogenesis (Gonzalez et al., 1990; Fu et al., 1991; Haub and Goldfarb, 1991; Niswander and Martin, 1992).

The human FGF6 gene was cloned by low stringency hybridization to an FGF4 probe (Marics et al., 1989). We have previously reported the presence of murine Fgf6 transcripts in adult muscles and during embryogenesis (deLapeyrière et al., 1990). In order to define better the pattern of expression of Fgf6, we have now performed in situ hybridization on embryo sections. This has revealed a striking tissue-specific and spatially regulated pattern of expression during mouse development. Fgf6 RNA specifically accumulated in skeletal muscles.

Understanding the developmental role of the FGF6 growth factor also requires precise knowledge of the distribution of its cognate receptor. Four high affinity FGF receptor (FGFR) genes belonging to the family of receptor-type tyrosine kinases have been cloned (Givol and Yayon, 1992, for review). Of the patterns of Fgfr expression that have been described (Orr-Urtreger et al., 1991; Peters et al., 1992; Stark et al., 1991; Yamaguchi et al., 1992), only that of Fgfr4 appears to overlap the distribution of Fgf6 transcripts. Furthermore, FGF6 is able to displace high affinity FGF1 binding to FGR4 (Vainikka et al., 1992). Thus, FGFR4 may constitute a putative receptor for FGF6. With this in mind, we have performed a direct comparison of the expression of Fgf6 and Fgfr4 in muscle masses during embryogenesis.

Our data suggest that the Fgf6 gene may play an impor-
tant role in muscle development. This role is discussed in
regard to the in vitro capability of the FGF6 protein to
repress the terminal differentiation of myoblast cells.

MATERIALS AND METHODS

Probes
Radiolabelled antisense probes were generated after linearization
of plasmid DNA by restriction enzyme digestion and in vitro tran-
scription, in the presence of 35 S-labelled UTP, in standard reac-
tions (Melton et al., 1984) using SP6, T3 or T7 RNA polymerase,
depending upon the vector DNA template used. A 434 bp EcoRV
fragment of the ‘dream’ plasmid containing the 3′ untranslated
part of Fgf6 cDNA (Ollendorff et al., 1992), was used as a probe
for Fgf6. A plasmid containing 120 bp of the 5′ non-coding
sequence of the murine α-cardiac actin gene (Sassoon et al., 1988)
and one containing 583 bp of the 3′ untranslated region of the
Fgf6 cDNA (Stark et al., 1991), were used as probes for com-
parative studies along with Fgf6.

Embryos
Embryos were obtained from C3H/He inbred mice. Noon of the
day on which the vaginal plug was detected was considered to be
0.5 day of gestation. Embryos were dissected from pregnant mice,
fixed in 4% buffered paraformaldehyde at 4°C and embedded in
paraffin wax.

In situ hybridization
Serial microtome sections (6 µm) were deparaffinized and
hybridized with 50,000-75,000 cpm/µl 35 S-labelled sense or anti-
sense cRNA probes, according to the protocol described by
Wilkinson et al. (1987), with minor modifications by Lyons et al.
(1990). After the washes, the slides were dipped into Kodak NTB-
2 nuclear track emulsion diluted 1:1 and autoradiographed (for 4-
5 weeks for Fgf6 and 1-2 weeks for the other probes). After pho-
tographic development, the slides were stained with toluidine blue
and analyzed using both bright- and dark-field optics of a Zeiss
Axiophot and a Nikon AXA microscopes.

Cells and culture conditions
C2 mouse muscle cells (clone C2.7) were obtained from C. Pinset
and D. Montarras (Institut Pasteur, Paris), and grown as myoblasts
at subconfluent density, in a medium made of 50% MCDB 202 -
50% Dulbecco’s modified medium (DME), supplemented with
20% FCS as described (Pinset et al., 1988). To test the activity
of FGF6 and FGF2, cells were plated at 100-200 cells per 90 mm
diameter culture dish in the presence of either 10 or 100 ng/ml
recombinant protein with or without 0.1 µg/ml heparin (Sigma).
Proteins were added every other day. After 8 days in culture, cells
were induced to differentiate in DME containing 1% FCS, and 10
µg/ml human insulin (Novo Nordisk Pharmaceutique). 48 or 72
hours later, cells were fixed. The number of differentiated colonies
was estimated by morphological observation and by counting the
number of troponin-T-positive colonies in a standard immunoflu-
orescence procedure using a monoclonal antibody against troponin
T (Amersham).

FGF2, produced by recombinant DNA technology, was a gift
from H. Prats (Laboratoire d’Endocrinologie Expérimentale,
Toulouse). Recombinant FGF6 was produced as previously
described (Pizette et al., 1991).

RNA isolation and northern analysis
RNAs were extracted by the guanidine isothyocyanate-caesium
chloride method from subconfluent C2 myoblasts and from C2
myotubes after 3 days in the differentiating medium. Equal
amounts of total RNAs, as assessed by ethidium bromide stain-
ing, were fractionated on 1% agarose/10% formaldehyde gels and
transferred onto nylon membranes (Nytran, Schleicher and
Schuell). The same fragments as for in situ hybridization were
used as probes after oligolabelling. The blot was first hybridized
with an Fgfr4 fragment at 68°C for 20 hours, according to the
method of Stewart and Walker (1989). The membrane was then
rehybridized with an α-cardiac actin probe to control for the myo-
genic differentiation.

RESULTS

In situ analysis of the fetal expression of FGF6
In order to localize Fgf6 expression during embryogenesis, sections
of prefixed mouse embryos at various postimplantation
stages of development were hybridized in situ to
either antisense or sense Fgf6 probes. No signal above
background was ever observed at any time using the sense probe
(data not shown). No Fgf6 signal was detected at stages
between 6.5 and 8.5 days post-conceptus (d.p.c.). Embry-
onic Fgf6 expression first occurred at 9.5 d.p.c. which is an
early stage of differentiation of the myotomes (Ott and
Buckingham, 1992) The Fgf6 signal appeared in the
myotomal compartment of the somites (Fig. 1A-D). The
dermatome and the sclerotome were not labelled in any of
the embryos examined. At 11.5 d.p.c., Fgf6 transcripts were
still abundant in the myotomes of the somites of the trunk
(Fig. 1E) and in those near the hindlimb bud; the latter was
negative (Fig. 1F). At 12.5 d.p.c., transcripts for Fgf6 were
detected in the somites of the tail (data not shown). At this
stage, Fgf6 mRNA was present in other regions as well, in
particular in the developing skeletal muscles of the neck
(Fig. 2A). However, Fgf6 transcripts were not detected in
all the areas where α-cardiac actin mRNA was present (Fig.
2A,B, and data not shown).

Fgf6 expression became more extensive between 13.5
and 14.5 days of gestation. Thorough examination of 13.5
and 14.5 d.p.c. embryos hybridized to the Fgf6 probe
showed that Fgf6 expression occurred only in developing
skeletal muscles (Fig. 2). This was confirmed by hybridiza-
tion of parallel sections to the α-cardiac actin probe. Fgf6
transcripts were detected in most skeletal musculature of the
embryo: in the facial regions including the tongue, the
jaw, the pharyngeal (Fig. 2C) and extrinsic ocular muscles
(Fig. 1E) and in those near the hindlimb bud; the latter was
negative (Fig. 1F). At 12.5 d.p.c., transcripts for Fgf6 were
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(Fig. 2C); in the neck region (Fig. 2C); in the trunk region
including the muscles surrounding the vertebral column, the
ribs, the shoulder (Fig. 2C, D), the pelvis (not shown), the
diaphragm and the thigh (Fig. 2J), and in the forelimbs (Fig.
2D) and hindlimbs (Fig. 2F). In limbs, there was a proxi-
mal-distal gradient of Fgf6 transcripts, whereas α-cardiac
actin expression was more distal (Fig. 2D,E), in keeping
with an earlier onset of expression. No Fgf6 expression was
observed in the heart at any stage, nor in smooth muscle.

The signal obtained with the Fgf6 probe differed from
that of the α-cardiac actin in two respects. First, a few
muscle masses, positive for α-cardiac actin, were negative
for Fgf6 (Fig. 2A,B,D,E). It is possible, however, that these
masses only express a low level of Fgf6 RNA, below the
threshold of detection. Second, in many muscle masses, the
α-cardiac actin probe hybridized all over the block, whereas
the Fgf6 probe showed a strong hybridization signal at the periphery only (Figs 2F,G, 3).

15.5 d.p.c. is the stage at which the mature pattern of muscle groups is formed, neuromuscular junctions are becoming established and secondary muscle fibres begin to appear (Ontell and Kozeka, 1984; Kieny et al., 1986). At this stage, Fgf6 transcripts were present in the muscle masses (Fig. 4), along the myotubes (Fig. 4C-F). Labelling with the Fgf6 probe began to decrease from 16.5 d.p.c. (data not shown).

Comparison of the fetal expression of Fgf6 and Fgfr4

FGFR4 could be a putative receptor for FGF6. To further investigate the relationship between Fgf6 and Fgfr4 expression, parallel sections of embryos were hybridized to Fgf6 and Fgfr4 probes. Comparison of both patterns of hybridization showed that Fgfr4 RNAs were more widely distributed than Fgf6 (Fig. 5). Thus, some muscle masses, such as the fibres surrounding the tongue, were positive for Fgfr4 and negative for Fgf6 (Figs 5A-D, 6). In addition, the distribution of the positive cells was different for each probe. Thus, Fgfr4 labelling was more extensive than that of Fgf6 which seemed, in contrast, highly restricted, like α-cardiac actin, to linear arrays of cells indicative of fibres (Fig. 6). Furthermore, in a given muscle mass, Fgfr4 mRNAs were evenly distributed over the whole mass whereas Fgf6 signal was stronger at the periphery of the mass (Fig. 5E, F).

The patterns of expression of Fgf6 and Fgfr4 in developing muscle are summarized in Table 1 and compared to that of other Fgfs, Fgfr receptors and muscle specific genes. Fgf6 appears at the same time as Fgfr4 and Fgfr4, whereas the onset of MyoD1 and Fgf5 expression is one day later. Fgf6 is the only FGF, studied by in situ hybridization, found to be expressed after 14.5 d.p.c., a time where Fgfr1 is also expressed.

The terminal differentiation of C2 myoblasts is repressed by FGF6

To verify that FGF6 was indeed able to regulate skeletal muscle differentiation, we treated C2 mouse myoblasts with recombinant FGF6 protein in conditions allowing the fusion of myoblasts into myotubes (see Materials and methods). 48-72 hours later, we looked at the number of morphologically differentiated colonies and the synthesis of muscle...
Fig. 2. *Fgf6* gene expression in developing skeletal muscles. (A) Dark-field micrograph of a section through the neck region of a 12.5 d.p.c. embryo hybridized to the *Fgf6* probe. (B) Section parallel to A hybridized to the α-cardiac actin probe. Comparison of both sections shows that *Fgf6* mRNAs are expressed in the neck muscle masses but to a lesser extent than α-cardiac actin. (C) Sagittal section in part of the head and neck region of a 14.5 d.p.c. embryo hybridized to the *Fgf6* probe showing expression in skeletal muscles including tongue (t), jaw (j), neck (nm), intercostal muscles (im) and rib (r). (D) Expression of *Fgf6* in a parasagittal section through the same embryo as in C and (E) expression of α-cardiac actin in a parallel section. The *Fgf6* gene is expressed in the proximal developing muscles of the forelimb (fl) and in the shoulder muscles (sh). (F) Expression of *Fgf6* in the hindlimb of a 14.5 d.p.c. compared with (G) expression of α-cardiac actin; *Fgf6* expression is restricted to muscle masses; bone (b) is negative; (H) bright-field photograph through the eye muscles at the same stage and (I) dark-field of the same section showing *Fgf6* transcripts in extrinsic oculomotor muscles (arrowheads). (J) Parasagittal section through the thigh of a 13.5 d.p.c. embryo hybridized to the *Fgf6* probe showing a positive signal in the diaphragm (d) and thigh muscles. Bars: (A, B,F,G,J) 100 μm; (C,D,E) 300 μm; (H,I) 50 μm.
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specific troponin T. Under these culture conditions, FGF6 affected the morphology of the cells; treated cultures developed a flattened, fibroblastic morphology characteristic of exponentially growing cells (not shown) and reminiscent of the influence of FGF2 on the differentiation of myoblasts in culture. FGF2 repressed the terminal differentiation of C2 myoblasts as reported previously (Lathrop et al., 1985; Clegg et al., 1987). This result is presented for comparison with the effect obtained with FGF6 (Fig. 7). Both growth factors repressed the differentiation of C2 cells, as defined by a reduced number of differentiated colonies (Fig. 7A), as well as a low synthesis of troponin T (Fig. 7B). FGF6 activity was lower than that of FGF2 and was increased in the presence of 0.1 µg/ml heparin. Similar effects of heparin upon FGF6 and various FGF activities have been reported previously (Pizette et al., 1991; Olwin and Rapraeger, 1992).

To determine whether this effect could be mediated through FGFR4, Fgfr4 expression in C2 cells was visualized by northern blot hybridization. The result indicates that Fgfr4 is indeed expressed in C2 myoblasts and downregulated in C2 myotubes whereas, as expected, α-cardiac actin is upregulated during myogenic differentiation (Fig. 8).

DISCUSSION

Fgf6 expression is restricted to developing skeletal muscles
The observed tissue specificity of Fgf6 expression during
Fgf6 expression was visualized by northern blot hybridization in skeletal muscle and very faintly in heart tissue (deLapeyrière et al., 1990). This discrepancy about the expression in the heart could be explained by a very low expression below the threshold of detection or by a different pattern of expression between adult and embryonic tissues. Fgf6-positive cells are distributed among the developing muscle masses whatever their embryonic origin since the extra-oculomotor muscles, which derive from the pre-chordal plate, are positive like other muscles derived from the dermamyotome or the myotome (Ott and Buckingham, 1992, for review). Fgf6 gene expression is first detected in the myotomes of 9.5 d.p.c. somites when transcripts coding for early isoforms of myosin begin to accumulate (Lyons et al., 1990). It continues in most but not all developing muscles, as the myotubes develop and are organized into muscle fibres. It begins to decrease from 16.5 d.p.c. The timing of detection of Fgf6 transcripts, and the analysis at high magnification both suggest that Fgf6 is expressed in muscle fibres. However, the precision of the in situ hybridization does not permit a clear distinction between tran-
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Scripts distributed in the fibres and those in the myoblasts bordering the myotubes.

Fgf6 expression is preferentially localized at the periphery of some muscle masses. This regionalization may reflect the state of maturation of the muscle fibres and/or their future myofibre type. Another possibility is that Fgf6 expression is induced or maintained by a signal located at the periphery.

**FGFR4 could be one of the in vivo receptors for FGF6**

To elucidate the role of FGF6 in myogenesis, it is neces-
necessary to identify the cells that may be competent to respond to an FGF6 signal, and therefore to determine the distribution of its cognate receptor. Among the known FGF receptors (Givol and Yayon, 1992), FGFR4 seems a good candidate for being an in vivo FGF6 receptor. This assumption is based on two lines of evidence: in competition experiments, FGFR4 binds FGF6 with a high affinity (Vainikka et al., 1992); the pattern of Fgfr4 expression overlaps with that of Fgf6 (Table 1). Fgfr4 is mainly expressed in the definitive endoderm and in developing muscles (Stark et al., 1991). Indeed, we show here that Fgf6 and Fgfr4 are coexpressed in the same muscle masses. However, close examination of hybridizations with both probes suggests that both genes are not always expressed in the same cells. Fgfr4-positive cells are evenly scattered in a muscle mass, while Fgf6 transcripts are distributed in a nonhomogenous manner, with a strong signal over the peripheral developing myotubes or on linear arrays of cells. These results suggest that FGF6 may act through autocrine and paracrine mechanisms. Alternatively, another FGF could interact with FGFR4 in the regions where Fgf6 is not expressed. Independently or simultaneously, FGF6 could interact with another FGF receptor; one of them could be FGFR1, since, like FGF6, FGFR1 is expressed in the tongue and in late developing muscles (Table 1; Orr-Urtreger et al., 1991; Peters et al., 1992).

In vitro differentiation of C2 myoblasts into myotubes is accompanied by a downregulation of Fgfr4. Similar results have been reported about the expression of Fgfr1 (Moore et al., 1991; Templeton and Haushka, 1992).

**Developing skeletal muscle, a major site of FGFs expression during later embryogenesis**

The analysis of the in vivo distribution of other members of the FGF family shows that FGF1, FGF2, FGF4 and FGF5 are also expressed in developing muscle. In contrast to FGF1 and FGF2, which have been studied at the level of the protein, and in species other than the mouse (Joseph-Silverstein et al., 1989; Gonzalez et al., 1990; Fu et al., 1991), extensive analyses of the spatial and temporal distribution of murine Fgf4 and Fgf5 transcripts have been performed (Haub and Goldfarb, 1991; Niswander and Martin, 1992). This enables comparative profiles to be established. The patterns of expression of Fgf4 and Fgf5 during myogenesis are more restricted than that of Fgf6: first, Fgf4 and Fgf5 transcripts are restricted to certain muscle masses (M. Goldfarb, personal communication); second, Fgf6 expression continues at later stages (after day

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**Fig. 6.** Expression of the Fgf6 gene in the tongue. (A) Bright-field and (B-D) dark-field photographs of neighbouring sagittal sections through the tongue of a 14.5 d.p.c. embryo; (A,B) hybridization to the Fgf6 probe, (C) α-cardiac actin probe and (D) Fgfr4 probe. Fgf6 transcripts are not detected in all the α-cardiac actin-positive cells particularly those forming the superficial muscle layer. Fgfr4 mRNAs are widely distributed throughout the tongue, even in non α-cardiac actin-positive cells. Arrows indicate fibres positive for the α-cardiac actin and Fgfr4 probes, and negative for the Fgf6 probe. Bar, 50 μm.
experiments and standard error) are expressed as a percentage of percentages of troponin-T-positive colonies.

After the initial period of growth, cells were shifted to a presence of protein/number of differentiated colonies in the mm dishes and grown in the presence of either FGF6 or FGF2 (A: 10 or 100 ng/ml; B: 100 ng/ml) supplemented (Hep +) or not (Hep −) with 0.1 µg/ml heparin. Proteins were added every other day. After the initial period of growth, cells were shifted to a differentiation medium containing 1% FCS and 10 ng/ml insulin and tested using a standard immunofluorescence procedure with a monoclonal antibody against troponin T. Results are expressed as means of three experiments and standard error.

Fig. 7. FGF6 represses the terminal differentiation of C2 myoblasts into myotubes. C2 cells were plated at 100-200 cells/90 mm dishes and grown in the presence of either FGF6 or FGF2 (A: 10 or 100 ng/ml; B: 100 ng/ml) supplemented (Hep +) or not (Hep −) with 0.1 µg/ml heparin. Proteins were added every other day.

Fig. 8. Fgfr4 is expressed in C2 myoblasts and downregulated in myotubes. Total RNAs (10 µg) were loaded in each lanes and analyzed by northern blot hybridization. A 3.6 kb transcript corresponding to Fgfr4 is observed in subconfluent myoblasts (Mb) and decreases in myotubes (Mt). As an indicator of the differentiation stage, a 1.6 kb transcript corresponding to α-cardiac actin is faintly visible in myoblasts and increases in myotubes. The ethidium-stained ribosomal RNA gives an indication of the quantity and integrity of the loaded RNAs.

14,5) whereas Fgf4 and Fgf5 are, at that time, down regulated.

Although several FGF genes are activated during myogenesis, their different patterns of expression suggest that they play distinct roles in the differentiation process. They may also act in synergy or be redundant for crucial steps. A possible functional redundancy of key players in myogenesis is supported by recent results (Braun et al., 1992; Rudnicki et al., 1992).

What is the actual function of FGF6 in myogenesis?

In vitro properties of the recombinant FGF6 protein are in agreement with a role of FGF6 in myogenesis. Indeed, FGF6 protein represses the terminal differentiation of C2 myoblasts in culture. This property is shared with other FGFs such as FGF1, FGF2, FGF4 and FGF5 (Lathrop et al., 1985; Clegg et al., 1987; Seed and Hauschka, 1988; M. Goldfarb, personal communication). It is, however, difficult to draw an exact parallel between the in vitro and in vivo activities of FGF6, since Fgfr6 transcripts seem present both in myocytes and myotubes. We could assume that FGF6 is synthesized by myocytes, that it increases their proliferation, and delays their own program of differentiation by an autocrine mechanism. In myotubes, FGF6 may exert a different function. It could inhibit the terminal differentiation of a subset of myogenic cells and be required for the fusion of others. Alternatively, FGF6 produced by the myotubes could, by a paracrine effect, stimulate the proliferation of adjacent myoblasts before their fusion into developing muscles in order to maintain a self-renewal capacity in the proliferating myoblast population. This latter activity of FGF6 could be important in the growth of muscle masses, and could account for continuous expression of Fgfr6 during development as well as in the skeletal muscles of the adult (deLapeyrière et al., 1990).

A family of myogenic regulatory genes has been described. Members of this family belong to the helix-loop-helix (HLH) superfamily of DNA binding proteins (see Olson, 1990; Weintraub et al., 1991 and Wright, 1992 for reviews). Several lines of evidence link the FGF family to these muscle-specific transcription factors. FGF2 can repress transcription of at least two of the myogenic regulatory genes, myogenin and MyoD1 (Vaidya et al., 1989; Brunetti and Goldfine, 1990). The comparison of the expression profile of Fgfr6 with those of the myogenic regulatory genes (Table 1) shows that Fgfr6 transcripts begin to accumulate in myotomes at the same time as Myf-6, when Myf-5 is already present, and one day earlier than MyoD1 and myogenin proteins accumulate (Cusella-De Angelis et al., 1992; Ott and Buckingham, 1992 and Buckingham, 1992, for reviews). The question of whether FGF6 regulates the expression of myogenic HLH proteins or whether these proteins regulate Fgfr6 expression could be answered by the study of Fgfr6 expression in mice lacking Myf-5 or MyoD1 (Braun et al., 1992; Rudnicki et al., 1992). Identification of both the regulatory elements and the targets of FGF6 and of other proteins of this family will provide clues to better understanding of the developmental mechanisms of myogenesis.

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