Available online at www.sciencedirect.com

SCIENCE DIRECT®

Biochimica et Biophysica Acta 1642 (2003) 97–105

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

www.bba-direct.com

Injection of FGF6 accelerates regeneration of the soleus muscle in adult mice

Anne-Sophie Armand[a], Thierry Launay[a,b], Claude Pariset[a], Bruno Della Gaspera[a], Frédéric Charbonnier[a,c], Christophe Chanoine[a,*]

[a] *Laboratoire de Biologie du Développement et de la Différenciation Neuromusculaire, LNRS UMR 7060, Centre Universitaire des Saints-Pères, Université René Descartes, 45 rue des Saints-Pères, F-75720 Paris Cedex 06, France*

[b] *UFR STAPS, Université René Descartes, France*

[c] *Département STAPS, Université d'Evry, Evry, France*

Received 3 June 2003; received in revised form 21 July 2003; accepted 22 July 2003

Abstract

FGF6, a member of the fibroblast growth factor (FGF) family, accumulated almost exclusively in the myogenic lineage, supporting the finding that FGF6 could specifically regulate myogenesis. Using FGF6 (−/−) mutant mice, important functions in muscle regeneration have been proposed for FGF6 but remain largely controversial. Here, we examined the effect of a single injection of recombinant FGF6 (rhFGF6) on the regeneration of mouse soleus subjected to cardiotoxin injection, specifically looking for molecular and morphological phenotypes. The injection of rhFGF6 has two effects. First, there is an up-regulation of cyclin D1 mRNA, accounting for the regulating role of a high FGF6 concentration on proliferation, and second, differentiation markers such as CdkIs and MHC I and Tn I increase and cellular differentiation is accelerated. We also show a down-regulation of endogenous FGF6, acceleration of FGFR1 receptor expression and deceleration of the FGFR4 receptor expression, possibly accounting for biphasic effects of exogenous FGF6 on muscle regeneration.

Keywords: FGF; FGF6; Growth factor; Muscle regeneration; Soleus

1. Introduction

Skeletal muscle regeneration occurs following injury and is sustained by mononucleated muscle cells, called satellite cells [1]. Following injury, these quiescent mononucleated stem cells are activated. After proliferation, the descendants of the satellite cells called muscle precursor cells (mpc) leave the cell cycle and fuse, forming multinucleated myotubes which consequently replace the damaged muscle. The proliferation and differentiation of the satellite cells into muscle fibers are controlled by a network of growth factors, signaling molecules and transcription factors [2].

Fibroblast growth factors (FGFs) make up a large family of polypeptide growth factors that have diverse roles, during embryonic development, in regulating cell proliferation, migration and differentiation [3]. In the adult organism, FGFs are homeostatic factors and function in tissue repair and response to injury [3]. The FGFs transduce their signals to the cell through transmembrane tyrosine kinase receptors (FGFRs) for which four distinct genes have been discovered (FGFR1–FGFR4) [4]. Among the FGF family members, FGF6 exhibits a restricted expression profile predominantly in the myogenic lineage in adult and developing skeletal muscle [5,6] that has suggested that it may be a component of signaling events associated with the somite [7] as well as the regeneration of the adult muscle [8]. FGF6 preferentially uses FGFR1 and FGFR4 for signal transduction [9,10]. Mutations in growth factor receptors expressed in skeletal muscle resulted either in early embryonic lethality (FGFR1) [11–13], thereby preventing analysis of their functions in muscle cells, or in no apparent phenotypic alterations of muscle development and its maintenance (FGFR4) [14]. In vitro studies indicated that FGF6 differentially regulated the expression of FGFR1 and FGFR4 in the C2 myoblast cells [8]. FGF6 stimulated the proliferation of C2 myoblasts and increased the expression of muscle cell differentiation markers or delayed differentiation into myotubes at low or

* Corresponding author. Fax: +33-1-42-86-21-19.
E-mail address: chanoine@biomedicale.univ-paris5.fr (C. Chanoine).

0167-4889/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0167-4889(03)00103-4

high concentrations, respectively [8]. However, the effect of exogenously supplied FGF6 has never been studied during myogenesis in vivo. To better understand the importance of FGF6 for muscle regeneration, mice with a homozygous disruption of the FGF6 gene have been generated in two laboratories and skeletal muscle regeneration has been studied in these FGF6 (− / −) mice, but this has given rise to contradictory results. In one case, a regeneration defect accompanied by fibrosis was associated with the absence of a functional FGF6 gene [15] whereas in the other case, the kinetics of muscle regeneration was not affected [16].

In this work, to provide a better insight into the involvement of FGF6 in muscle regeneration, we examined the molecular and morphological effects of a single injection of recombinant FGF6 on the regeneration of mouse soleus subjected to cardiotoxin injection. Using semi-quantitative RT-PCR assays, we monitored the expression of regulatory genes involved in proliferation, cell cycle sorting and differentiation in relation to morphological changes in rhFGF6-injected regenerating soleus versus regenerating control soleus.

2. Materials and methods

2.1. Animals and muscle injury

Studies were carried out on adult *Mus musculus* C3H (about 30 g) originating from the breeding center R. Janvier (Le Genest Saint-Isle, France). Animals were anesthetized by intraperitoneal injection of 3.5% chloral hydrate (1.7 ml/100 g). The skin was cut and 35 μl of cardiotoxin (10^{-5} M in 0.9% NaCl) from *Naja mossambica nigricollis* venom (Latoxan; Valence, France) with 3 μl of recombinant human FGF6 (rhFGF6) (0.1 mg/ml in PBS 0.1% BSA; Sigma), unless indicated otherwise, 6 μl of heparin (0.1 mg/ml; Sigma) and 26 μl of PBS 0.1% BSA was injected into the left soleus muscle. In the right control soleus muscle, we injected 35 μl of cardiotoxin (10^{-5} M in 0.9% NaCl, Latoxan) with 6 μl of heparin (0.1 mg/ml; Sigma) and 29 μl of PBS 0.1% BSA. In order to follow the regeneration process, soleus muscles were collected at a 12-h interval for a total duration of 196 h following injection.

2.2. Semi-quantitative RT-PCR and Southern blotting

Total RNA was isolated from mouse soleus samples as described by Chomczynski and Sacchi [17]. First-strand cDNAs were synthesized from 1 μg of total RNAs by oligo (dT) priming using Superscript reverse transcriptase (Invitrogen) at 37 °C for 1 h. Complementary DNA was then used as a template for the PCR in a 50-μl reaction volume including 0.25 μM of each primer, 100 μM dNTPs, Taq buffer and 1 μl of Taq polymerase (ATGC). The primers used for amplification are listed in Table 1. The PCR conditions for the analysis of the expression of each gene were designed to avoid PCR saturation and to enable semiquantitative determination. Each data point was normalized by the abundance of glyceraldehyde phosphate dehydrogenase (GAPDH). For Southern blot analysis, 15 μl of the products of each PCR was loaded on a 1% agarose gel, transferred onto Hybond-N nylon membrane (Amersham Biosciences), and hybridized overnight at 45 °C with ^{32}P-labeled 20-mer primers. The primers (see Table 2) were ^{32}P-labeled at their 3′-ends by incorporation of [^{32}P] dCTP using terminal transferase (Invitrogen) according to the

Table 1
Primers used in PCR

Gene	Product size (bp)	Forward primer	Reverse primer
GAPDH	377	5′ TCCTGCACCACCAACTGCTTAGCC 3′	5′ TAGCCCAAGATGCCCTTCAGTGGG 3′
MRF4	475	5′ TTAGAAGTGGCAGAGGGCTC 3′	5′ AGGTGCGCAGGAAATCCGCA 3′
Myf-5	428	5′ GGGCAGAATACGTGCTTTTC 3′	5′ TATACCATTAGGCACAACTG 3′
Myogenin	485	5′ TGTCCTGATGTCCAGAAAAC 3′	5′ TTGGCAGACGGCAGCTTTAC 3′
MyoD	408	5′ GGATGGTGCCCCTGGTTCTT 3′	5′ TACACCTGAGACCTGAGTGG 3′
MEF2A	545	5′ GGATAAGCAGTTCTCAAGCC 3′	5′ TATTAGCAGGTCGGCCAAGC 3′
MEF2C	467	5′ AAGAAACACGGGGACTATGG 3′	5′ CTGGACACTGGGATGGTAAC 3′
FGFR1	472	5′ CTAACCGCAGAACTGGGATG 3′	5′ AGCTACAGGCCTACGGTTTG 3′
FGFR2	430	5′ AGTGTTCACTACTCGCCAGC 3′	5′ CGGTAATCCCATCTGCACAC 3′
FGFR3	398	5′ TCTCCACAGAGGCGTTCTCC 3′	5′ GGGAGGCATTTAGCACTTGC 3′
FGFR4	467	5′ CTCACGTGGACAACAGCAAC 3′	5′ CACAGCCACGGTGCTGGTTT 3′
Cycline D1	489	5′ TGTGCTGCGAAGTGGAGACC 3′	5′ GGCATTTTGGAGAGGAAGTG 3′
Cdk4	489	5′ TGGGACGGTGTACAAAGCCC 3′	5′ GTACCAGAGCGTAACCACCA 3′
P15	408	5′ CCACCCCGCCTATTTGTCTC 3′	5′ TTCAGGGCGTTGGGATCTGC 3′
P16	421	5′ GATTGGGCGGGCACTGAATC 3′	5′ CGCACGATGTCTTGATGTCC 3′
P21	417	5′ ACTCCTACTTCTGTGGACAT 3′	5′ CAGGGCTCAGGTAGACCTTG 3′
P27	482	5′ CAGAAATCTCTTCGGCCCGG 3′	5′ TTCTTGGGCGTCTGCTCCAC 3′
P57	482	5′ AGCAGAACAGCGATGGAACG 3′	5′ CTCCGGAATTGGGTCTGAGG 3′
MHC-I	373	5′ CGGTGAAGGGCATGAGGAAG 3′	5′ TAAAGGGCTGTTGCAAAGGC 3′
Myoglobine	264	5′ CTGTGCCTGGCAGAGTCCGG 3′	5′ CTAAGCCCAGCTGAAAGTGG 3′
TnIs	426	5′ TGTCTCAGCCGATGCCATGC 3′	5′ GTACCATAAGCCCACACTGC 3′

Table 2
Primers used for Southern blot experiments

Gene	Sequence
GAPDH	5′ AAAGCTGTGGCGTGATGGCC 3′
MRF4	5′ ACATTGAGCGTCTACAGGAC 3′
Myf-5	5′ GGAGGCAATTAATTGACAGT 3′
Myogenin	5′ CACATAAGGCTAACACCCAG 3′
MyoD	5′ AAGGCCACTTGCACTCTGGC 3′
MEF2A	5′ CTGGAGGGCAGTTATCTCAG 3′
MEF2C	5′ GTGACTGTGAGATTGCACTG 3′
FGFR1	5′ CGACCTGCTACAGCTTCGCT 3′
FGFR2	5′ AGGAGCGCTGCCATTCAAGT 3′
FGFR3	5′ TGGTCCAGAGCAGCGAGTTG 3′
FGFR4	5′ GAGGTCCTCTGGCAAGTCAA 3′
Cycline D1	5′ TGAACTACCTGGACCGCTTC 3′
Cdk4	5′ CAAGGTCACCCTAGTGTTTG 3′
P15	5′ AGAGACCAGGCTGTAGCAAT 3′
P16	5′ TCTGGAGCAGCATGGAGTCC 3′
P21	5′ ATGTCCAATCCTGGTGATGT 3′
P27	5′ CGGTGCCTTTAATTGGGTCT 3′
P57	5′ CAGGATGTGCCTCTTCGAGG 3′
MHC-I	5′ AGTTCCGCAAGGTGCAGCAC 3′
Myoglobine	5′ GGAGCATGGGAGCACAACCT 3′
TnIs	5′ CGACGCTGCTAAGTCCCCGA 3′

manufacturer's recommendations. The blots were washed twice at room temperature with buffer containing 2 × SSC and 0.1% SDS. Signals were detected by autoradiography. All the RT-PCR experiments were repeated five times in the same conditions, and for each gene expression analysis, the PCR was repeated twice with comparable results.

2.3. Histology

Muscles were fixed in 4% paraformaldehyde in PBS, dehydrated and infiltrated with paraffin. Then 7-μm-thick serial sections were mounted on TESPA-coated glass slides. Sections were deparaffinized in xylene, rehydrated through an ethanol series, and stained with hemalun and eosin for structural analysis. One section of each stage originating from six distinct experimental muscles was analyzed.

2.4. Immunoblotting analysis of FGF6 protein

Soleus muscles collected were frozen in liquid nitrogen, pulverized and homogenized in 5 volumes of ice-cold buffer, consisting of 100 mM KCl, 20 mM Tris–HCl (pH 7.5), 1 mM DTT and protease inhibitors (50 μg ml^{-1} PMSF (phenylmethylsulfonyl fluoride, Sigma), 50 g ml^{-1} aprotinin (Sigma), 50 μg ml^{-1} leupeptin (Sigma)), and centrifuged (4 °C, 20 min, 13,500 rpm). The resulting pellets were reacted with Laemmli SDS sample buffer (1 × final concentration [18]). The final tissue extracts were heated at 95 °C for 5 min, separated on a 12% SDS-PAGE and transferred to PVDF membrane (Bio-Rad, France). The nonspecific binding sites were blocked in PBST (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) 1% nonfat milk powder for 1 h. Membranes were probed overnight at 4 °C with the anti-FGF6 antibody (1:2000; TEBU). After washing for 30 min in several changes in PBST, immunoblots were incubated for 1 h at room temperature with the secondary antibody HRP-conjugated anti-goat sheep immunoglobulin (1:5000; Sigma). Several washes in PBST were performed for 30 min each, and antibody binding was revealed using the ECL system (Amersham Biosciences Europe). This experiment was repeated three times with comparable results.

3. Results

3.1. Morphological effects of FGF6 injection on regeneration of soleus

The sequence of histological changes observed in regenerating mouse muscle following snake toxin injury has been previously described [19,20]. A single injection of cardiotoxin causes an almost complete degeneration of the myofibers within 24 h. Following a significant proliferation of myoblasts, forming myotubes were observed from 3 days post-injection (Fig. 1A and B). Numerous young myotubes with central nuclei, as well as myoblasts that lined up and fused, were identified at 4 days P-I (Fig. 1C and D). Comparison of the transverse sections of control regenerating soleus with those of recombinant human FGF6 (rhFGF6)-injected soleus clearly showed an acceleration of the differentiation process in rhFGF6-injected regenerating muscles. This was particularly visible at 4 days P-I, when myotubes with central nuclei were the predominant muscle cells observed in rhFGF6-injected regenerating soleus (Fig. 1C). Moreover, these myotubes exhibited a larger surface area in rhFGF6-injected muscle in comparison to corresponding control muscle. Indeed, the area of the individual regenerating fibers in the rhFGF6-injected muscle is significantly greater (1.5-fold) than the area of the individual myotubes in the control muscle.

3.2. Detection of FGF6 protein in control and FGF6-injected regenerating muscles

The effect of rhFGF6 injection on the morphological changes of regenerating soleus led us to further investigate the timing of accumulation of FGF6 protein in regenerating soleus. The expression pattern of FGF6 protein was determined in control as well as in rhFGF6-injected regenerating muscles. As first shown by Kästner et al. [21], FGF6 could solely be extracted from the particulate fraction but not from the soluble fraction of the muscle extract. Fig. 2 depicts a Western blot analysis of soleus particulate fractions at different times after cardiotoxin injection. Two signals were detected. The higher molecular mass (20 kDa) form corresponds to the murine native FGF6 protein, whereas the lower molecular mass signal (14 kDa) might arise from the native form by an N- and C-terminal truncation of the endogenous native peptide, as suggested by Kästner et al.

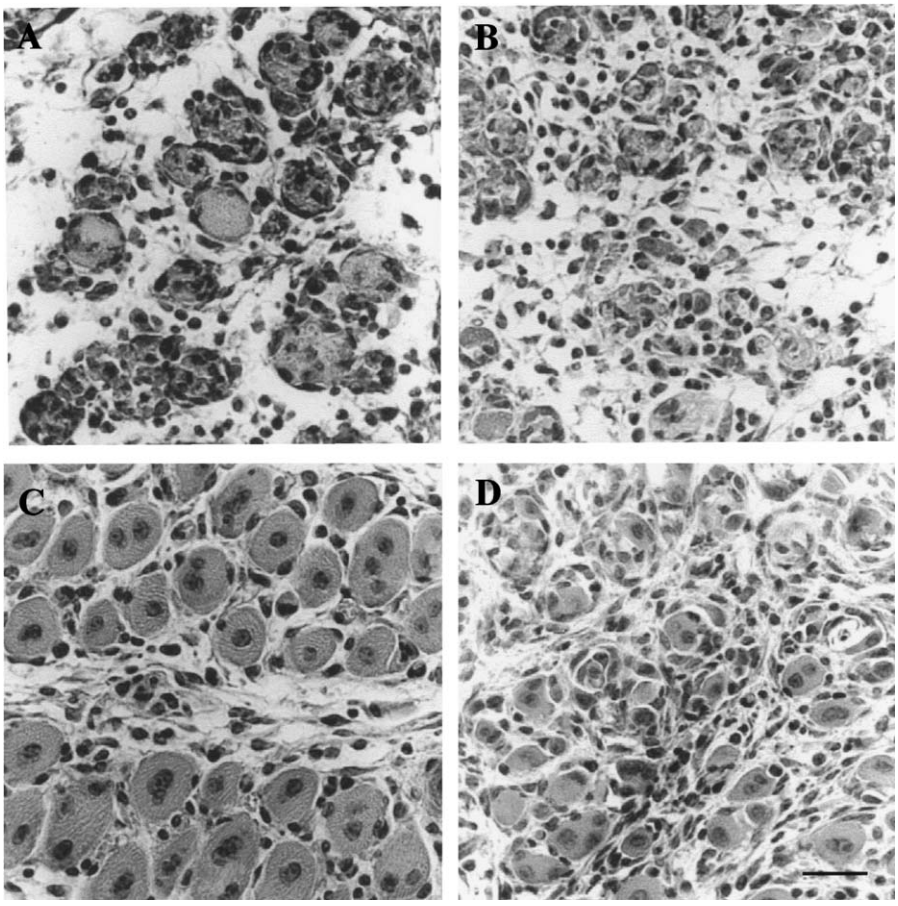

Fig. 1. Hematoxylin–eosin histological stains of transverse section of regenerating soleus muscle 3 days (A, B) and 4 days (C, D) following cardiotoxin injury. (A, C) rhFGF6-injected regenerating muscles. (B, D) uninjected regenerating muscles. Scale bar, 8 μm.

[21]. The endogenous FGF6 protein was detected from 24 h P-I and peaked at 72 to 84 h P-I in soleus regenerating muscle. The recombinant human FGF6 used in our injection experiments has a predicted molecular mass of 14 kDa according to the supplier (Fig. 3). Following a co-injection of cardiotoxin and 0.3 μg of rhFGF6, the exogenous FGF6 was still highly detected 6 h following injection in the regenerating soleus and a barely detectable 14-kDa signal could still be seen 12 h after injection (data not shown) (Fig. 3). During the same period, the endogenous FGF6 was not yet detected in regenerating soleus as already shown in Fig. 2. Analysis by Western blot of the accumulation of the endogenous FGF6 protein after the injection of 0.3 μg of rhFGF6 revealed down-regulation of the endogenous FGF6 accumulation (Fig. 2). Indeed, the endogenous FGF6 protein was both barely detectable and belatedly expressed (at 48 h P-I) in the rhFGF6-injected regenerating soleus.

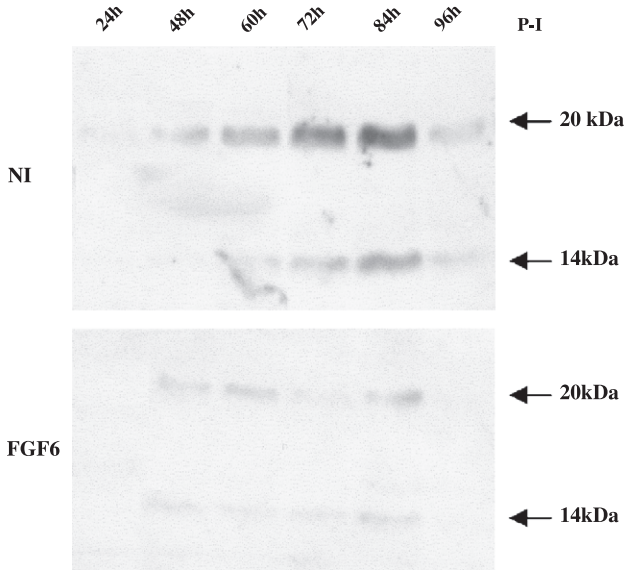

Fig. 2. Western blotting analysis of the endogenous FGF6 protein at different stages of soleus regeneration, from 24 to 96 h P-I. (FGF6) rhFGF6-injected regenerating muscles. (NI) Uninjected regenerating muscles. (P-I) Post injection of cardiotoxin. Two bands of 14 and 20 kDa were detected.

3.3. Analysis of gene expression by RT-PCR

Given that FGFs are involved in myoblast proliferation in vitro [22], we suspected that the expression of one or more genes encoding cell cycle control proteins may be affected in

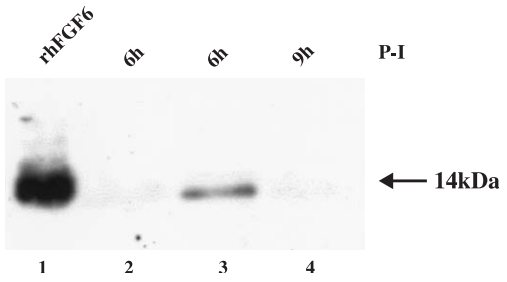

Fig. 3. Accumulation pattern of the injected rhFGF6 protein in the first stages of soleus regeneration. Lane 1, 0.75 μg of rhFGF6 (control); lane 2, the particulate fraction of uninjected regenerating soleus at 6 h following cardiotoxin injury. Lanes 3 and 4, the particulate fractions of rhFGF6-injected regenerating soleus at 6 and 9 h following cardiotoxin injury, respectively. Only one band of 14 kDa was detected at these stages of regeneration. (P-I) Post injection of cardiotoxin.

regenerating soleus following FGF6 injection. For this reason, we chose to analyze the expression of the different FGF receptors, the Cdks, their associated cyclins and the cyclin-Cdk inhibitors (CdkIs), which are players regulating various phases of cell cycle. It is well established that cell cycle arrest can be mediated through the inactivation of cyclin-dependent kinases (cdks) by cdk inhibitors (cdkIs), of which there are two families ($p16^{INK4a}$ and $p21^{Cip1}$).

The analysis of the accumulation of the four FGFR transcripts during soleus regeneration showed that the expression patterns of FGFR2 and FGFR3 were not modified by the injection of rhFGF6 (Fig. 4). In contrast, FGF6 treatment up-regulated and down-regulated the expression of FGFR1 and FGFR4, respectively. Indeed, FGFR1 transcripts were detected more precociously in rhFGF6-injected regenerating muscles, at 48 h P-I instead of at 60 h P-I as in control regenerating soleus. The appearance of FGFR4 mRNA was delayed by 12 h in FGF6-injected regenerating soleus and was detected at 72 h P-I instead of 60 h P-I as in control regenerating muscle.

Cyclin D1 transcripts were detected from 36 h P-I in the regenerating soleus (Fig. 5). A dose-dependent effect on the accumulation of cyclin D1 mRNAs was observed for rhFGF6 intramuscular injection. Whereas the injection of 0.2-μg rhFGF6 had no detectable effect on the expression of cyclin D1 transcripts, the injection of 0.3-μg and of 0.6-μg rhFGF6 resulted in a more precocious accumulation of the cyclin D1 transcripts 24 and 12 h, respectively, after cardiotoxin injection instead of 36 h P-I as for control injected soleus

RT-PCR analysis showed that whereas Cdk gene expression was not affected (data not shown), members of the two families of CdkIs, the $p16^{INK4a}$ and $p21^{Cip1}$ families, were up-regulated in rhFGF6-injected regenerating soleus (Fig. 6). $p21^{Cip1}$ mRNA was detected very early, 6 h following cardiotoxin injection in control regenerating soleus, and the level of $p21^{Cip1}$ transcript was up-regulated in the first stages of regeneration in rhFGF6-injected soleus. It should be noted that the other members of the $p21^{Cip1}$ family,

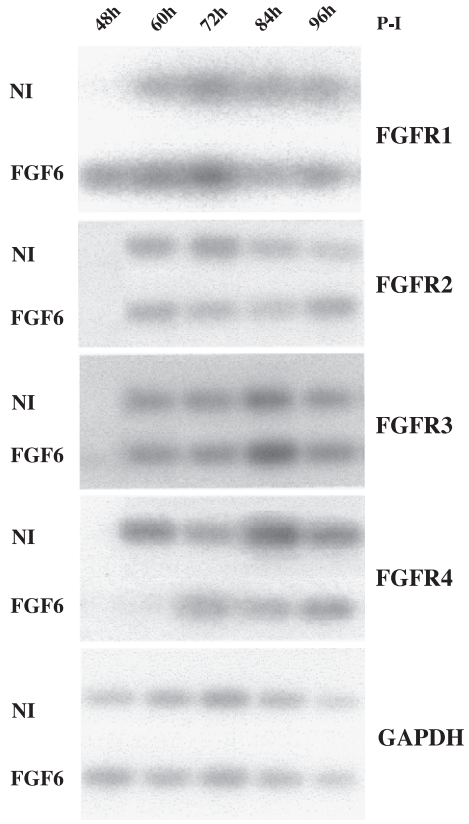

Fig. 4. RT-PCR analysis of FGFR gene expression in rhFGF6-injected regenerating soleus (FGF6) versus uninjected regenerating muscles (NI). (P-I) Post injection of cardiotoxin. FGFR1 and FGFR4 were the only two FGFRs subjected to a regulation by FGF6.

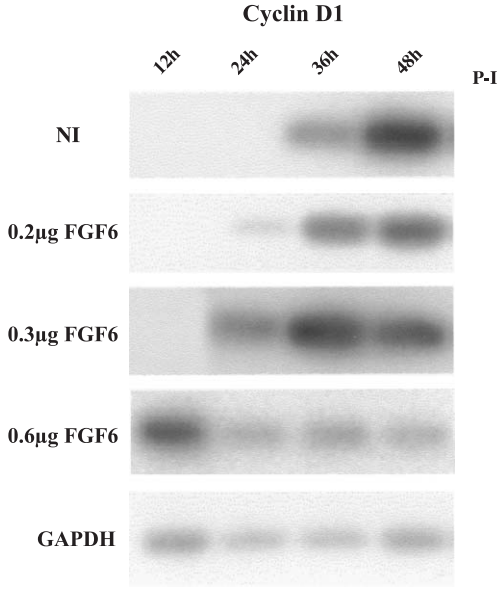

Fig. 5. RT-PCR analysis of Cyclin D1 gene expression in 0.2 μg rhFGF6 (0.2μg FGF6), 0.3 μg rhFGF6 (0.3μg FGF6) and 0.6 μg rhFGF6 (0.6μg FGF6)-injected regenerating soleus versus uninjected regenerating muscles (NI). (P-I) Post injection of cardiotoxin.

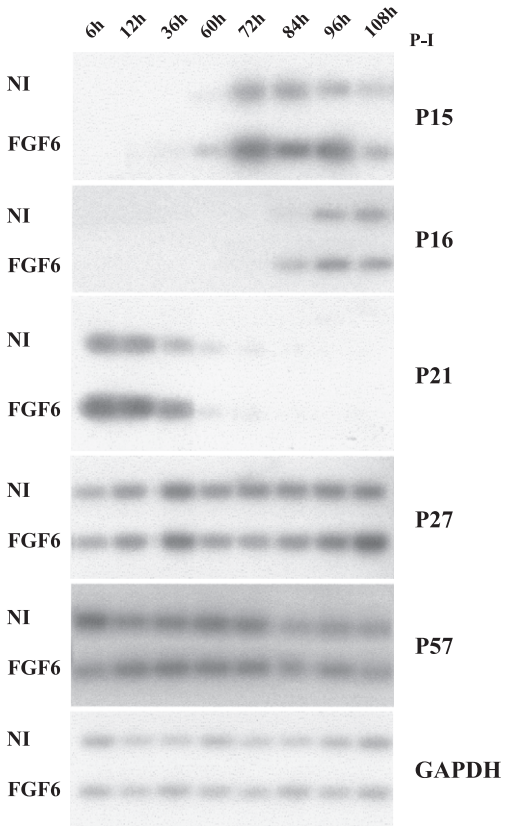

Fig. 6. RT-PCR analysis of CdkIs gene expression in rhFGF6-injected regenerating soleus (FGF6) versus uninjected regenerating muscles (NI). (P-I) Post injection of cardiotoxin.

$p27^{Kip1}$ and $p57^{Kip2}$, were not regulated by the injection of rhFGF6, showing the specificity of the regulating control of FGF6. $p15^{INK4b}$ and $p16^{INK4a}$ mRNA accumulated later than $p21^{Cip1}$ in regenerating soleus at 72 and 96 h P-I, respectively. They appeared more precociously and the level of $p15^{INK4b}$ mRNA was positively regulated in regenerating rhFGF6-injected soleus as shown in Fig. 6. Thus, these results indicated a positive effect of the injection of FGF6 on the expression of several CdkIs involved in cell cycle withdrawal.

In myogenic tissue culture systems, the link between proliferation and differentiation has been proposed to be via MyoD and $p21^{Cip1}$. Members of the MyoD family (MyoD, Myf-5, myogenin and MRF4) are muscle transcription factors required for the determination and/or the differentiation of skeletal myoblasts [23]. Upon serum withdrawal, 10T1/2 cells transfected with MyoD can transcriptionally up-regulate $p21^{Cip1}$ expression, causing cell cycle arrest and subsequent myotube fusion [24–26]. For this, we performed RT-PCR analysis for the four members of the MyoD family and for members of the myocyte enhancer factor 2 family, MEF2A and MEF2C, which also control muscle development [27].

In regenerating soleus, the MRF expression pattern accounted for the previous in situ hybridization studies

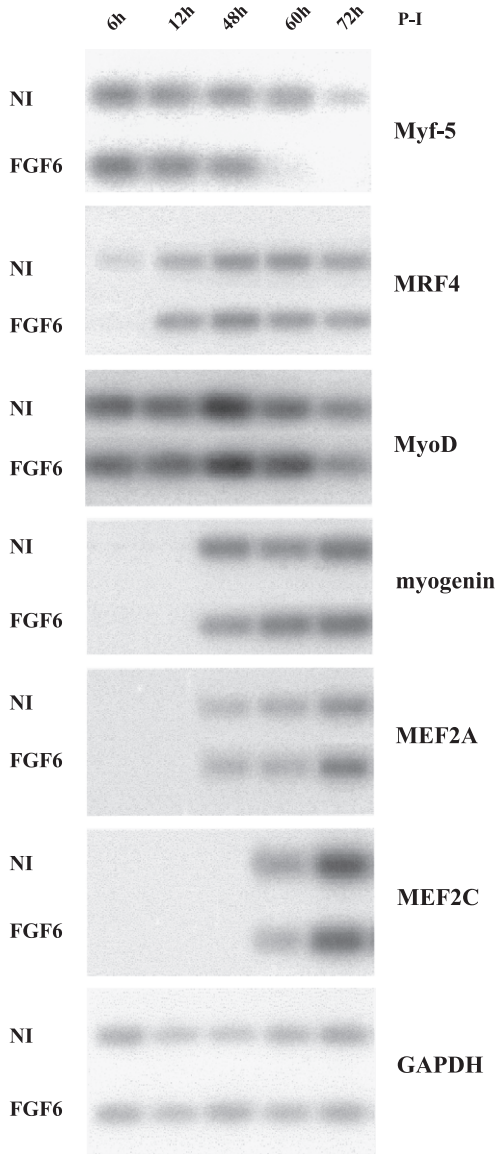

Fig. 7. RT-PCR analysis of MRF and MEF2 gene expression in rhFGF6-injected regenerating soleus (FGF6) versus uninjected regenerating muscles (NI). (P-I) Post injection of cardiotoxin.

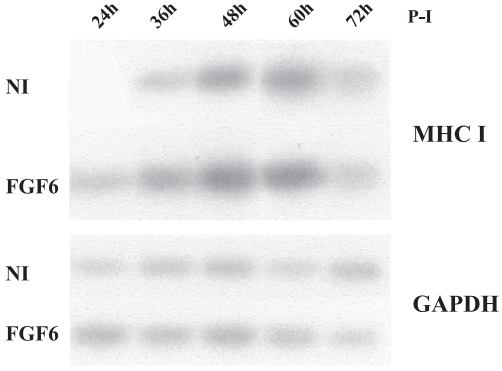

Fig. 8. RT-PCR analysis of MHC I gene expression in rhFGF6-injected regenerating soleus (FGF6) versus uninjected regenerating muscles (NI). (P-I) Post injection of cardiotoxin.

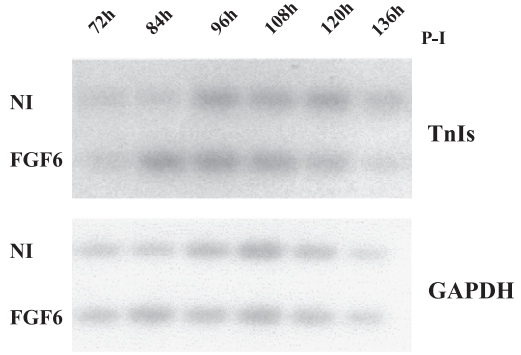

Fig. 9. RT-PCR analysis of Tn I gene expression in rhFGF6-injected regenerating soleus (FGF6) versus uninjected regenerating muscles (NI). (P-I) Post injection of cardiotoxin.

[20]. rhFGF6 injection did not modify the pattern of expression of MyoD (or that of myogenin and MRF4) (Fig. 7); the only significant change was the more precocious disappearance of Myf-5 transcripts in rhFGF6-injected regenerating soleus (Fig. 1). No changes were detected in the accumulation of MEF2 mRNAs in regenerating soleus following rhFGF6 injection as compared to control regenerating soleus.

Since our results showed that rhFGF6 injection accelerated cell cycle withdrawal as well as cell differentiation (see Figs. 1 and 6) in the regenerating soleus, we analyzed the effect of the injection of FGF6 on the expression of two slow muscle structure genes: the myosin heavy chain I (MHC I) (Fig. 8) and the slow isoform of Troponin I (Tn I) (Fig. 9). In both cases, the injection of rhFGF6 accelerated the appearance of the transcripts during regeneration, the MHC I mRNA was detected at 24 h P-I instead of 36 h P-I as in control regenerating soleus and the Tn I mRNA was strongly up-regulated at 84 h P-I instead of 96 h P-I as in control regenerating soleus.

4. Discussion

The investigations described here were carried out to begin defining the FGF6 signaling pathways that act during regeneration of mouse soleus. We have taken a comprehensive approach of analyzing at a histological level the effects of a single injection of FGF6 on the regeneration of mouse soleus following cardiotoxin injury, in combination with exploring the gene expression patterns of FGF receptors, different players regulating phases of cell cycle, as well as myogenic markers. We also performed an analysis of the accumulation of FGF6 protein in control and rhFGF6-injected regenerating muscles with the aim of better understanding the regulatory role of FGF6 in myogenesis.

In vitro analysis showed that FGFs, like many growth factors, induced the proliferation of myoblasts [28,29]. The injection of neutralizing antibodies against bFGF into regenerating muscle reduced the number and diameter of regenerating myofibers, suggesting a delay in proliferation and/or fusion of activated satellite cells [30]. In one model, FGF6 (−/−) mutant mice showed a severe regeneration defect with fibrosis and myotube degeneration. The number of MyoD- and myogenin-expressing activated satellite cells after injury was significantly reduced in these mutants, suggesting to the authors that FGF6 is a critical component of the muscle regeneration machinery in mammals, possibly by stimulating or activating satellite cells [15]. The present report showed that a single injection of FGF6 in regenerating soleus has a biphasic effect, involving, first, proliferation characterized by an up-regulation of cyclin D1, followed secondly by an increase of differentiation markers (such as CdkIs and MHCI and TnI) as well as of cellular differentiation.

Whereas FGF6 protein was not detected in soleus of the adult rat [31], we showed that FGF6 protein transiently accumulated in regenerating mouse soleus and that the injection of rhFGF6 down-regulated the accumulation of the endogenous FGF6. Floss et al. [15] previously reported that FGF6 transcripts were up-regulated after muscle injuries, which is consistent with our observations. It is well established that growth factors of the FGF family are able to regulate their own signaling pathways [32,33]. However, to our knowledge, the down-regulation of one endogenous FGF by its own exogenous injection, as shown here for FGF6, has not been reported; this could account for the biphasic effect of the injection of FGF6 reported in this in vivo study.

The fact that rhFGF6 injection up-regulated and down-regulated the expression of FGFR1 and FGFR4, respectively, in vivo in regenerating soleus, as previously shown in vitro [8], should be discussed in relation to the potential involvement of both FGFR1 in proliferation and FGFR4 in differentiation. Both FGF6 and FGFR4 were uniquely expressed by myofibers and satellite cells; FGFR1 was ubiquitously expressed by myogenic and nonmyogenic cells [21]. FGF receptors have different signaling and mitogenic potentials [34,35]. In skeletal muscle satellite cells in culture, it has been shown that FGFR1 is required for proliferation and to repress differentiation by an ERK1/2 signaling cascade [36]. In contrast, in vivo inhibition of FGFR4 signal resulted in an arrest of muscle progenitor differentiation [37].

The correlation between the FGF6 expression when satellite cells initiate proliferation and the effect of FGF6 on enhancing the number of proliferating satellite cells in vitro [21] suggested that FGF6 is also involved in vivo in the proliferation of satellite cells. The positive regulation of the cyclin D1 mRNA induced by rhFGF6 injection in regenerating soleus, similar to that observed for other growth factors such as FGF2 [38], accounted for the involvement of FGF6, at high concentration, on proliferation, probably by a signaling pathway emanating from FGFR1. At a later time, following the down-regulation of the endogenous FGF6, the FGF6 signaling through

FGFR4 could have a positive effect on muscle differentiation, increasing CdkIs as well as myogenic markers such as slow Tn I and MHC I. In accounting for the specific myogenic expression of FGF6 and FGFR4 [21], we suggest that, in regenerating soleus, FGFR1 may regulate ongoing proliferation of myoblasts, acting in a similar manner to its action in other cell systems whereas FGFR4 might be involved in a myogenic-specific pathway. The up-regulation of $p15^{INK4b}$, $p16^{INK4a}$ and $p21^{Cip1}$ by rhFGF6 injection would account for growth arrest and the subsequent acceleration of myogenic differentiation observed in regenerating soleus. The $p16^{INK4a}$ family ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$) specifically inhibits cdk4 and cdk6, while the $p21^{Cip1}$ family ($p21^{Cip1}$, $p57^{Kip2}$ and $p27^{Kip1}$) inhibits all cdks involved in the G1/S transition (reviewed by Sherr and Roberts [39]). Considering the role of MRFs and MEF2 proteins in muscle differentiation [23,27], it appears surprising that neither MRF nor MEF2 were positively regulated in regenerating soleus following FGF6 injection. These results contrast with those of Pizette et al. [8] that indicated that FGF6 added at low levels increased the expression of MyoD and myogenin in culture of C2 myoblasts. This accounted for the requirement of myogenin in the myoblast-to-myotube transition, including myoblast fusion and terminal differentiation [23]. Using a MyoD (−/−) mutant, Megeney et al. [40] showed that MyoD plays a crucial role in satellite cell function, the transition from proliferation to differentiation being delayed in satellite cells from mice lacking MyoD [41]. The more precocious disappearance of Myf-5 transcripts in rhFGF6-injected versus non-injected regenerating soleus could reflect the acceleration of the myogenic differentiation in rhFGF6-injected animals, since we have previously shown that Myf-5 mRNA transiently accumulated in the first stages of soleus regeneration followed by a significant decrease in forming myotubes [20]. MRFs and MEF2 proteins are subjected to posttranslational regulation and numerous studies point out the crucial role of the phosphorylations on the functional activity of these transcription factors [42–44]. For this reason, a better understanding of the involvement of MRFs as well as members of the MEF2 family in the FGF6 signaling pathways should be considered in relation to their phosphorylation status.

Among the members of the different growth factor families (TGFs, IGFs, FGFs) regulating muscle regeneration, to our knowledge, IGF1 was the only growth factor able to stimulate both mpc proliferation and muscle hypertrophy (increase in protein content and size of myofibers) during muscle regeneration [2]. This role was supported by experiments in which direct infusion of IGF1 into the tibialis anterior muscles of adult rat led to increased total muscle protein and DNA content, demonstrating skeletal muscle hypertrophy concomitant with satellite cell activation [45,46]. Here, using an in vivo model, we strongly suggested that one member of the FGF family, FGF6, also has a dual function in proliferation and muscle differentiation. This study permitted us to identify clearly a subset of genes regulated by FGF6 during muscle regeneration. Future analysis of the expression pattern of these FGF6-regulated genes in regenerating muscles of the FGF6 (−/−) mouse in combination with rescue experiments by injection of FGF6 is likely to provide new insights into the function of FGF6 in adult muscle.

Acknowledgements

Anne-Sophie Armand held a doctoral fellowship from the Ministère de l'Education Nationale de la Recherche et de la Technologie (MENRT). We thank R. Cassada for critical reading of the manuscript.

References

[1] A. Mauro, Satellite cells of skeletal muscle fibers, J. Biophys. Biochem. Cytol. 9 (1961) 493–498.
[2] P. Seale, M.A. Rudnicki, A new look at the origin, function, and "stem-cell" status of muscle satellite cells, Dev. Biol. 218 (2000) 115–124.
[3] D.M. Ornitz, N. Itoh, Fibroblast growth factors, Genome Biol. 2 (2001) 3005–3012.
[4] G. Szebenyi, J.F. Fallon, Fibroblast growth factors as multifunctional signaling factors, Int. Rev. Cytol. 185 (1999) 45–106.
[5] O. deLapeyriere, V. Ollendorff, J. Planche, M.O. Ott, S. Pizette, F. Coulier, D. Birnbaum, Expression of the Fgf6 gene is restricted to developing skeletal muscle in the mouse embryo, Development 118 (1993) 601–611.
[6] J.K. Han, G.R. Martin, Embryonic expression of FGF6 is restricted to the skeletal muscle lineage, Dev. Biol. 158 (1993) 549–554.
[7] S. Grass, H.H. Arnold, T. Braun, Alterations in somite patterning of Myf-5-deficient mice: a possible role for FGF-4 and FGF6, Development 122 (1996) 141–150.
[8] S. Pizette, F. Coulier, D. Birnbaum, O. deLapeyriere, FGF6 modulates the expression of fibroblast growth factor receptors and myogenic genes in muscle cells, Exp. Cell Res. 224 (1996) 143–151.
[9] S. Vainikka, J. Partanen, P. Bellosta, F. Coulier, D. Birnbaum, C. Basilico, M. Jaye, K. Alitalo, Fibroblast growth factor receptor-4 shows novel features in genomic structure, ligand binding and signal transduction, EMBO J. 11 (1992) 4273–4280.
[10] D.M. Ornitz, J. Xu, J.S. Colvin, D.G. McEwen, C.A. MacArthur, F. Coulier, G. Gao, M. Goldfarb, Receptor specificity of the fibroblast growth factor family, J. Biol. Chem. 271 (1996) 15292–15297.
[11] C.X. Deng, A. Wynshaw-Boris, M.M. Shen, C. Daugherty, D.M. Ornitz, P. Leder, Murine FGFR-1 is required for early postimplantation growth and axial organization, Genes Dev. 8 (1994) 3045–3057.
[12] C. Deng, M. Bedford, C. Li, X. Xu, X. Yang, J. Dunmore, P. Leder, Fibroblast growth factor receptor-1 (FGFR-1) is essential for normal neural tube and limb development, Dev. Biol. 185 (1997) 42–54.
[13] T.P. Yamaguchi, K. Harpal, M. Henkemeyer, J. Rossant, FGFR-1 is required for embryonic growth and mesodermal patterning during mouse gastrulation, Genes Dev. 8 (1994) 3032–3044.
[14] M. Weinstein, X. Xu, K. Ohyama, C.X. Deng, FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung, Development 125 (1998) 3615–3623.
[15] T. Floss, H.H. Arnold, T. Braun, A role for FGF6 in skeletal muscle regeneration, Genes Dev. 11 (1997) 2040–2051.
[16] F. Fiore, A. Sebille, D. Birnbaum, Skeletal muscle regeneration is not

impaired in Fgf6 −/− mutant mice, Biochem. Biophys. Res. Commun. 272 (2000) 138–143.
[17] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[18] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[19] R. Couteaux, J.C. Mira, A. d'Albis, Regeneration of muscles after cardiotoxin injury: I. Cytological aspects, Biol. Cell 62 (1988) 171–182.
[20] T. Launay, A.S. Armand, F. Charbonnier, J.C. Mira, E. Donsez, C.L. Gallien, C. Chanoine, Expression and neural control of myogenic regulatory factor genes during regeneration of mouse soleus, J. Histochem. Cytochem. 49 (2001) 887–899.
[21] S. Kästner, M.C. Elias, A.J. Rivera, Z. Yablonka-Reuveni, Gene expression patterns of the fibroblast growth factors and their receptors during myogenesis of rat satellite cells, J. Histochem. Cytochem. 48 (2000) 1079–1096.
[22] I. Husmann, L. Soulet, J. Gautron, I. Martelly, D. Barritault, Growth factors in skeletal muscle regeneration, Cytokine Growth Factor Rev. 7 (1996) 249–258.
[23] M.E. Buckingham, Which myogenic factors make muscle? Curr. Biol. 4 (1994) 61–63.
[24] K. Guo, J. Wang, V. Andres, R.C. Smith, K. Walsh, MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation, Mol. Cell. Biol. 15 (1995) 3823–3829.
[25] O. Halevy, B.G. Novitch, D.B. Spicer, S.X. Skapek, J. Rhee, G.J. Hannon, D. Beach, A.B. Lassar, Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD, Science 267 (1995) 1018–1021.
[26] S.B. Parker, G. Eichele, P. Zhang, A. Rawls, A.T. Sands, A. Bradley, E.N. Olson, J.W. Harper, S.J. Elledge, p53-independent expression of $p21^{Cip1}$ in muscle and other terminally differentiating cells, Science 267 (1995) 1024–1027.
[27] B.L. Black, E.N. Olson, Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins, Annu. Rev. Cell Dev. Biol. 14 (1998) 167–196.
[28] S.M. Sheehan, R.E. Allen, Skeletal muscle satellite cell proliferation in response to members of the fibroblast growth factor family and hepatocyte growth factor, J. Cell. Physiol. 181 (1999) 499–506.
[29] Z. Yablonka-Reuveni, M.A. Rudnicki, A.J. Rivera, M. Primig, J.E. Anderson, P. Natanson, The transition from proliferation to differentiation is delayed in satellite cells from mice lacking MyoD, Dev. Biol. 210 (1999) 440–455.
[30] J.P. Lefaucheur, A. Sebille, Basic fibroblast growth factor promotes in vivo muscle regeneration in murine muscular dystrophy, Neurosci. Lett. 202 (1995) 121–124.
[31] K. Sakuma, K. Watanabe, M. Sano, I. Uramoto, T. Totsuka, Differential adaptation of growth and differentiation factor 8/myostatin, fibroblast growth factor 6 and leukemia inhibitory factor in overloaded, regenerating and denervated rat muscles, Biochim. Biophys. Acta 1497 (2000) 77–88.
[32] G. Minowada, L.A. Jarvis, C.L. Chi, A. Neubuser, X. Sun, N. Hacohen, M.A. Krasnow, G.R. Martin, Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed, Development 126 (1999) 4465–4475.
[33] A. Sasaki, T. Taketomi, T. Wakioka, R. Kato, A. Yoshimura, Identification of a dominant negative mutant of Sprouty that potentiates fibroblast growth factor—but not epidermal growth factor—induced ERK activation, J. Biol. Chem. 276 (2001) 36804–36808.
[34] Z.Q. Wang, M.R. Fung, D.P. Barlow, E.F. Wagner, Regulation of embryonic growth and lysosomal targeting by the imprinted Igf2/Mpr gene, Nature 372 (1994) 464–467.
[35] S. Vainikka, V. Joukov, S. Wennstrom, M. Bergman, P.G. Pelicci, K. Alitalo, Signal transduction by fibroblast growth factor receptor-4 (FGFR-4). Comparison with FGFR-1, J. Biol. Chem. 269 (1994) 18320–18326.
[36] N.C. Jones, Y.V. Fedorov, R.S. Rosenthal, B.B. Olwin, ERK1/2 is required for myoblast proliferation but is dispensable for muscle gene expression and cell fusion, J. Cell. Physiol. 186 (2001) 104–115.
[37] I. Marics, F. Padilla, J.F. Guillemot, M. Scaal, C. Marcelle, FGFR4 signaling is a necessary step in limb muscle differentiation, Development 129 (2002) 4559–4569.
[38] S.S. Rao, D.S. Kohtz, Positive and negative regulation of D-type cyclin expression in skeletal myoblasts by basic fibroblast growth factor and transforming growth factor beta. A role for cyclin D1 in control of myoblast differentiation, J. Biol. Chem. 270 (1995) 4093–4100.
[39] C.J. Sherr, J.M. Roberts, CDK inhibitors: positive and negative regulators of G1-phase progression, Genes Dev. 13 (1999) 1501–1512.
[40] L.A. Megeney, B. Kablar, K. Garrett, J.E. Anderson, M.A. Rudnicki, MyoD is required for myogenic stem cell function in adult skeletal muscle, Genes Dev. 10 (1996) 1173–1183.
[41] L.A. Sabourin, A. Girgis-Gabardo, P. Seale, A. Asakura, M.A. Rudnicki, Reduced differentiation potential of primary MyoD −/− myogenic cells derived from adult skeletal muscle, J. Cell Biol. 144 (1999) 631–643.
[42] L. Li, R. Heller-Harrison, M. Czech, E.N. Olson, Cyclic AMP-dependent protein kinase inhibits the activity of myogenic helix-loop-helix proteins, Mol. Cell. Biol. 12 (1992) 4478–4485.
[43] P.L. Puri, V. Sartorelli, Regulation of muscle regulatory factors by DNA-binding, interacting proteins, and posttranscriptional modifications, J. Cell. Physiol. 185 (2001) 155–173.
[44] H. Wu, B. Rothermel, S. Kanatous, P. Rosenberg, F.J. Naya, J.M. Shelton, K.A. Hutcheson, J.M. DiMaio, E.N. Olson, R. Bassel-Duby, R.S. Williams, Activation of MEF2 by muscle activity is mediated through a calcineurin-dependent pathway, EMBO J. 20 (2001) 6414–6423.
[45] G.R. Adams, F. Haddad, The relationships among IGF-1, DNA content, and protein accumulation during skeletal muscle hypertrophy, J. Appl. Physiol. 81 (1996) 2509–2516.
[46] G.R. Adams, S.A. McCue, Localized infusion of IGF-I results in skeletal muscle hypertrophy in rats, J. Appl. Physiol. 84 (1998) 1716–1722.