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FGFR4 signaling is a necessary step in limb muscle differentiation

Irène Marics*, Françoise Padilla*, Jean-François Guillemot, Martin Scaal and Christophe Marcelle†

Developmental Biology Institute of Marseille, Laboratoire de Génétique et de Physiologie du Développement (LGPD), University Aix-Marseille II, Campus de Luminy, Case 907, 13288 Marseille Cedex 09, France

*These authors contributed equally to this work
†Author for correspondence (e-mail: marcelle@ibdm.univ-mrs.fr)

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SUMMARY

In chick embryos, most if not all, replicating myoblasts present within the skeletal muscle masses express high levels of the FGF receptor FREK/FGFR4, suggesting an important role for this molecule during myogenesis. We examined FGFR4 function during myogenesis, and we demonstrate that inhibition of FGFR4, but not FGFR1 signaling, leads to a dramatic loss of limb muscles. All muscle markers analyzed (such as Myf5, MyoD and the embryonic myosin heavy chain) are affected. We show that inhibition of FGFR4 signal results in an arrest of muscle progenitor differentiation, which can be rapidly reverted by the addition of exogenous FGF, rather than a modification in their proliferative capacities. Conversely, over-expression of FGF8 in somites promotes FGFR4 expression and muscle differentiation in this tissue. Together, these results demonstrate that in vivo, myogenic differentiation is positively controlled by FGF signaling, a notion that contrasts with the general view that FGF promotes myoblast proliferation and represses myogenic differentiation. Our data assign a novel role to FGF8 during chick myogenesis and demonstrate that FGFR4 signaling is a crucial step in the cascade of molecular events leading to terminal muscle differentiation.

Key words: Skeletal muscle, FGF8, FGFR4, FGFR1, Quail, Chick

INTRODUCTION

Somites give rise to all skeletal muscles of the body, with the exclusion of head muscles (reviewed by Brand-Saberi and Christ, 2000; Christ and Ordahl, 1995). Trunk and neck muscles are subdivided into epaxial muscles, which derive from the medial portion of the somite, and hypaxial and appendicular muscles, which arise from the lateral half of the somite (Ordahl and Le Douarin, 1992). Muscle formation in the trunk is different to that in the limbs. Myogenesis in the trunk is dependent upon at least two distinct waves of muscle progenitor cell production. The first emanates from the medial and lateral borders of the dermomyotome, and is composed of post-mitotic muscle precursors (myocytes) that readily express basic helix-loop-helix (bHLH) myogenic determination factors such as MyoD. The second wave is formed by progenitors that translocate from the anterior and posterior borders of the dermomyotome and express the avian fibroblast growth factor FREK/cFGFR4[§]. These cells have the capacity to proliferate before undergoing terminal myogenic differentiation (Denetclaw et al., 2001; Kahane et al., 2001; Kahane et al., 1998; Marcelle et al., 1995; Ordahl et al., 2001). In contrast, early myogenesis in the limb derives from a single wave of progenitors which migrate from the lateral somites into the limb mesenchyme and sequentially express Pax3, FGFR4 and Myf5, then MyoD, and finally muscle structural proteins, such as the embryonic myosin heavy chain (MyHC) (Bober et al., 1994; Delfini et al., 2000; Goulding et al., 1994; Marcelle et al., 1995; Williams and Ordahl, 1994).

Because the dermomyotome, which is the sole source of trunk and limb muscles, disappears around E5 of chick development, the muscle progenitors that are present within the limb and somite muscle masses must be able to provide the continuous supply of cells that is required during embryonic muscle growth. The high expression of FGFR4 in all proliferative myoblasts present within skeletal muscles throughout embryogenesis, as well as its expression in mouse and chick satellite cells (which are the only cells of the adult muscle capable of proliferation and differentiation upon activation), strongly suggest that this molecule not only represents a specific marker for this muscle stem cell population, but might also play an important role during myogenic differentiation (Halevy et al., 1994; Kastner et al., 2000; Marcelle et al., 1994; Marcelle et al., 1995). However, its function during this process is unknown.

There is a wealth of data describing the importance of FGF-like molecules during myogenesis. Although numerous studies have demonstrated that FGF promotes continuous cell proliferation and represses the onset of terminal differentiation, it has also been shown that early myoblast precursors require FGF exposure in order to subsequently express their myogenic phenotype (Clegg et al., 1987; Olwin and Hauschka, 1986;

[§]Although we previously reported notable differences in sequence and expression pattern between FREK and the mammalian FGFR4 (Marcelle et al., 1994), Southern blot analysis of the chick genome indicates that FREK represents the fourth member of the avian FGF receptor family and should therefore be renamed cFGFR4 (unpublished).

Seed and Hauschka, 1988; Templeton and Hauschka, 1992) (reviewed by Pownall and Emerson, 1992). In addition, genetic studies recently conducted in *C. elegans* have shown that FGF signaling plays an important function in myoblast migration (reviewed by Boilly et al., 2000; Montell, 1999). Therefore, under different conditions, FGF can promote the proliferation, the differentiation or the migration of myogenic precursors. In search of a receptor that might mediate FGF activities in vertebrate skeletal muscles, it was observed that FGFR1 transcription gradually decreases as muscle cell lines differentiate in vitro (Moore et al., 1991; Olwin and Hauschka, 1988; Olwin and Hauschka, 1990; Templeton and Hauschka, 1992). This led to the assumption that, in vivo, FGFR1 might play an important role during myogenesis. Subsequent expression studies did not support this hypothesis, as it became clear that FGFR1 is poorly expressed in muscles (Orr-Urtreger et al., 1991; Peters et al., 1992; Yamaguchi et al., 1992; Edom-Vovard et al., 2001) (this study). In vivo, a transmembranal, dominant-negative form of FGFR1 was shown to inhibit muscle progenitor migration; in addition, this molecule induced a disruption of skeletal muscle development, which was attributed to a premature activation of terminal differentiation (Itoh et al., 1996; Flanagan-Steet et al., 2000). However, because the transmembranal, dominant-negative receptors that were used in these studies can heterodimerize with heterologous FGF receptors (including FGFR4), and thereby inhibit all FGF signaling (Bellot et al., 1991), it is possible that the effects that were observed are not specific to FGFR1 signaling. Therefore, it was important to readdress this problem by selectively inhibiting the function of FGFR1 and FGFR4 during muscle differentiation.

We used secreted forms of FGFR4 and FGFR1 to challenge limb myogenesis in vivo. We demonstrate that inhibition of FGFR4 signaling leads to a dramatic, and in some cases total, loss of limb muscles that can be rapidly reverted by the addition of exogenous FGF. Conversely, over-expression of FGF8 in somites promotes FGFR4 expression and muscle differentiation in this tissue. All muscle markers analyzed (such as Myf5, MyoD and the myosin heavy chain) are affected, indicating that FGFR4 signaling acts close to the top of a molecular cascade that controls overt muscle differentiation in the limb. In sharp contrast, inhibition of FGFR1 signaling has no visible effect on muscle differentiation. Finally, we demonstrate that the inhibition of myogenesis does not result in an arrest of muscle progenitor proliferation. Rather, the observation that this inhibition can be overcome by the addition of exogenous FGF, implies that the progenitors have retained their ability to respond to FGF stimulation.

MATERIALS AND METHODS

Production of retroviruses carrying the secreted forms of FGFR4 and FGFR1

A secreted form of the FGFR4/FREK was constructed as follows: the extracellular portion of the quail FGFR4 (amino acids 1 to 282 of the GenBank Accession Number, X76885) (Marcelle et al., 1994) was PCR-amplified and fused to a genomic clone coding for the Fc fragment of the human immunoglobulin gamma-1 heavy chain constant region (nucleic acids 906-1803 of the GenBank sequence Accession Number, J00228) (Takahashi et al., 1982); two amino acids (G and S) introduced between FGFR4 and Fc serve as a molecular hinge for correct protein conformation. This construct (named S-FR4-Fc) was transferred into the Slax shuttle vector, which contains the *src* 5′ leader sequences upstream of a Kozak's consensual ATG translation initiation site, surrounded by ClaI restriction sites convenient for subcloning into the RCAS-BP(B) retroviral vector (Hughes et al., 1987). Similarly, a secreted, Fc-tagged form of FGF receptor-1 (named S-FR1-Fc) was constructed by fusing the same Fc fragment to the extracellular portion of the quail FGF receptor 1 (C. M., unpublished). This quail cDNA clone corresponds to the chick FGFR1 IgG 3c isoform, which is the major functional isoform of FGFR1 (Lee et al., 1989; Pasquale and Singer, 1989; Partanen et al., 1998); it was PCR amplified (the amplified fragment corresponds to amino acids 1 to 373 of the cFGFR1/CEK1 GenBank Accession Number, TVCHFG). This was fused to Fc, transferred into the Slax shuttle vector and subcloned into RCAS-BP(A). When secreted in the extracellular space of embryonic tissues, both molecules should bind their cognate ligand(s) and titer them from the endogenous FGF receptors. Therefore, unless in vivo FGFR1 and FGFR4 bind the same ligand(s), S-FR1-Fc should not inactivate endogenous FGFR4 signaling. Naturally occurring forms of secreted FGFR1 have been previously described (Johnson et al., 1990) (reviewed by Johnson and Williams, 1993) and are thought to act as endogenous inhibitors of FGF signaling. The human alkaline phosphatase gene cloned into the RCAS-BP (B) vector (Morgan and Fekete, 1996) served as control.

Although line O chick primary fibroblasts (SPAFAS) were used in early stages of this research, we found that the recently developed chicken embryonic fibroblast cell line UMNSAH/DF-1 (purchased from ATCC) (Himly et al., 1998) gave more reproducible infections of chick embryos; thus, they were used for the remaining experiments. These were cultured in DMEM, 10% fetal calf serum, 2% chicken serum medium. Cells were transfected with the lipofectamine reagent (Gibco BRL) according to the manufacturer's instructions. After 5-7 days, we tested that the cell cultures were fully infected by performing an immunohistochemistry reaction against the viral core protein p27 (using a rabbit anti-p27 polyclonal antibody – SPAFAS). We verified that all infected cells expressed the chimeric FGF receptor molecule by performing immunohistochemistry using a goat anti-human immunoglobulin Fc fragment antibody (Jackson Laboratory). The infected fibroblasts stably produced the chimeric molecules for at least two months in culture.

In vivo electroporation of somites

Qiagen EndoFree purified plasmid cDNA was injected into the somitocoel of the (4-5) newly formed somites of (Hamburger-Hamilton) stage 15 chick embryos. These somites correspond to the prospective interlimb level (somites 22-27). FGF8 cDNA (for details, see Dubrulle et al., 2001) was co-electroporated with a plasmid containing the eGFP coding sequence (Clontech) under the control of an SV40 promoter and enhancer region (C. M., unpublished). Final electroporation solution was 4 μg/μl FGF8; 1.5 μg/μl eGFP; carboxymethylcellulose 0.33% (Sigma); Fast Green 1% (Sigma); $MgCl_2$ 1 mM; PBS 1X. Platinum (+) and tungsten (–) electrodes were placed on both sides of the embryo and pulsed five times at 80 V for 20 msec with an Intracell TSS 10 electroporator. By placing the positive electrode on the right-hand side of the embryo, we electroporated the lateral side of somites. Three days later, embryos were analyzed by in situ hybridization.

Chick embryos micromanipulation

Fertilized White Leghorn eggs were purchased from a local provider; they were incubated at 38°C in a humid chamber. For cell injections, embryos were windowed at Hamburger and Hamilton (HH) stage 10 (Hamburger and Hamilton, 1951).

Infected cells produce not only the recombinant replication-competent viruses that are used to infect embryonic tissues, but they

also secrete the proteins encoded by the chimaeric cDNAs. We have used these two properties in separate experiments:

(1) Infected cells injected into virus-sensitive chick embryos serve as a continuous source of viruses, which after a few days infect large areas of embryonic tissues. To infect limb bud muscle progenitors, pellets of infected cells were pressure-injected into the presomitic mesoderm at the level of the presumptive somites 15 to 21, which contain the progenitors of the fore limb muscles (Chevallier et al., 1977; Christ et al., 1977). Thus, limb bud muscle progenitors that subsequently migrate out of the somites should be infected by the retroviruses. Although there is no particular tropism of the retroviruses towards muscle progenitors, we observed in several cases (after staining for efficiency of infection) that the dorsal and ventral muscle masses – which are easily discernable at the time of the analysis (around E6) – were preferentially infected over the limb bud mesenchyme. Embryos were further incubated for four days and then dissected out.

(2) Infected cells injected into virus-insensitive chick embryos serve as a source of protein, which locally perturbs the signaling of endogenous receptors. To inhibit endogenous FGFR4 signaling, pellets of S-FR4-Fc-producing cells were pressure-injected into the limb muscles of E5 chick embryos. These were analyzed after an overnight incubation.

Injections were performed using a Picospritzer (General Valve Corporation) and glass micropipettes.

FGF-loaded beads

Heparin-immobilized acrylic beads (Sigma) were saturated overnight at 4°C in a solution of 1 μg/μl of either FGF2 (Sigma) or FGF8 (R&D systems) diluted in PBS 0.2% BSA. Beads were then implanted in embryos. For the rescue experiments, FGF-soaked beads were introduced in chick wing either 48 hours or 8 hours prior to dissection (i.e. at E4 or E6). Control beads were incubated in PBS, 0.2% BSA overnight at 4°C.

FGF-binding assay

To test whether S-FR4-Fc and S-FR1-Fc bind FGF, FGF-loaded beads (prepared as described above) were incubated for 2 hours at room temperature with 500 μl of cell supernatant. The beads were washed, resuspended in 20 μl of protein-loading buffer, boiled and loaded onto a protein gel. Western blot analysis was performed as described (Lambert et al., 2000). After protein transfer, the membrane was probed with an alkaline phosphatase-conjugated goat anti-human immunoglobulin Fc fragment antibody (Jackson Laboratory). Color development was performed using Western Blue® substrate for alkaline phosphatase (Promega).

In situ hybridization, immunohistochemistry

Whole mount in situ hybridizations on chick embryos were performed as described (Henrique et al., 1995). The probes used in this study are (1) two quail FGFR4 probes directed against the extracellular or the cytoplasmic portion of the molecule, which were used to recognize only the endogenous or both the endogenous and exogenous FGFR4; (2) a quail Pax3 probe directed the 3′ end of the coding region of the quail Pax3 gene [probe ‘c’ in Stark et al. (Stark et al., 1997)]; (3) a quail Myf5 probe corresponding to a 850 bp portion of the Myf5 cDNA (Pownall and Emerson, 1992); (4) a chick MyoD probe, corresponding to the complete 1518 bp MyoD cDNA (Lin et al., 1989); (5) a probe directed against the Fc portion allowed us to follow the expression of the exogenous qFGFR4 and qFGFR1. Note that quail probes recognize the corresponding chick mRNAs.

In most experiments, we performed double in situ hybridizations: unless otherwise stated, the first probe was directed against the Fc portion of the constructs and allowed us to monitor that the limb buds had been efficiently infected. After fixation and removal of the first red color reaction with methanol (that was obtained after alkaline phosphatase reaction with a INT-BCIP substrate, Roche), the embryos that had been satisfactorily infected were processed through a second round of in situ reaction to detect markers of myogenic differentiation (i.e. Pax-3, FGFR4 and MyoD).

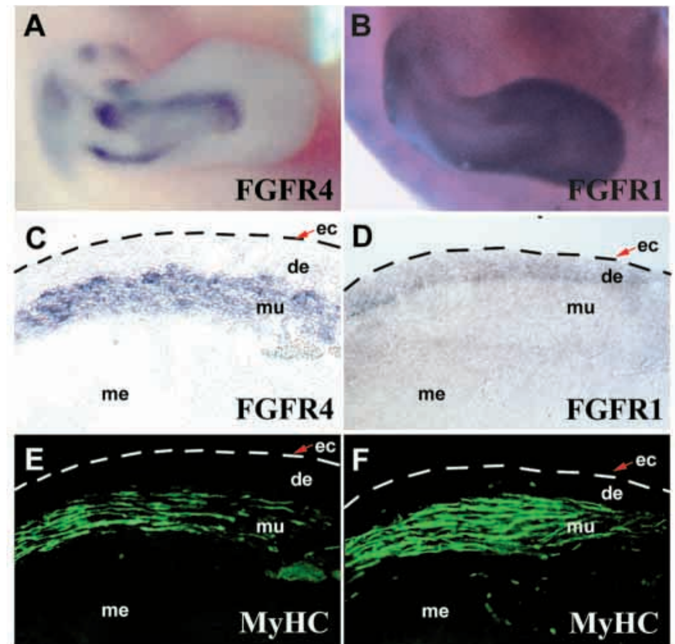

Fig. 1. FGFR4, but not FGFR1 is expressed in embryonic limb skeletal muscles. E5 embryos were hybridized to a FGFR4-specific RNA probe (A,C,E), or to a FGFR1-specific probe (B,D,F). Whole-mount in situ indicate that FGFR4 is specifically expressed in the developing muscles (A), whereas FGFR1 is widely expressed in the entire limb (B). Sections confirm that FGFR4 is specifically expressed in the skeletal muscles (C), which are recognized after immunohistochemistry with an embryonic myosin heavy chain-specific monoclonal antibody (E). In contrast, FGFR1 is strongly expressed in the dermis (D) and poorly, if at all, in the muscles (F). ec, ectoderm; de, dermis; mu, skeletal muscles; me, mesenchyme.

Whole mount embryo immunohistochemistry was performed as described (Marcelle et al., 1999). For whole mount immunohistochemistry, embryos were incubated overnight at 4°C in a 1:10 dilution of an MF-20 hybridoma supernatant directed against the embryonic myosin heavy chain; this supernatant was obtained from the Developmental Studies Hybridoma Bank (maintained by the Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD, USA and the Department of Biology, University of Iowa, Iowa City, IA, USA), under contract N01-HD-2-3144 from the NICHD. BrdU labelling of embryos was performed as described (Sechrist and Marcelle, 1996).

Nuclear staining using 4′,6-diamidino-2-phenylindole (DAPI) has been shown to be an easy and efficient way to monitor cells in the terminal phase of apoptosis, which display fragmented nuclei (Marcellus et al., 1998; Marazzi et al., 1997; Yardin et al., 1998). To monitor for tissues undergoing cell death, sections of infected embryos were incubated for 10 minutes in a 1 μg/ml concentration of DAPI-HCl solution (Calbiochem). Sections were then mounted in Mowiol solution.

RNA injection in *Xenopus laevis* embryos

The S-FR1-Fc and S-FR4-Fc constructs were subcloned into the pCS2 expression vector commonly used for frog injections. RNA production and whole-mount in situ hybridization was performed as previously described (Gawantka et al., 1995). Injections were done at the four-cell stage: 5 nl of a 0.2 ng/nl (high dose) or 0.02 ng/nl (low

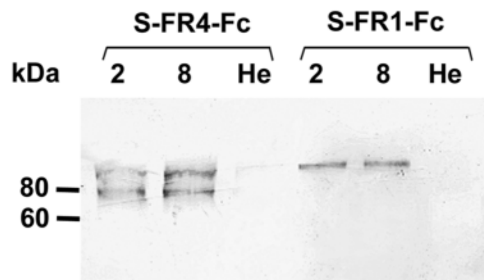

Fig. 2. S-FR4-Fc and S-FR1-Fc bind to FGF in vitro. Heparin acrylic beads were first soaked with FGF2 (2) or FGF8 (8) and then incubated with 300 μl of cell supernatant from DF-1 cells infected with the S-FR4-Fc or S-FR1-Fc constructs. Control beads (He) were incubated with 2% BSA prior to exposure to the cell supernatants. Proteins adsorbed on these beads were separated by SDS-Page and blotted onto a membrane; they were recognized using an anti-human Fc fragment antibody. Both S-FR4-Fc and S-FR1-Fc bind to FGF2- and FGF8-loaded beads, but not heparin beads.

dose) RNA solution were injected into both dorsal blastomeres at the level of the marginal zone which corresponds to the presumptive territory of the mesoderm. To follow the injected cells, β-galactosidase RNA was co-injected at a 0.15 ng/nl concentration. Control embryos were injected with β-galactosidase RNA only. Whole-mount β-galactosidase staining was performed as described (Sanes et al., 1986).

RESULTS

FGFR4 is the only FGF receptor expressed at high level during chicken muscle differentiation

Although in vitro data have suggested that FGFR1 might play a role during myogenesis (Olwin and Hauschka, 1988; Templeton and Hauschka, 1992), expression studies question this hypothesis, because they have shown that FGFR1 expression is high in most embryonic tissues, but appears absent from early avian or mammalian myotome (Orr-Urtreger et al., 1991; Patstone et al., 1993; Peters et al., 1992; Yamaguchi et al., 1992). Because the latter studies have been done several years ago with less sensitive in situ techniques, it was possible that low FGFR1 expression in muscles had been overlooked. Therefore, we re-examined FGFR1 expression pattern during muscle formation. Chick embryos at six days of development (i.e. the embryonic stage at which most of the experimental embryos used in the present study have been analyzed) were hybridized to an FGFR1 probe (Fig. 1B,D). As a point of comparison, embryos at the same embryonic age were hybridized to a quail FGFR4 probe (Fig. 1A,C). In whole mount in situ hybridization, FGFR4 expression clearly delineates the differentiating muscle masses (Marcelle et al., 1995); in contrast, FGFR1 expression pattern is readily different. To determine the tissues that express this molecule, these embryos were sectioned. On sections, FGFR1 is most prominently expressed in the sub-ectodermal mesenchyme (i.e. the developing dermis), and is faintly expressed in the limb bud core mesenchyme (where long bones will later form). In the muscle masses, which are recognized with an antibody specific for the embryonic myosin heavy chain, MF20 (which labels mature muscle fibers), FGFR1 expression is almost absent

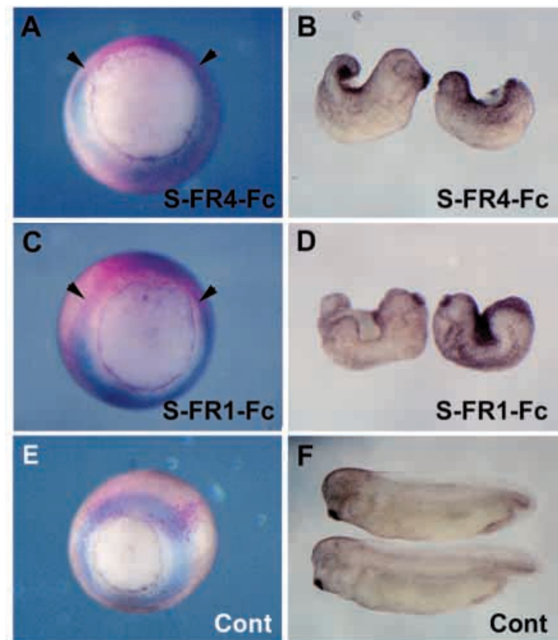

Fig. 3. S-FR4-Fc and S-FR1-Fc disrupt gastrulation in *Xenopus* embryos. At gastrula stage, the expression of the mesodermal marker Brachury (in blue), which is normally expressed as a ring around the blastopore (E), is downregulated in cells co-injected with β-galactosidase and S-FR4-Fc (A) or S-FR1-Fc (C) RNA. β-galactosidase expression is detected in red. Arrowheads in A,C indicate the region in which Brachyury is downregulated. Overexpression of S-FR4-Fc (B) or S-FR1-Fc (D) in the dorsal blastomeres of early *Xenopus* embryos produce gastrulation defects, which lead later in development (around stage 28) to grossly abnormal embryos, when compared with control embryos injected with β-galactosidase RNA only (F). Note that high and low doses of injected RNA were comparable with those used by Amaya et al. (Amaya et al., 1991; Amaya et al., 1993).

(Fig. 1D,F), confirming earlier reports (Patstone et al., 1993; Peters et al., 1992). Various probes directed against different regions of the FGFR1 gene have led to the same results, indicating that the very low level of FGFR1 expression observed in muscles is not artefactual. It is important to note, however, that the low level of FGFR1 expression might result from non-muscle cells (notably connective tissue and endothelial cells) present within muscle masses. Because FGFR2 and FGFR3 are not expressed in developing muscles (Patstone et al., 1993; Peters et al., 1992) (our observation), these observations confirm that FGFR4 is the only FGF receptor prominently expressed in developing skeletal muscles and suggest an important role for FGFR4 during avian myogenesis.

Secreted forms of FGFR1 and FGFR4 act as competitive inhibitors of FGF-receptor signaling

We have constructed a specific inhibitor of FGFR4 signaling (S-FR4-Fc) by fusing the extracellular portion of the molecule to the Fc fragment of the human immunoglobulin (see Materials and Methods). To test the specificity of the inhibition of FGFR4 signaling by S-FR4-Fc, we compared its activity to that of a chimaeric molecule made in a similar way with the

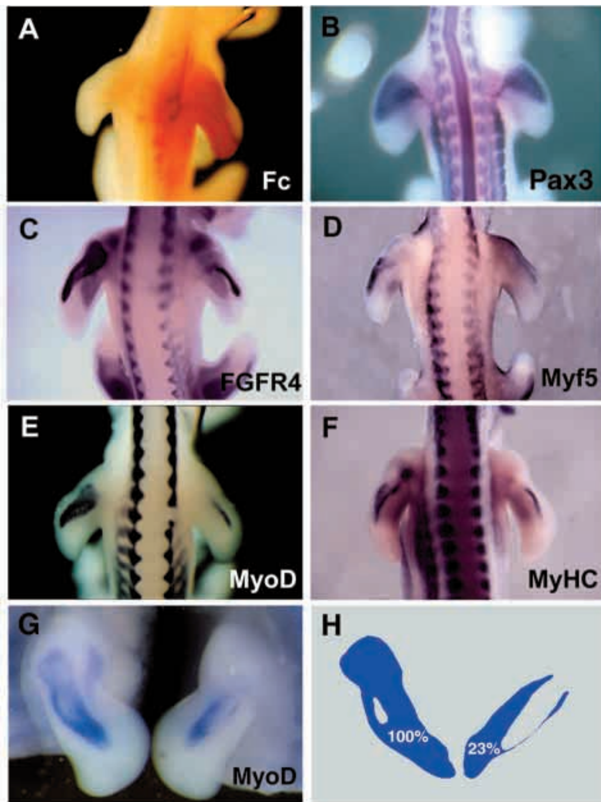

Fig. 4. Inhibition of FGFR4 signaling results in a block of limb bud myogenesis. Two-day-old embryos were injected in the prospective limb bud domain with cells infected with a secreted form of FGFR4. Four days later, infected embryos were processed for double in situ hybridization (A-E,G) or whole-mount immunohistochemistry with a monoclonal antibody directed against the embryonic form of the myosin heavy chain (MyHC, F). In a first round of in situ hybridization, a Fc-specific probe enabled us to determine which embryos had been efficiently infected (such an embryo is presented in A). These were then destained and a second in situ reaction was performed with probes specific for various stages of myogenic differentiation. Although none of the infected embryos displayed a variation in Pax3 expression (B), all muscle markers (Myf5, D; MyoD, E), the embryonic myosin heavy chain (F) and FGFR4 itself (C) were strongly downregulated. (G,H) To estimate the amplitude of the inhibition, embryos were separated in two parts after in situ hybridization, and then photographed (G); the stained dorsal muscle masses were delineated manually with Adobe Photoshop, and their surfaces were compared by pixel counting (H). In the case presented here, MyoD staining was decreased by 77%. When control embryos were counted in a similar manner, a difference of no more than 8% was observed between both limbs.

extracellular portion of the FGFR1 (i.e. S-FR1-Fc). Various controls were made to ensure that S-FR4-Fc and S-FR1-Fc constructs are biologically active.

First, we tested that both molecules were actively secreted by a chick fibroblast cell line (see Materials and Methods) infected with the constructs. Western blot analysis of cell supernatants probed with a monoclonal antibody specific for the human Fc confirmed a robust secretion of both S-FR4-Fc and S-FR1-Fc (not shown).

Second, we verified that S-FR4-Fc and S-FR1-Fc actively bind FGF. We incubated FGF2- and FGF8-coated heparin beads with the supernatant of transfected cells. The proteins adsorbed on the beads were then analyzed by Western blot analysis, using an anti-Fc monoclonal antibody. Our results demonstrate that S-FR4-Fc and S-FR1-Fc bind to FGF2 and FGF8 in vitro (Fig. 2). As a control, neither S-FR4-Fc nor S-FR1-Fc bound to uncoated heparin beads.

Third, we tested both constructs in a well-characterized biological assay, the mesoderm formation in Xenopus embryos. The role of FGFR1 signaling in frog mesoderm formation has been well-documented. It was shown that the injection of a transmembranal, dominant-negative form of FGFR1 (TM-FR1) in the prospective dorsal marginal zone of embryos results in grossly abnormal embryos. The expression of the mesodermal marker Brachyury, which is normally observed in a ring of cells around the blastopore, is inhibited in the region of the injection. This leads during gastrulation to defects of axis formation in a dose-dependent manner (Amaya et al., 1991; Amaya et al., 1993). S-FR4-Fc and S-FR1-Fc were injected in the marginal zone of the two dorsal blastomeres of four-cell stage embryos. Two days after injection, 70% of the embryos (n>100) injected with high doses of the secreted form of FGFR1 exhibited a default in dorsal closure. This percentage dropped to 33% with low doses of S-FR1-FC (n>100) (Fig. 3C,D). Similarly, S-FR4-Fc induced 50% of abnormal gastrulation defects when injected at high dose (n>100), and 24% at low dose (n>100) (Fig. 3A,B). As a control, β-galactosidase injection at high dose resulted in only a small percentage (5%) of gastrulation defects (Fig. 3E,F). Injection of both constructs resulted in the inhibition of Brachyury expression in the region of the injection (Fig. 3A,C). These phenotypes are entirely consistent with those obtained with TM-FR1 (Amaya et al., 1991; Amaya et al., 1993), and demonstrate that S-FR4-Fc and S-FR1-Fc act as efficient competitive inhibitors of FGF signaling in vivo.

FGFR4, but not FGFR1 signaling is a necessary step in the limb bud myogenic differentiation program

Early limb bud myogenesis is the result of a migration of Pax-3-positive, proliferative muscle progenitors from the lateral portion of the dermomyotome. Subsequently, they start expressing FGFR4 and Myf5 and only later do they exit from the cell cycle while initiating terminal myogenic differentiation, which can be monitored by the expression of the bHLH molecule, MyoD (Delfini et al., 2000; Marcelle et al., 1995). Thus, FGFR4 expression represents an early step in the limb bud myogenic differentiation program. To test whether FGFR4 signaling is a functionally significant step along this pathway, we have inhibited its function during fore limb myogenesis. Three to four days after injection of DF-1 cells infected with a S-FR4-Fc construct, embryos were

Table 1. Result of the inhibition of FGFR4 and FGFR1 signaling

Probes	S-FR4-Fc	S-FR1-Fc
MyoD	50% n=45	0% n=17
Pax3	0% n=37	ND
FGFR4	50% n=50	0% n=26

Percentages were calculated as the ratio of embryos displaying a visible inhibition over the number of embryos tested (n).
ND, not determined.

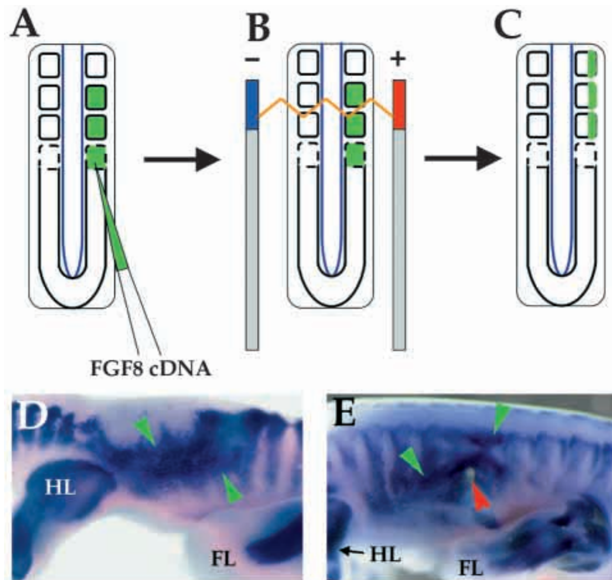

Fig. 5. Overexpression of FGF8 in somites promotes myogenic differentiation and FGFR4 activation. (A-C) Protocol used for electroporation of somites in vivo. FGF8 plasmid cDNA was injected into the somitocoel of the newly formed somites of (Hamburger-Hamilton) stage 15 chick embryos (A). These somites correspond to the prospective interlimb level (somites 22-27). By placing the positive electrode on the right-hand side of the embryo, we electroporated the lateral side of somites (B,C). Three days later, embryos were analyzed by in situ hybridization for MyoD (D) and FGFR4 (E) expression. Massive overexpression was observed with both probes (green arrowheads) along the entire mediolateral axis of somites (i.e. prospective epaxial and hypaxial domains). Red arrowheads in E indicate an overgrowth that, in some embryos, developed into a partial ectopic limb. HL, hindlimb; FL, forelimb.

dissected out, and double in situ hybridizations were performed (see Materials and Methods). The first probe was directed against the Fc portion of the construct (Fig. 4A); the embryos that had been satisfactorily infected were destained for the first colour, then processed through a second round of in situ reaction to detect markers of myogenic differentiation (i.e. Pax3, FGFR4 and MyoD). None of the tested embryos displayed a decrease in Pax3 expression (*n*=37) (Fig. 4B; see also Table 1), indicating that inhibition of FGFR4 signaling does not significantly impede the migration of Pax3-positive muscle progenitors into the limb mesenchyme and their proliferation in this tissue.

In contrast, we observed in 50% of the embryos infected in the same experimental series a marked decrease of FGFR4 (*n*=50) (Fig. 4C) and MyoD (*n*=45) (Fig. 4E) expression. In control embryos injected with the human alkaline phosphatase gene, we never observed any modification of Pax-3, MyoD or FGFR4 expression level (data not shown). Downregulation of MyoD expression is accompanied by a decrease of muscle structural gene expression, as observed after whole-mount immunohistochemistry with the MF20 antibody specific for the embryonic myosin heavy chain (*n*=30) (Fig. 4F). The overall level of expression of these genes is not decreased in the muscles; rather, it is the area in which they are expressed that is reduced. By comparing the surface of the area stained in the infected versus normal, contralateral limb, we could estimate that the amplitude of the decrease of FGFR4 and MyoD expression ranged from 10 to 100%, with half of the embryos exhibiting a decrease larger than 50% (Fig. 4G,H).

Because Myf5 and FGFR4 are expressed at approximately the same time in limb muscle progenitors, it was important to determine whether inhibition of FGFR4 signaling would affect Myf5 expression as well. We performed double in situ hybridization on S-FR4-Fc-infected embryos, using MyoD as a first probe. The embryos that displayed an important decrease of MyoD expression were destained with methanol and re-stained for Myf5 expression. In all examined cases (*n*=9) we observed in these embryos a marked decrease of Myf5 expression (Fig. 4D).

Because FGFR4-null mice display no muscle phenotype (Weinstein et al., 1998), a hypothesis is that other FGF receptors can compensate for the loss of FGFR4 transcription. A likely candidate for such activity is FGFR1. Therefore, we determined whether FGFR1 expression might be modified in S-FR4-Fc-infected embryos. Double in situ hybridization was performed with MyoD as a first probe. Those that displayed a marked decrease of MyoD were processed for FGFR1 expression. No difference was observed between the infected and the non-infected limb (not shown), indicating that, within the timeframe of the experiment, FGFR1 expression was not upregulated to compensate for the loss of FGFR4 activity.

Finally, to test the specificity of the S-FR4-Fc-mediated inhibition of muscle differentiation, we compared its activity to that of S-FR1-Fc, using the same experimental protocol. In sharp contrast with the situation observed after S-FR4-Fc infection, we never observed any decrease in MyoD (*n*=17) or FGFR4 (*n*=26) expression in embryos infected with S-FR1-Fc construct (Table 1), reinforcing the assumption that FGFR4, but not FGFR1 signaling regulates limb embryonic muscle differentiation in vivo.

Together, these results indicate that the specific inhibition of FGFR4 signaling in the limb bud impedes the entire muscle differentiation program in this tissue, suggesting that the activation of FGFR4 signaling represents a necessary step during limb myogenesis. The observation that expression of S-FR4-Fc leads to a downregulation of Myf5 expression suggests that Myf5 is located downstream of FGFR4 in a cascade of molecular events which lead to overt myogenic differentiation. Finally, the observation that S-FR4-Fc-infected embryos display a marked decrease of endogenous FGFR4 expression indicates (as was already shown in vitro (Halevy et al., 1994) that this molecule regulates its own transcription, presumably through a feedback loop mechanism.

FGF signaling promotes somitic myogenesis

In the experiments described above, although limb bud myogenesis was inhibited in 50% of the experimental embryos, downregulation of somite myogenesis was observed in only 10-20% of the injected embryos (see Fig. 4D,F, in which the downregulation of muscle markers in the limbs is not paralleled by a similar decrease in the somites, although the embryo in Fig. 4C,E displays a marked decrease in both the limb and the ipsilateral somites). We interpreted this partial failure as an indication that somites or their surrounding tissues express higher levels of FGF signal than the limb bud, such that this signal is blocked by S-FR4-Fc only when massive infection of somitic tissues was obtained. In addition, mechanical

disruption of the somites at the site of injection rendered the interpretation of the somitic phenotypes difficult. Therefore, we decided to address the role of FGF signaling during somite myogenesis using a different approach (i.e. the over-activation of FGF signaling in these tissues). We electroporated an FGF8 cDNA in the lateral dermomyotome of interlimb somites (Fig. 5A-C). Three days later, a massive over-expression of MyoD was observed in the region of electroporation (*n*=10) (Fig. 5D). These results demonstrate that myogenic differentiation in somites is promoted by FGF signaling and are entirely consistent with the data obtained in the limb. Moreover, the observation that activation of MyoD is paralleled by a strong up-regulation of FGFR4 (*n*=20) (Fig. 5E) indicates that this receptor is implicated in this process. At present, we cannot determine whether the activation of muscle differentiation by FGF8 in somites is mediated by FGFR4 as it seems to be the case in the limb, or indirectly through another FGF receptor.

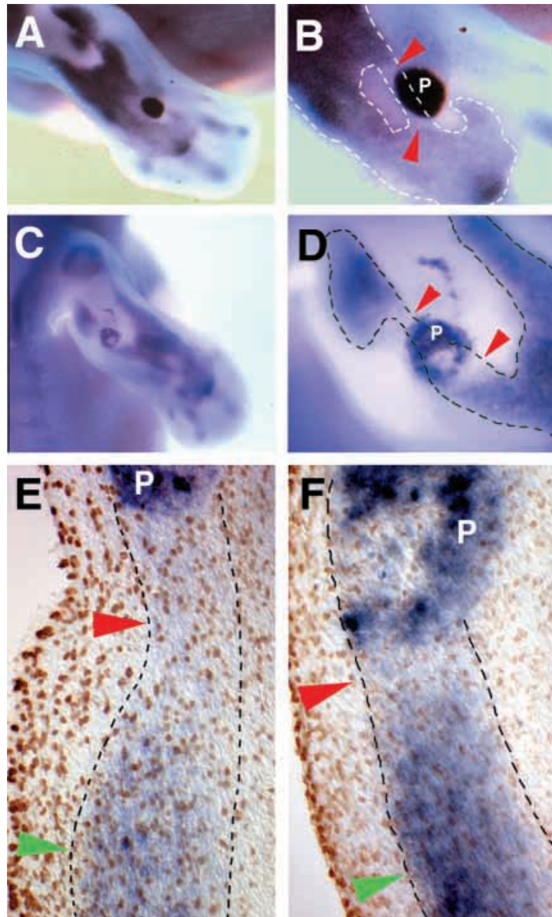

Fig. 6. Local inhibition of FGFR4 signaling does not modify muscle progenitor proliferation. S-FR4-Fc-expressing cells were injected into the limb buds of E6 embryos. These cells serve as a source of protein, which locally perturbs the signaling of endogenous FGFR4. After overnight incubation, the embryos were exposed to BrdU for 1 hour and analyzed for FGFR4 expression. The choice of the probe enabled us to examine the expression of endogenous FGFR4 and the position of the injected cell pellet (P). Two independent experiments are shown. (A,B,E) The first embryo; (C,D,F) the second embryo. (A,C) General views of the injected limbs. (B,D) Close up of the views presented in A,C. Broken lines in B,D represent the outline of the muscle bundles as they are observed in the contralateral, uninjected side. In B,D, a muscle bundle crosses the cell pellet. We observed that the muscles immediately adjacent to the injected cells display a strong downregulation of FGFR4 expression (red arrowheads), indicative of an efficient inhibition of muscle differentiation. (E,F) Sections of the embryos presented in B,D. Around the cell pellet, light, but clearly visible, blue staining (red arrowhead) enabled us to identify the position of affected muscle progenitors, which we delineated with a broken line. At 10-20 cell diameters away from the injected cells, FGFR4 expression was normal (dark blue, green arrowhead). BrdU counting was carried out by arbitrarily choosing a similar surface in the affected and unaffected regions and comparing the number of BrdU-positive nuclei in each. This was done in four embryos and in at least two adjacent sections each. No differences in BrdU-positive cells were observed between the two regions, demonstrating that an arrest of cell division cannot explain the disappearance of myogenic markers.

FGFR4 signaling regulates muscle progenitor differentiation, but not proliferation

The observation that FGFR4 is expressed in proliferating myoblasts, and yet post-mitotic muscle fibers never express this gene has led us to hypothesize that FGFR4 signaling might play a role in muscle stem cell proliferation and/or differentiation (Marcelle et al., 1995). The results obtained after inhibition of FGFR4 signaling in the limb clearly demonstrate that indeed FGFR4 is a necessary step during limb bud myogenesis. However, the reduction in size of the MyHC-, MyoD- Myf5- and FGFR4-positive cell populations that was observed in infected embryos could be because of either an arrest of cell proliferation (coupled or not to an increase in cell death) or a blockage of differentiation. To test the role of FGFR4 on proliferation, we locally inhibited its signaling and tested whether muscle progenitor proliferation was modified. We injected pellets of S-FR4-Fc-infected cells in the limb buds of E4-E5 chick embryos. After overnight incubation, embryos were exposed to BrdU for one hour and analyzed. The cell pellets injected in the muscle masses operate as local sources of S-FR4-Fc which should act on FGFR4-expressing cells; the progenitors that express Myf5, MyoD or the MyHC at the time of injection should not be affected by FGFR4 inhibition. Thus, to identify the embryos in which the inhibition had been successful, we screened them with a FGFR4-specific probe. The probe was chosen to recognize both the exogenous (i.e. the cell pellet) and endogenous FGFR4. After whole mount in situ hybridization, we observed that FGFR4 expression was downregulated in the muscles surrounding the injected pellets (*n*=17) (Fig. 5A-F) when compared to the normal non-injected limb, thereby confirming the results of the long-term infections described above. On sections, we could estimate that the inhibition of FGFR4 expression extended over 10-20 cell diameters around the pellet (Fig. 6E,F). After immunostaining for BrdU-positive cells, the number of BrdU-positive cells was compared in the region where FGFR4 is downregulated to that where it is unaffected. No difference was found in the number of BrdU-positive nuclei between the two regions (Fig. 6E,F). These experiments indicate that the inhibition of FGFR4 signaling does not significantly affect the rate of proliferation of muscle progenitor cells; thus, an arrest of cell division cannot explain the disappearance of the myogenic markers.

Long-term inhibition of FGFR4 signaling does not seem to modify cell survival either: embryos infected by S-FR4-Fc and

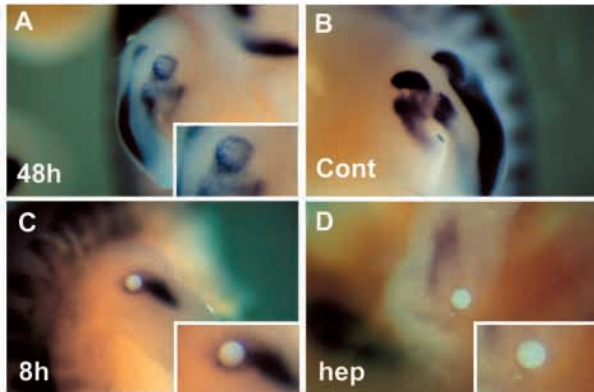

Fig. 7. Muscle progenitors inhibited by S-FR4-Fc retain their ability to respond to FGF stimulation. S-FR4-Fc cells are injected in the segmental plate of two-day-old embryos. Two or 4 days later, heparin beads coated with FGF2 or FGF8 were implanted into the developing limb of embryos. In a first set of experiments (A,B), the beads were grafted in E4 embryos and re-incubated for an additional 2 days. (B) The contralateral, non-infected, non-implanted forelimb of the embryo shown in A. In a second set of experiments (C,D), the beads were implanted in E6 embryos and re-incubated for only 8 hours. We observed 2 days (A) or 8 hours (C) after implantation of the beads a rescue of MyoD expression immediately around implanted beads. No rescue was observed with control heparin beads (D).

which displayed a strong downregulation of MyoD expression (such as the sample presented in Fig. 4G) were sectioned, and stained with the nuclear stain DAPI (which recognizes fractionated nuclei indicative of cell death). Although fractionated nuclei were readily observed in tissues that undergo cell death, such as spinal cord motoneurons, no difference was observed between the infected versus non-infected muscle masses (not shown). Thus, cell death is not the cause of the extensive disappearance of myogenic markers.

If a decrease in cell proliferation or cell death cannot explain the inhibition of myogenesis in S-FR4-Fc-injected embryos, an alternative hypothesis is that the blockage of FGFR4 signaling inhibits the myogenic differentiation of muscle progenitors. Thus, introducing exogenous FGF in infected embryos should rescue the normal differentiation of muscle progenitors. To test this, heparin beads coated with FGF2 or FGF8 were implanted into the developing limb of embryos infected with S-FR4-Fc viruses. In a first set of experiments, the beads were grafted in embryos that had been injected two days earlier; these were re-incubated for an additional two days; in a second set of experiments, the beads were implanted four days after initial injection and embryos were re-incubated for only eight hours (see Fig. 7). A successful experiment requires that the muscle differentiation of the infected limbs would be efficiently inhibited (so that the normal MyoD expression domain is almost absent) and that the bead would be positioned in the muscle masses. Clearly, there is no way to recognize whether muscle progenitor differentiation is inhibited before we implanted the beads in the limbs. Thus, we discarded several embryos in which implanted beads did not give sufficiently clear results. We observed after two days (n=4) or after eight hours (n=4) a clear rescue of MyoD expression immediately around implanted beads (Fig. 7A,C). In contrast, control heparin beads never reinitiated MyoD expression around them (Fig. 7D). The upregulation of MyoD expression that was observed after eight hours of exposure to FGF rules out the possibility that cells that expressed MyoD migrated towards or proliferated around the beads. These results indicate that at least a portion of the limb bud progenitor cells which are arrested in their myogenic program are still competent to rapidly reinitiate muscle differentiation upon exposure to FGF. How can this myogenic response be initiated in such a short time? We have shown previously in vitro that although starved embryonic myoblasts display almost undetectable levels of FGFR4 RNA, they strongly upregulate its transcription upon addition of 1 ng/ml FGF in as little as two hours (Marcelle et al., 1994). The result is that, through this regulatory loop, a greater number of receptor molecules are produced, thus putatively leading to a strong amplification of the cellular response. Given these observations, it is conceivable that a similar response could take place in our experiments: in embryos infected with S-FR4-Fc, in which FGFR4 expression is greatly reduced; addition of ectopic FGF leads to a fast upregulation of FGFR4 transcription, which could in turn lead, eight hours after bead implantation, to the response we observed (i.e. myogenic differentiation).

Together, our data support a model whereby FGFR4 signaling promotes the differentiation rather than the proliferation of muscle progenitors present within the muscle masses.

DISCUSSION

This study was initiated by the observation that most, if not all, replicating myoblasts within the skeletal muscle masses of the developing embryo express high levels of FGFR4 (Marcelle et al., 1994; Marcelle et al., 1995). Together with the observation that no other FGF receptor displays such a strong and specific expression pattern in muscles, this suggested an important role for FGFR4 during myogenesis.

The data presented here clearly demonstrate that inhibition of FGFR4 signaling leads to a dramatic reduction in limb bud myogenic differentiation. This decrease varied between a slight (10%) and a total (100%) inhibition. Although this difference is likely to be because of different levels of infection, the observation that, in some embryos, we can completely abolish muscle differentiation, indicates that FGFR4 signaling is required for the differentiation of all limb bud myoblasts. In agreement with these observations, activation of FGF signaling in trunk somites leads to a strong activation of myogenesis differentiation.

FGFR4, but not FGFR1 signaling regulates limb bud myogenic differentiation

Although FGFR4 expression levels in skeletal muscles is at least an order of magnitude higher than that of FGFR1 in this tissue, it was possible that low FGFR1 expression might nonetheless be functionally significant. Therefore, it was important to specifically inhibit FGFR4 but not FGFR1 signaling during myogenesis. We have chosen to use a secreted competitive-inhibitor form of FGFR4, rather than a transmembranal dominant-negative form, because this might have heterodimerized with heterologous endogenous FGF receptors and thereby inhibit all FGF receptor signaling (Bellot

et al., 1991). The specificity of the phenotype we obtained is supported by the observation that a similar construct made with the extracellular portion of FGFR1 did not lead to any noticeable muscle phenotype, although both constructs are active in another biological assay (mesoderm formation in frogs). Our data support the assumption that FGFR4, but not FGFR1 signaling regulates limb bud myogenic differentiation. Evidently, our results imply that the ligand that mediates FGFR4 function during limb bud muscle differentiation does not bind to FGFR1 (Ig3c isotype). A multitude of splice variants have been described for all FGF receptors, and in particular for FGFR1. These isotypes bind to different FGFs with varying affinities (Johnson and Williams, 1993). Thus, although FGFR1 Ig3c isotype seems to be the prominent isotype of FGFR1 that is functional during embryogenesis (Partanen et al., 1998), it remains possible that other FGFR1 variants would be able to block myogenesis in our experimental model. Recently, a transmembranal inhibitory form of FGFR1 (Isotype 3c as well) has been used to challenge the differentiation of limb muscles; this led to a partial reduction of the muscle masses (Flanagan-Steet et al., 2000). In the light of the results presented here, it is likely that the molecule used in that study heterodimerized with the endogenous FGFR4 to block its signaling. A similar situation was encountered in mouse, where it was demonstrated that the FGFR3-dependent lens differentiation can be inhibited by a transmembranal, but not a secreted form of FGFR1 (Govindarajan and Overbeek, 2001; Robinson et al., 1995). Thus, the use of secreted forms of FGF receptors represents a technique of choice to test whether FGF receptors (and probably most growth factor receptors) have specific or redundant roles in vivo (Celli et al., 1998).

Because in mouse, as in chick, FGFR4 is highly expressed in all skeletal muscles of the developing embryo (Stark et al., 1991) (our observations), it is surprising that FGFR4-null mutant mice do not display any obvious muscle (or any other) phenotype (Weinstein et al., 1998). This indicates that FGFR4 function can be compensated for during myogenesis, most likely by another FGF receptor. Recently, it was shown that ectopic expression of FGF4 in limb muscles leads to a downregulation of FGFR4 expression and the concomitant upregulation of FGFR1 (Edom-Vovard et al., 2001), indicating a possible cross-talk between FGFR1 and FGFR4. In the timeframe of our experiments, we did not observe any upregulation of FGFR1 expression when FGFR4 signaling was inhibited. However, it would be informative to examine whether long-term inhibition of FGFR4 signaling in chick leads to any recovery of normal muscle differentiation, and whether this is paralleled by the up-regulation of FGFR1 or any of the other FGF receptors.

FGFR4 signaling is regulating myoblast differentiation

Upon ligand binding, FGF receptors can elicit a variety of responses, such as modifications in cell proliferation, differentiation and migration (reviewed by Boilly et al., 2000; Klint and Claesson-Welsh, 1999; Martin, 1998; Powers et al., 2000). All FGF receptors are able to trigger these biological responses, given the right experimental context. Not only can distinct ligands initiate specific responses, but also changing the time of exposure to a ligand or its concentration can modify these responses. Multiple FGFs are expressed in the developing limb bud: FGF6 and FGF2 are expressed by the limb muscle masses; FGF2, FGF5, FGF10 and FGF18 are present in the mesenchyme of the progress zone and FGF2, FGF4 and FGF8 in the AER and/or the limb ectoderm (deLapeyriere et al., 1993; Dono and Zeller, 1994; Fallon et al., 1994; Haub and Goldfarb, 1991; Isaac et al., 2000; Niswander and Martin, 1992; Ohuchi et al., 1997; Savage and Fallon, 1995; Crossley and Martin, 1995). Most of these ligands can bind to FGFR4 and FGFR1 (reviewed by Powers et al., 2000). Despite this multiplicity of signals, our results demonstrate a specific role for FGFR4 in vivo, such that its inhibition leads to an arrest of muscle progenitor differentiation, while their migration and proliferation is unaffected. The inhibition of FGFRF4 signaling by S-FR4-Fc is not phenocopied on addition of S-FR1-Fc. This reflects a tight regulation of the cellular response to FGFR4 signaling in muscle tissues. The molecular or cellular mechanisms that regulate this specificity in vivo are unknown. The microenvironment to which muscle progenitors are confronted (such as microgradients of signaling molecules), the extracellular matrix, which contains the glycosaminoglycan heparan sulfate critical to FGFR signaling, might play a role in the regulation of this specificity. Such variables might not be easily reconstituted in an in vitro system. Therefore, discrepancies between the in vitro and in vivo activity of FGF receptors are not surprising. Indeed, using an in vitro culture system, we observed results that were entirely in contradiction with the observations we made for S-FR4-Fc and S-FR1-Fc function in vivo. Thus, in vitro it appears that FGFR1 signaling seems to regulate myoblast differentiation (as reported in similar in vitro conditions) (Flanagan-Steet et al., 2000; Itoh et al., 1996), whereas FGFR4 signaling does not affect this process (C. M., unpublished). This emphasizes the importance of addressing the role of these molecules in vivo.

FGF8 is a myogenic differentiation factor in somites

Upon electroporation of FGF8 in trunk somites, we observed a strong upregulation of FGFR4 expression and a robust activation of myogenesis. Together with the observation that FGF8 can bind to S-FR4-Fc in vitro, and can partially rescue myogenesis in vivo after specific inhibition of FGFR4 signaling, this indicates that FGF8 might be one of the signals that triggers myogenic differentiation through FGFR4 signaling in vivo. FGF8 is strongly expressed by all myocytes (i.e. elongated, mononucleated, post-mitotic fibers) present in the somitic myotome (Crossley and Martin, 1995) (reviewed by Martin, 1998) (our observation). This strongly supports the notion that FGF8 is a major player in the muscle differentiation program of somites. Thus, as they enter the myotomal compartment of the somite, FGFR4-expressing muscle progenitors are placed in direct contact with myocytes that secrete a factor that promotes their myogenic differentiation. Interestingly, somitic myocytes also express FGF4; however, this molecule seems to have an opposite effect on myogenic differentiation, because its over-expression leads to a repression of myogenic differentiation (Edom-Vovard et al., 2001). These observations raise several interesting issues: an attractive hypothesis is that FGF4 and FGF8 maintain the balance between differentiating and non-differentiating populations in the growing muscle masses. Evidently, the respective roles of FGF8 and FGF4 will need to be clarified

further in the somitic environment. Another crucial point is to determine whether different FGF ligands mediate antagonistic responses through a single receptor, FGFR4, or through the activation of distinct receptors. It is important to note that neither FGF8 nor FGF4 are expressed by early limb muscle fibers, although muscle differentiation mediated by FGFR4 signaling is taking place. It is possible that yet unidentified members of the FGF family exert FGF8-like activities in the limb muscle fibers. Alternatively, the multiplicity of FGF molecules expressed around the muscle masses (see above) might play this role in the limb.

In past years, induction of epaxial versus hypaxial, somitic versus limb muscles have been the subject of intense and often confusing debates. Through the analysis of FGFR4 function, we provide here a unifying view on how all skeletal muscles of the body differentiate, once FGFR4-expressing cells have appeared within the different muscle-forming units of the embryo. It will become important to determine the tissue and molecular mechanisms that regulate the emergence of FGFR4-expressing muscle progenitors within the embryo.

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