

Developmentally Regulated Expression and Localization of Fibroblast Growth Factor Receptors in the Human Muscle

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ABSTRACT Fibroblast growth factors (FGFs) are believed to play a key role in tissue differentiation and maturation. Thus, the expression of the four members of the high-affinity tyrosine kinase FGF receptor family (FGFRs) and of the low-affinity heparan sulphate proteoglycan binding sites, syndecan-1 and perlecan, was studied in the human skeletal muscle during development. Northern blot analysis demonstrated a developmentally regulated expression of the mRNAs for FGFR-1, FGFR-3, FGFR-4, whereas only traces of FGFR-2 mRNA were found. Each receptor type had a different developmental pattern, suggesting an independent regulation. Signal for FGFR-3 was retained only in the adult muscle. Among the low-affinity FGF binding sites, perlecan was absent, whereas RNA transcript for syndecan-1 peaked at week 13 of gestation, after which a significant decrease was observed.

Immunohistochemistry for FGFRs revealed that their localization changed with muscle maturation. At early embryonic stages, FGFR-3 and FGFR-4 had a scattered distribution in the tissue, and FGFR-1 was found on myotube and myofiber plasma membranes. At later stages, FGFR-1 positivity decreased and was found in a few areas of the muscle, FGFR-3 was concentrated in the nuclei of some, but not all, muscle fibers, and FGFR-4 maintained an association with plasma membrane. In adult tissue, weak positivity for FGFR-3 and FGFR-4 was observed in the connective tissue only.

When immunocytochemistry was performed on human fetal myoblasts in culture, confocal microscope analysis revealed a nonhomogeneous cell membrane distribution of FGFRs. Taken together, the data strongly suggest that developmentally regulated expression and cell distribution of FGFRs play a role during muscle maturation. *Dev. Dyn.* 1998;211:362-373. © 1998 Wiley-Liss, Inc.

Key words: fibroblast growth factors; receptors; syndecan-1; muscle; muscle proteins; human fetus; development

INTRODUCTION

Fibroblast growth factors (FGFs) represent a family of closely related polypeptide mitogens (Burgess and Maciag, 1989; Miller and Rizzino, 1994). They exhibit a variety of properties, including modulation of differentiation, developmental effects, and neurotrophic and angiogenic activities (Miller and Rizzino, 1994). In particular, several lines of evidence support a potential role for FGFs in muscle and limb development (Joseph-Silverstein et al., 1989; Niswander and Martin, 1992; Suzuki et al., 1992; Pizette et al., 1996). At least two FGFs (FGF-4 and FGF-5) are expressed in embryonic muscle precursors of the mouse (Haub and Goldfarb, 1991; Stark et al., 1991; Niswander and Martin, 1992), whereas a differential expression of FGFs has been demonstrated in proliferating or differentiated skeletal muscle cells (Hannon et al., 1996). Moreover, FGFs have been shown to inhibit differentiation of myoblasts in vitro (Lathrop et al., 1985; Clegg et al., 1987) and to regulate the transcription of important myogenic regulatory genes (Vaidya et al., 1989; Brunetti and Goldfine, 1990).

FGFs exert their activity through interaction with high-affinity tyrosine-kinase receptors (FGFRs). Four distinct FGFR genes have been identified, and their protein products differentially bind FGFs (Johnson and Williams, 1993; Miller and Rizzino, 1994). Expression of FGFRs follows a developmental pattern in different human tissues (Partanen et al., 1991). Although no data are available about the developmental expression of FGFR-2 and FGFR-3 in muscle, FGFR-2 is more prominent in mesenchyme (Orr-Urtreger et al., 1991; Peters et al., 1992). Myoblasts express FGFR-1, but its level gradually decreases as muscle fibers develop both in vivo and in vitro (Olwin and Hauschka, 1988, 1990; Moore et al., 1991; Templeton and Hauschka, 1992).

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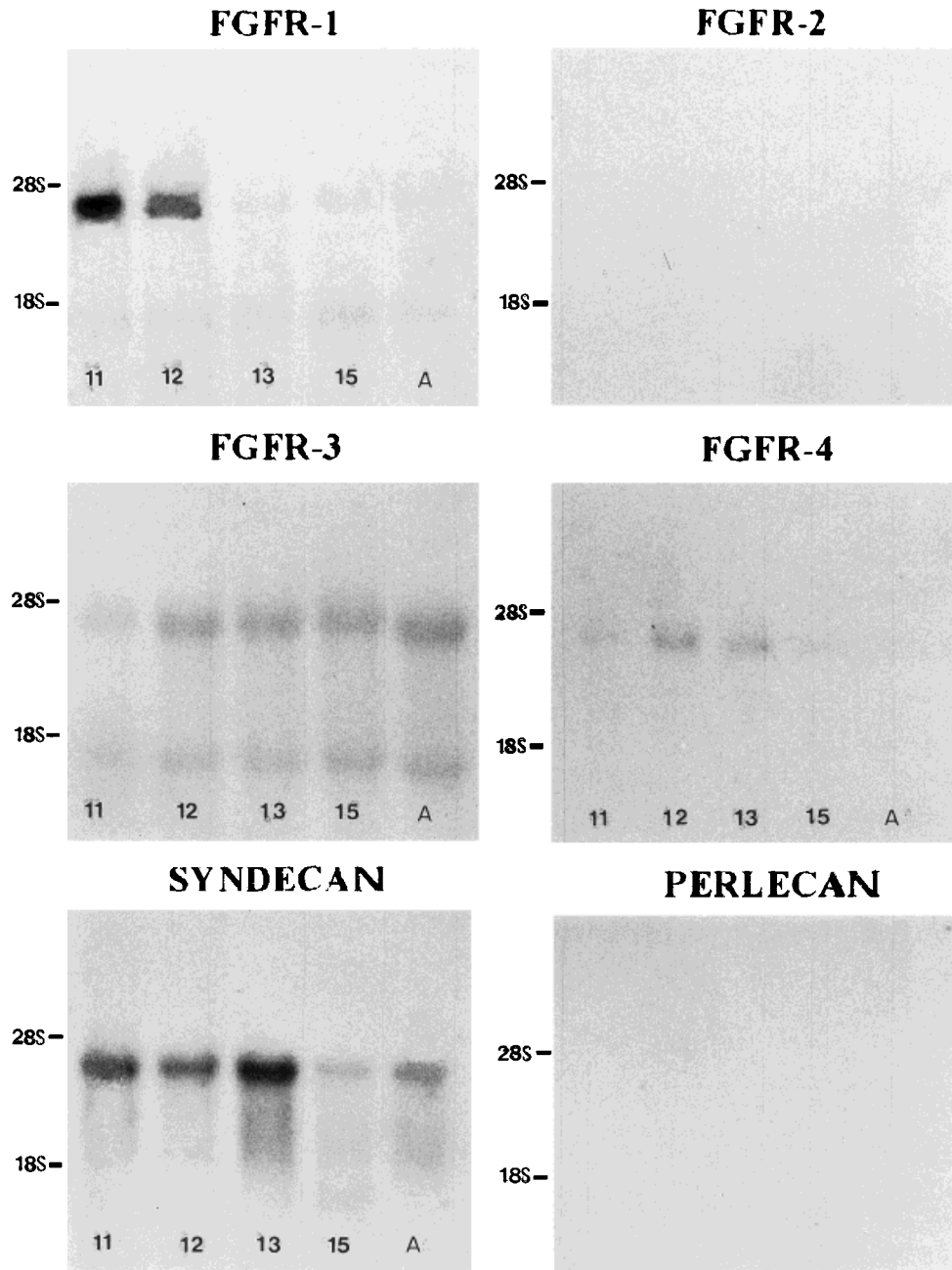


Fig. 1. Northern blot analysis of fibroblast growth factor (FGF) receptor (FGFR) mRNAs in human muscle during development. Whole leg tissue (embryos at 11, 12, 13, and 15 weeks of gestation) or quadriceps bioptic samples (adult; A) were used.

Moreover, it has been demonstrated recently that loss of FGFR-1 is necessary for terminal differentiation of embryonic limb muscle (Itoh et al., 1996). On one hand, transcripts for FGFR-4 are most conspicuous in the myotome, in muscle precursors, and in differentiated skeletal muscle (Stark et al., 1991). On the other hand, mouse EC cells do not express transcripts for FGFR-4 until they are induced to differentiate into cultures containing myoblasts (Stark et al., 1991).

The interaction of FGFs with FGFRs is also modulated by low-affinity proteoglycans containing heparan sulphate polysaccharides (HSPGs), leading to the formation of FGF/HSPG/FGFR ternary complexes (Rusnati and Presta, 1996). Prevention of their formation leads to reduced stimulation in cell proliferation (Rapraeger et al., 1991). In addition, HSPGs may induce FGF oligomerization, which facilitates FGFR dimerization and signal transduction (Spivak-Krolzman et al., 1994).

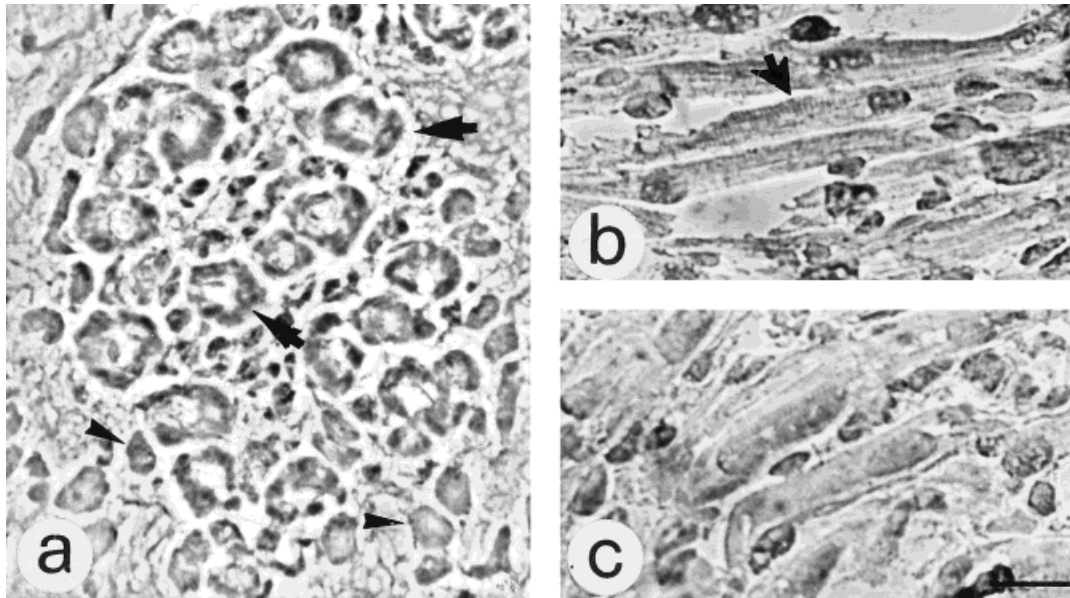


Fig. 2. Representative muscle sections used for staining (phase contrast). **a:** Transverse section of myofibers (arrows) and myotubes (arrowhead) from a developing human muscle (15 weeks of gestation) showing the thick peripheral cytoplasm associated with the membrane and the typical central cavity (more evident in myofibers). **b:** Longitudinal

section of myofibers from an embryonic muscle (12 weeks of gestation). Myofibrils are evident (arrow). **c:** Oblique section from a 12-week-old muscle in which the central position of nuclei is evident. Scale bar = 10 μ m.

Accordingly, *in vitro* myoblast differentiation is dependent on cellular heparan sulphate (Rapraeger et al., 1991; Olwin and Rapraeger, 1992). Among the cell-associated HSPG species responsible for the induction of FGF/FGFR interaction, syndecan (Elenius et al., 1992; Chernousov et al., 1993), betaglycan (Andres et al., 1992), and perlecan (Aviezer et al., 1994) are all able to bind FGF-2 but with different roles in modulating the biological activity of the growth factor. Interestingly, developmental regulation of HSPG expression causes a change in their binding specificity for different members of the FGF family (Nurcombe et al., 1993).

All of these data strongly suggest that both FGFRs and HSPGs are involved in muscle maturation. However, no data are available about the contemporary expression of these receptors in a tissue such as striated muscle, in which, even if precursors are already committed to their final fate, the building up of the tissue involves events such as cell proliferation and cell fusion that span over a long period of the intrauterine life. Consequently, we studied FGFR and HSPG developmental expression as well as their localization in the human fetal muscle.

RESULTS

Expression of FGFR mRNAs

Figure 1 shows that Northern blot analysis demonstrated the presence of transcripts for FGFR-1, FGFR-3, and FGFR-4 in the human muscle from 11 weeks of gestation. Their expression was developmentally regulated. In particular, the most intense signal for FGFR-1 was found between week 11 and 12 of gestation, after

which a sharp decrease could be observed, and only a very weak signal was detectable in adult muscle. On the other hand, FGFR-4 mRNA was clearly detectable in embryonic muscle, peaked between weeks 12 and 13, then decreased, and was absent in the adult. FGFR-3 transcript showed a different pattern, increasing between weeks 11 and 12 and then remaining steady. Only traces of FGFR-2 mRNA were found in both embryonic and adult muscle.

For HSPG expression, we found a peak of syndecan-1 mRNA at week 13 followed by a significant decrease. Perlecan mRNA was not detectable throughout development.

Localization of FGFRs

The distribution of FGFR-1, FGFR-3, and FGFR-4 followed a different pattern throughout muscle development (for tissue orientation, see Fig. 2).

In earlier embryos (9–10 weeks of gestation), FGFR-1 was distributed mainly on the surface of myotubes and myofibers in discrete areas of the immature muscle (Fig. 3a), even though some cytoplasmic staining could not be excluded. In parallel with the lower mRNA levels, a progressive decrease of FGFR-1 immunoreactivity was observed at later stages of development (Fig. 3b,c), with the exception of randomly distributed areas at week 18 of gestation, when some positivity associated to plasma membrane was observed (Fig. 3c, inset).

FGFR-3 was distributed differently in myotubes and myofibers throughout development. In earlier stages (9–10 weeks of gestation), labeling was scattered within the tissue with no clear-cut association with plasma

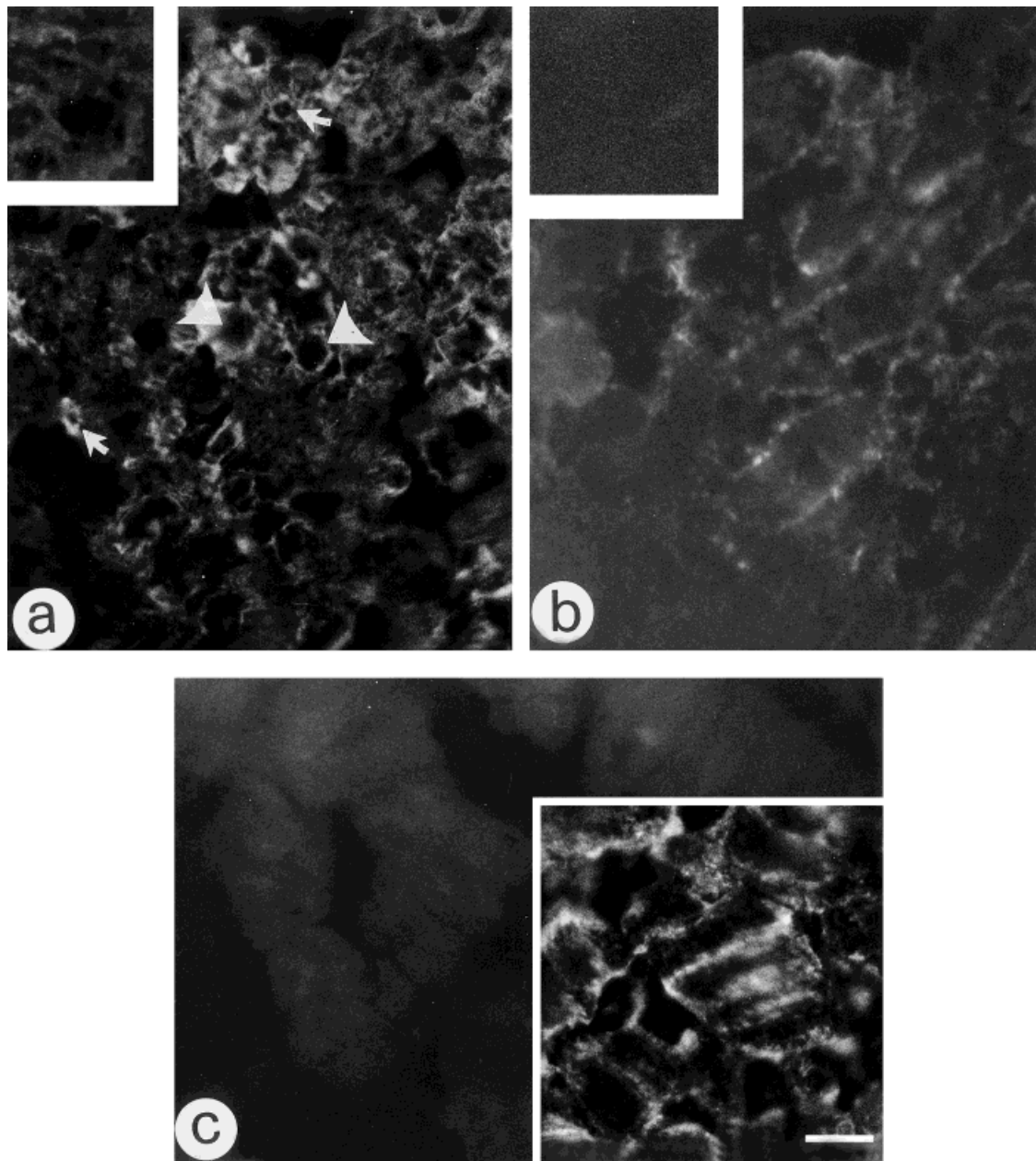


Fig. 3. FGFR-1 immunocytochemistry in human embryonic muscle. **a:** Transverse section from a 9-week-old fetus: Labeling is associated with the plasma membrane of myotubes (arrows) and myofibers (arrowheads). Preincubation (PI) of the antibody with the receptor leads to faint, nonspecific staining (*inset*). **b:** Oblique section from an 11-week-old fetus:

Labeling is weaker and is not distributed homogeneously on the surface of myofibers. *Inset:* Negative control in which the specific antibody was omitted. **c:** Transverse section from an 18-week-old fetus: The tissue shows faint and diffuse staining except in randomly scattered areas (*inset*, oblique section). Scale bar = 10 μ m.

membrane of myotubes and myofibers (Fig. 4a). In 13-week-old embryos, the immunoreactivity was found mainly associated to the plasma membrane of myofibers, where, however, its distribution was not homogeneous, and some cytoplasmic staining could be found (Fig. 4b). Then, a shift toward an intense staining in myofiber nuclei was observed at 18 weeks of gestation (Fig. 4c). However, nuclear counterstaining showed that not all of the nuclei were labeled (Fig. 5).

In early embryonic stages, FGFR-4 surrounded myofibers in scattered areas of the tissue (Fig. 6a). Then, a heavy staining could be observed in myofibers at 12 weeks of gestation at both membrane and cytoplasmic levels (Fig. 6b). Later (15–18 weeks of gestation), positivity was still found on plasma membrane together with some punctuate staining in the cytoplasm (Fig. 6c).

Adult muscle tissue was negative for FGFR-1, FGFR-3, and FGFR-4, but the connective tissue endomy-

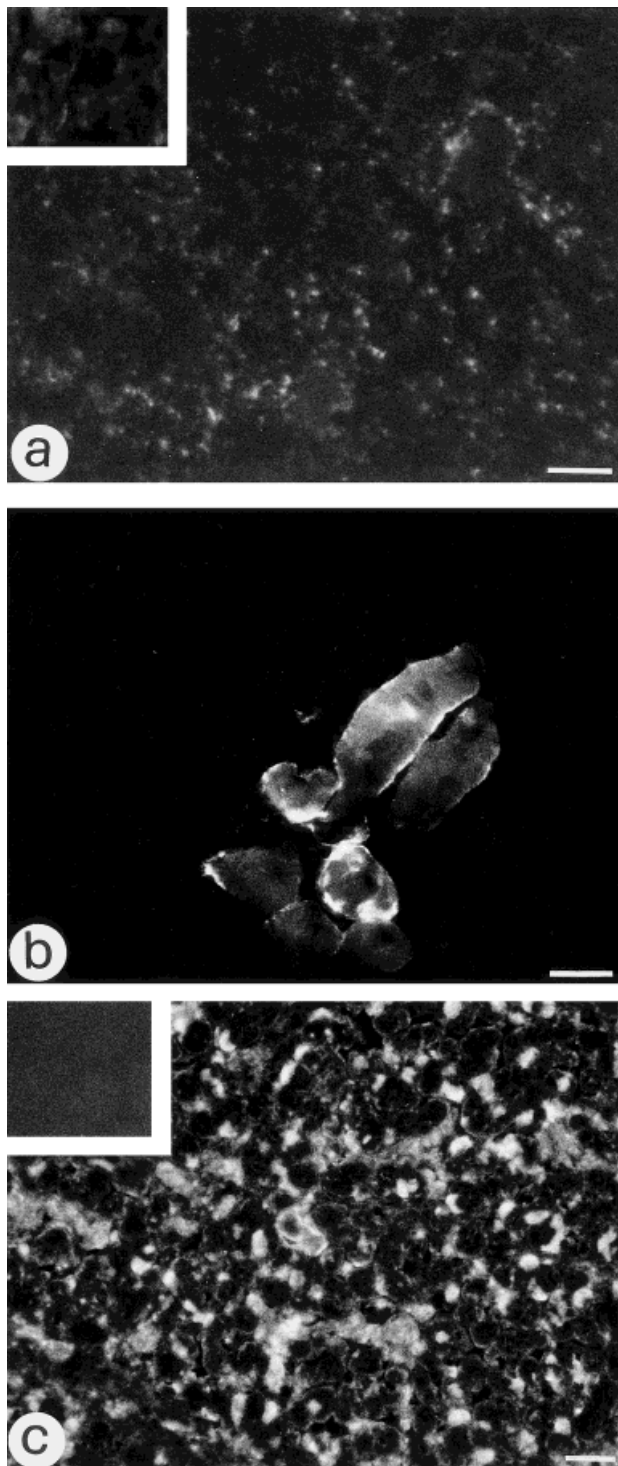


Fig. 4. FGFR-3 immunocytochemistry in human embryonic muscle. **a:** Transverse sections of a 10-week-old fetal muscle: Positivity is punctate and scattered among the tissue, with no clear-cut association with myotubes or myofibers. **Inset:** PI control. **b:** Longitudinal section of a 13-week-old fetal muscle shows no homogeneous membrane staining. **c:** Transverse section from an 18-week-old fetus: Labeling is localized mainly to the cell nuclei. **Inset:** Negative control. Scale bars = 10 μ m.

sium showed weak, punctuated FGFR-3 and FGFR-4 staining (Fig. 7).

Despite the absence of the corresponding mRNA, we found some staining with FGFR-2 antibody in embryonic muscle. This might have been due either to cross reactivity of the antibodies or to a low turnover of the receptor protein, which might have been synthesized in earlier embryos. Further investigations are required to test this hypothesis. However, preincubation of the antibody with the specific receptor fragment did not completely abolish immunoreactivity (see also Experimental Procedures).

FGFR Localization and Muscle Maturation

To correlate FGFR expression with muscle maturation, we performed double staining for various muscle proteins, including fast and slow myosin molecules, embryonic and adult myosin chains, and muscle dystrophin.

Muscles from early embryos, as expected, showed a diffuse staining for the embryonic type of myosin (not shown). On the other hand, samples from 15- and 18-week-old fetuses had a few muscle fibers bearing adult type of myosin as well as fast and slow myosin-positive fibers. For all of these proteins, no clear-cut association between positive or negative fibers and FGFR staining could be observed (Fig. 8). Similarly, both dystrophin-negative and dystrophin-positive myofibers stained for FGFRs (data not shown).

Interestingly, the embryonic connective tissue surrounding the fiber bundles (identified by Azan-Mallory staining; not shown here) was generally devoid of FGFR labeling, with the exception of 13-week-old muscle (Fig. 8b).

FGFR Localization in Cultured Myoblasts

In agreement with the *in vivo* observations, immunocytochemistry performed on cultured human embryonic myoblasts showed positivity for FGFR-1, FGFR-3, and FGFR-4. FGFRs, as observed under the confocal microscope, had a fine distribution on cell surfaces (Fig. 9). In all cases, the distribution was not homogeneous, and concentration of the receptors in discrete areas was observed. No nuclear staining was found with anti-FGFR-3.

DISCUSSION

Our data represent the first demonstration of the presence and the developmentally regulated, differential expression of the high-affinity tyrosine kinase FGFRs and of the low-affinity HPSG syndecan-1 in human muscle. They suggest an independent regulation for each receptor due to their different developmental pattern of expression. In fact, FGFR-1, FGFR-4, and syndecan-1 mRNA levels peaked at different times in the embryo, whereas FGFR-3 was clearly expressed from week 12 of gestation and then remained steady.

The question arises about the physiological significance as well as the role played by each FGFR in human muscle maturation. FGFRs differentially bind the vari-

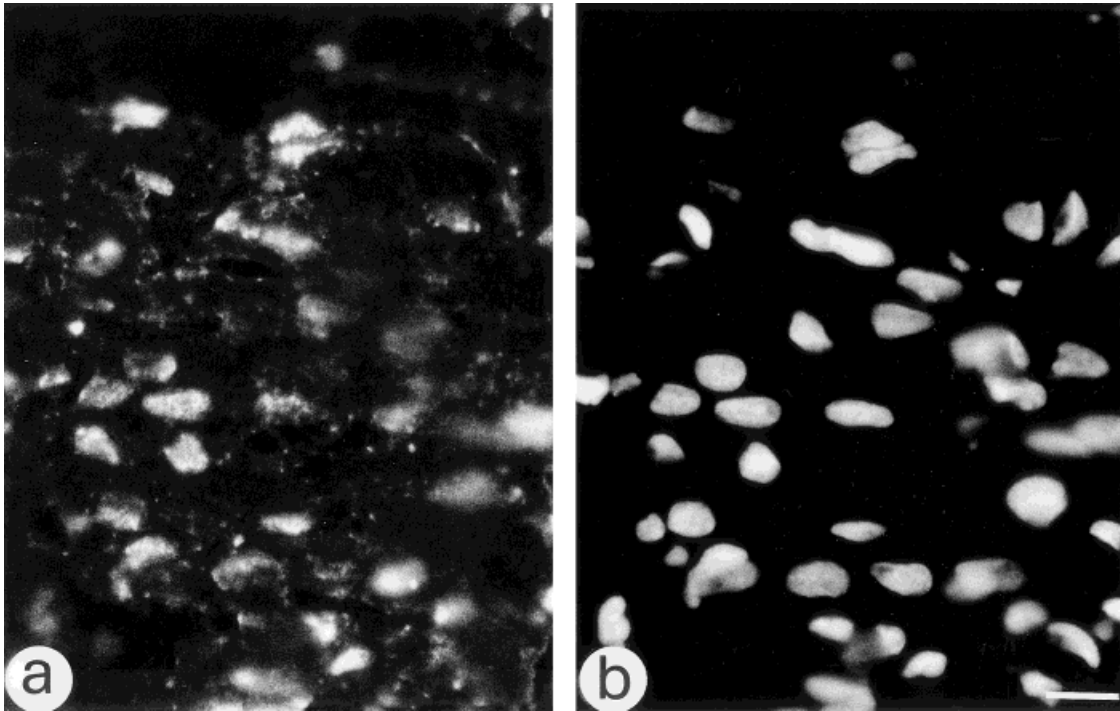


Fig. 5. Immunocytochemical localization of FGFR-3 in human fetal muscle at 18 weeks of gestation. **a:** Longitudinal sections show labeling of myofiber nuclei. **b:** Counterstaining for nuclear chromatin shows that not all of the nuclei are positive for FGFR-3. For controls, see Figure 4a,c. Scale bar = 10 μ m.

ous members of the FGF family (Miller and Rizzino, 1994). Because a differential expression of FGFs has been demonstrated *in vitro* during muscle cell differentiation (Hannon et al., 1996), the hypothesis can be advanced that human fetal muscle has a diverse response to FGFs during maturation through a differential expression of FGFRs. Indeed, FGF receptors display both common and distinct signal-transduction pathways (Shaoul et al., 1995), so that each receptor might subserve a different function in muscle.

Overexpression of FGFR-1 has been associated with proliferation (Shaoul et al., 1995) and lack of differentiation (Itoh et al., 1996) in muscle cell lines. On the other hand, FGFR-1 transcription was greatly reduced in the mouse skeletal muscle cell line MM14 upon differentiation (Olwin and Hauschka, 1988; Templeton and Hauschka, 1992), and loss of FGFR-1 has been observed in association with terminal myoblast differentiation *in vitro* or *in vivo* (Olwin and Hauschka, 1988, 1990; Moore et al., 1991; Templeton and Hauschka, 1992; Itoh et al., 1996). Thus, the abundance of FGFR-1 mRNA in early human embryonic muscle, its decrease with maturation, and its disappearance in adult muscle are in line with these observations.

The data on the developmentally regulated FGFR-3 localization are intriguing. The delayed expression of FGFR-3 compared with FGFR-1, its persistence throughout fetal and adult life, and its shift toward nuclear localization suggest that this receptor is re-

quired for advanced muscle maturation. Nuclear FGFR-3 localization has been described recently in breast epithelial cells (Johnston et al., 1995), so that an active role for FGFR-3 in mediating FGF nuclear function(s) has been hypothesized (Hawker and Granger, 1994). If this hypothesis is correct, then the shift of FGFR-3 localization during development might imply a critical role for nuclear FGFs in advanced muscle maturation. On the other hand, FGFR-3 localization data demonstrate that connective tissue rather than muscle is the FGF target in the adult.

Interestingly, our data also show a major expression of FGFR-4 in human muscle, which decreased during development. FGFR-4 shows a great deviation from the expression patterns of the other FGFRs in several human tissues (Partanen et al., 1991). Moreover, signal transduction triggered by FGFR-4 differs from that of other FGFRs (Vainikka et al., 1994). FGFR-4 is unable to mediate cell proliferation in the rat myoblast L6E9 cell line, suggesting that this receptor may function in maintaining the nondifferentiated status of myoblasts rather than promoting proliferation (Shaoul et al., 1995). Thus, the abundance of FGFR-4 on the surface of maturing myofibers might prevent signal(s) to terminal differentiation to affect the immature muscle.

The observation that not all myotubes and myofibers are positive for FGFRs at the same time is not surprising. Muscle development is a complex phenomenon that is characterized not only by cell proliferation and fusion

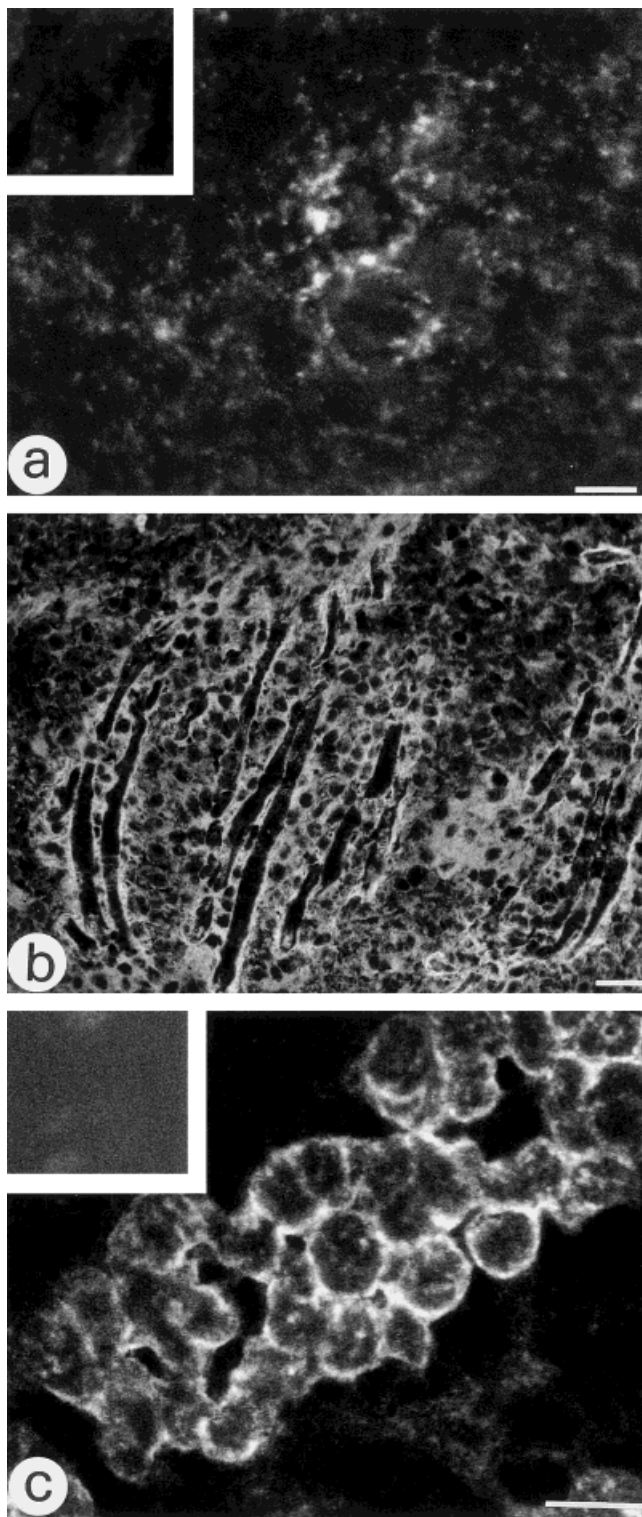


Fig. 6. FGFR-4 immunocytochemistry in human fetal muscle. **a:** Transverse section from a 10-week-old embryo: Positive myofibers are found in scattered areas. **Inset:** PI control. **b:** Longitudinal sections from 12-week-old embryos show both membrane and cytoplasmic staining in most fibers. **c:** Confocal microscope analysis of a transverse section at 18 weeks of gestation demonstrates membrane labeling as well as punctuate cytoplasmic staining. **Inset:** Negative control. Scale bars = 10 μ m.

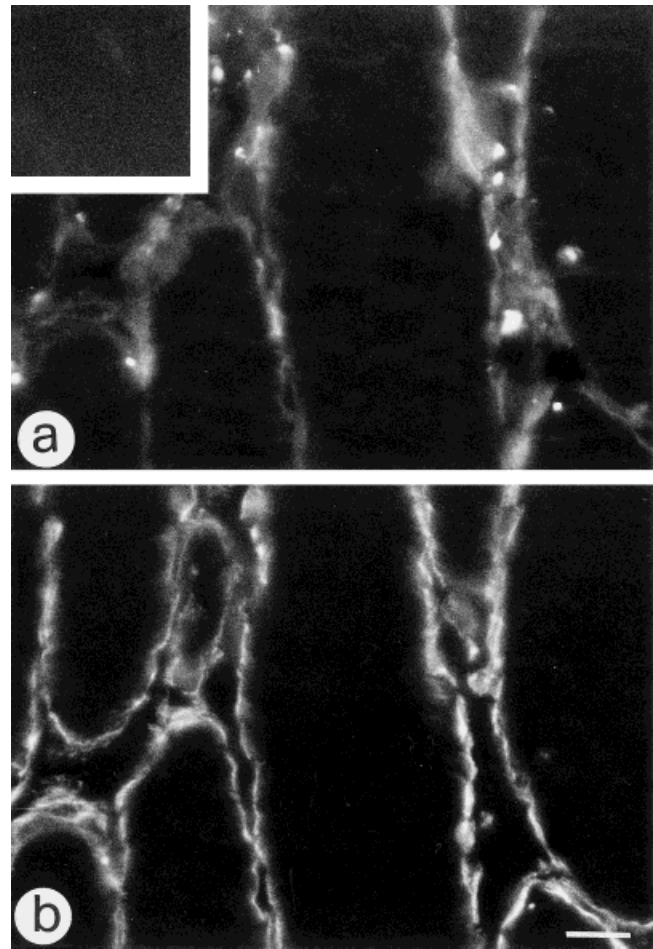


Fig. 7. Double staining for FGFR-3 and dystrophin in adult human muscle. **a:** Receptor staining is present in the endomysium but is absent in muscle. **Inset:** Negative control. **b:** Dystrophin antibody labels muscle membrane. These observations overlap the results obtained with FGFR-4 antibodies (not shown here). Scale bar = 10 μ m.

but also by asynchronous differentiation of successive fiber generation, as recently reviewed by Schiaffino and Reggiani (1996). Thus, the differential distribution of FGFRs within the tissue can be also a consequence of this developmental pattern. In apparent disagreement with this hypothesis, FGFRs were found to be associated to myofibers and myotubes regardless of whether they bear embryonic or adult myosin as well as slow or fast myosin isoforms. However, fast- and slow-type isogenes are coexpressed during development (Schiaffino and Reggiani, 1996), and muscle maturation is far from being completed at the stages considered.

The expression of FGFRs in human embryonic muscle was confirmed by immunostaining of cultured human embryonic myoblasts. The data showed a nonhomogeneous membrane localization for the different receptors, suggesting the possibility of a "spotted" FGF signaling, the physiological significance of which requires further investigation. Relevant to this point, recent observations have indicated the presence

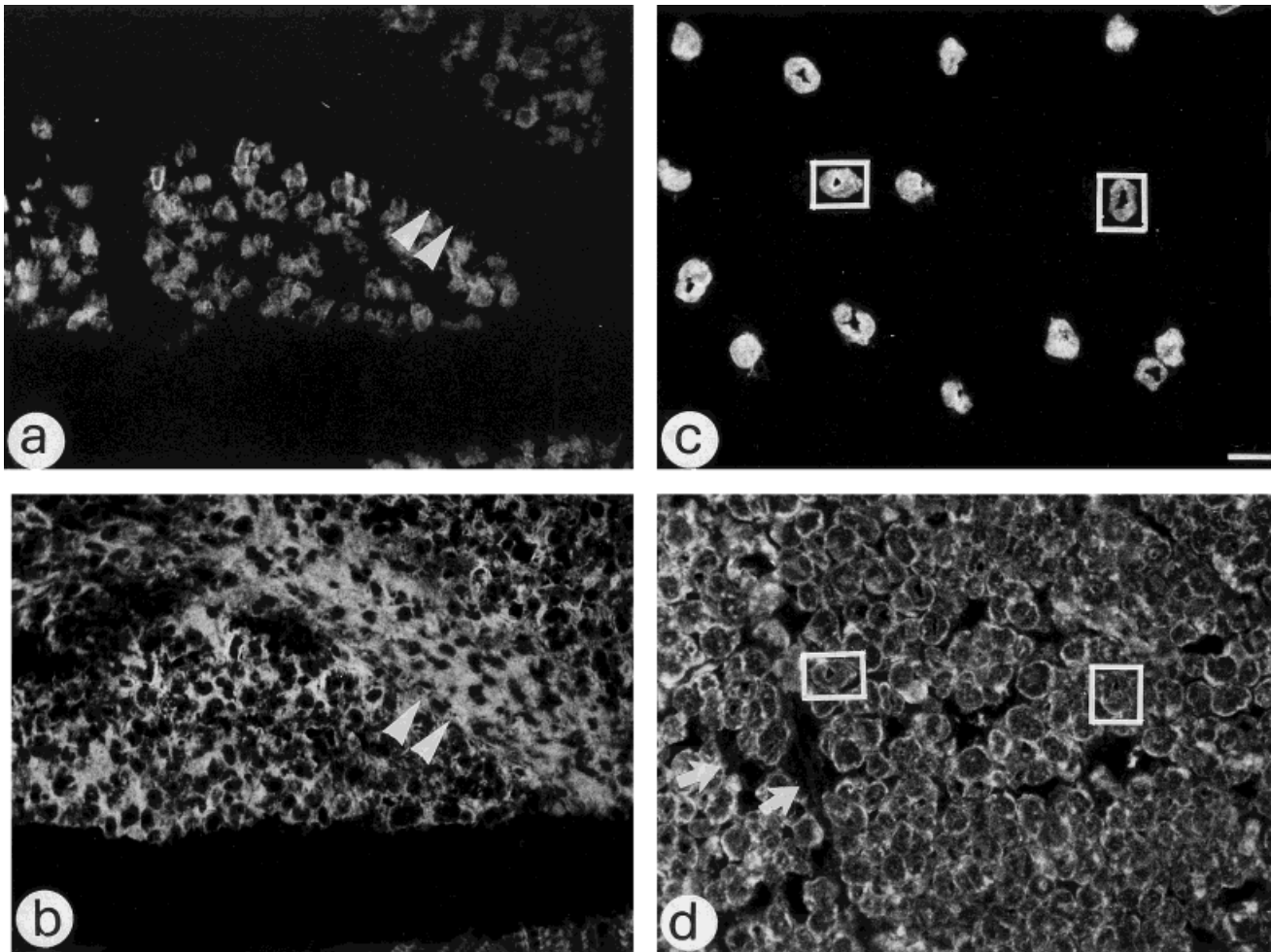


Fig. 8. Double staining for FGFRs and muscle proteins. **a,b:** Muscle transverse section from a 13-week-old fetus: Embryonic myosin staining (a) labels both myofibers and myotubes, all of which bear FGFR-3 (b). At this stage, only connective tissue (negatively stained for myosin; arrowheads in a) is positive for FGFR-3 (arrowheads in b). **c,d:** Muscle

transverse section from an 18-week-old fetus: Slow adult myosin staining (c) shows that only a few myofibers bear this protein (squares), even though all of them are positive for FGFR-4 (d). At this stage, connective tissue (arrows in d) is negative for FGFR-4. Scale bar = 10 μ m.

of FGFR-1 in cell-adhesion plaques (Plopper et al., 1995).

Also, the observation that syndecan-1, but not perlecan, has a developmentally regulated expression in human muscle is of interest. The physiological significance of the interaction of FGFs with HSPGs is manifold (for a recent review, see Rusnati and Presta, 1996). Previous observations have demonstrated that the effects of FGFs on *in vitro* myoblast differentiation are dependent on cellular HSPGs (Rapraeger et al., 1991; Olwin and Rapraeger, 1992). The developmental regulation and contemporary presence of high- and low-affinity FGF binding sites in the maturing muscle and their disappearance at terminal differentiation support the hypothesis for a role of FGFs during human muscle development.

EXPERIMENTAL PROCEDURES

Tissue Collection

Fetal tissues were obtained from either spontaneous or medically induced abortions. The use of these tissues has been approved by the local Ethical Committee.

Northern Blot Analysis

Northern blot analysis of total RNA extracted from whole leg muscles (embryos) or quadriceps biopsies (adult; 20 μ g/sample) was performed according to standard procedures (Maniatis et al., 1990). Uniform loading of the gels was assessed by ethidium bromide staining. FGFR-1, FGFR-2, and syndecan-1 probes were kindly provided by A. Mansukhani (New York University, New York, NY), FGFR-3 and FGFR-4 probes were provided by J. Partanen (University of Helsinki,

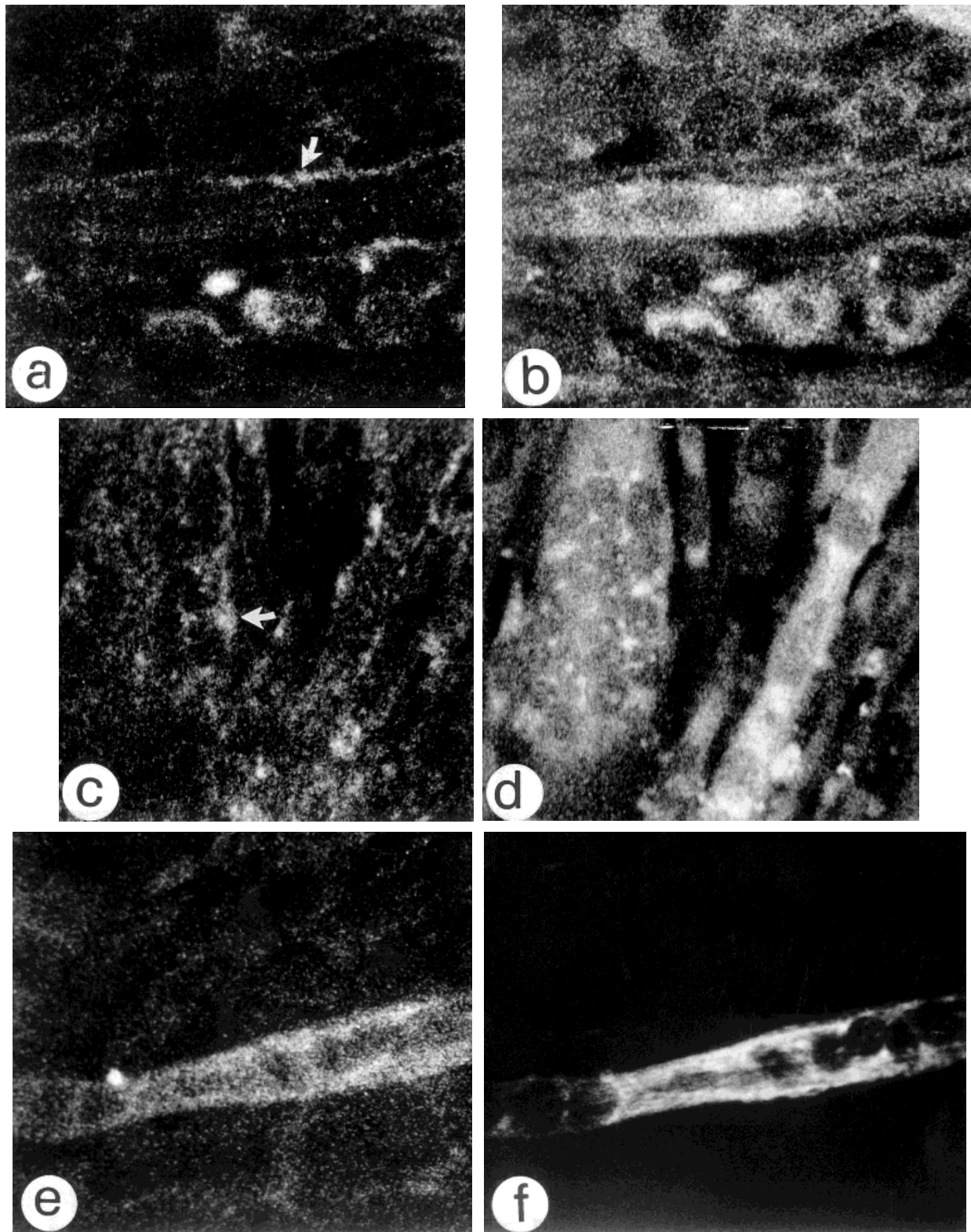


Fig. 9. **a–l**: Confocal microscope analysis of FGFR staining in human fetal cultured myoblasts. FGFR-1 (a,c), FGFR-3 (e,g), FGFR-4 (i,k), dystrophin (b,d), myosin (f,h,j,l). Not all receptors are distributed homoge-

neously on cell membranes, but they tend to concentrate in some cell areas (arrows). Some FGFR-3 and FGFR-4 cytoplasmic staining is detectable. Scale bar = 10 μ m.

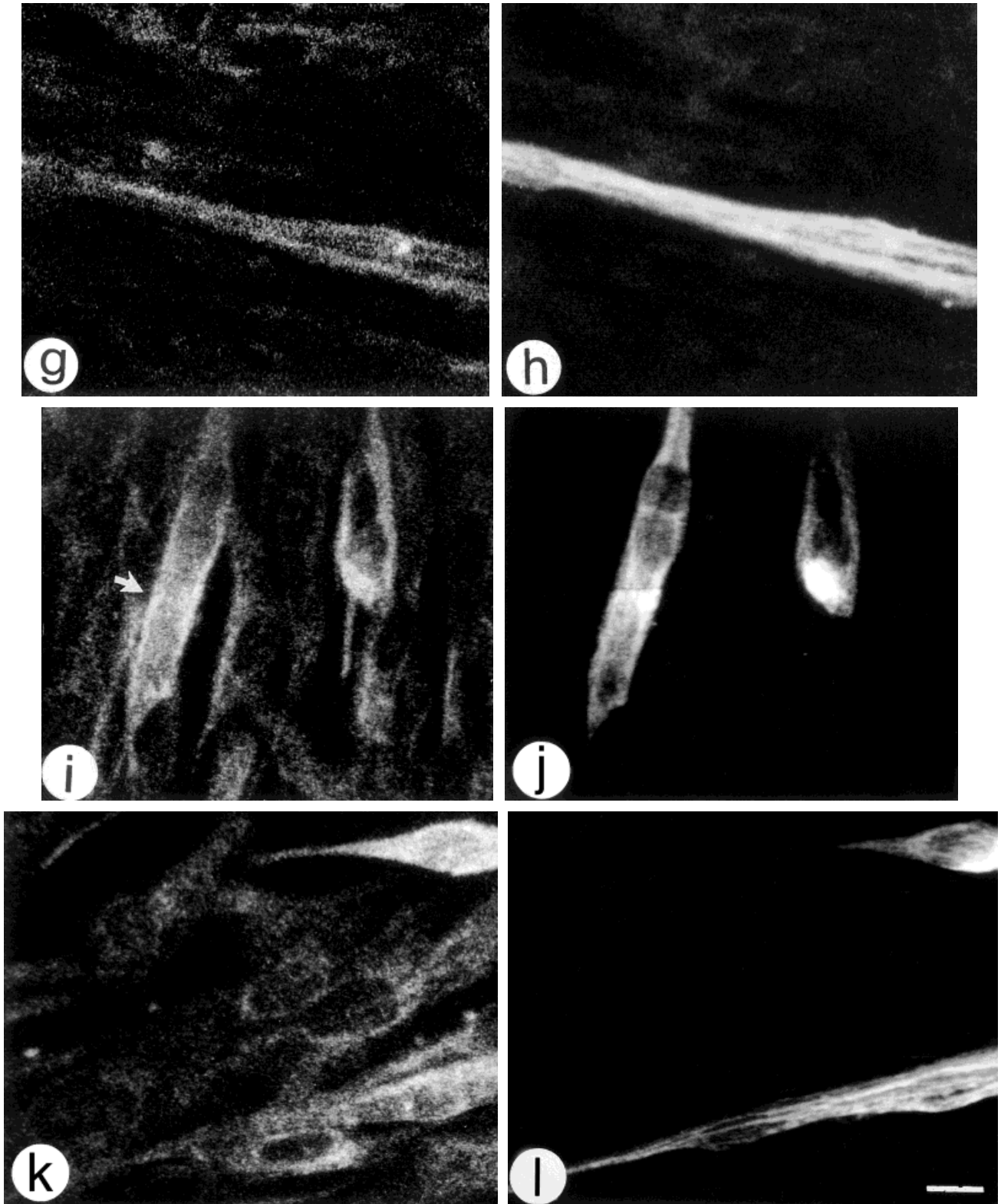


Fig. 9. (Continued.)

Helsinki, Finland), and perlecan probe was provided by D. Noonan (IST, Genova, Italy).

Immunocytochemistry

Intact leg muscles were obtained from 10–18-week-old normal human fetuses. Normal adult muscle samples were obtained from quadriceps biopsies. Fresh muscle tissue was frozen at -80°C and sectioned at $25\ \mu\text{m}$ in a cryostat (for tissue orientation, see Fig. 2). The sections collected on gelatin-coated glass slides were fixed in cold methanol (-20°C for 4 min) and then rehydrated in phosphate-buffered saline (PBS) when used. Immunocytochemical localization of FGFRs was performed by fluorescence staining. In brief, after several washes in PBS, samples were preincubated with human AB serum (1:10) for 15 min and then overlaid with anti-FGFR-1/flg monoclonal antibody (1:40) or anti-FGFR-2, anti-FGFR-3, and anti-FGFR-4 polyclonal antibodies (1:100; all from Santa Cruz Biotechnology, Santa Cruz, CA) for 45 min in a humid chamber. The antibodies used have been shown to specifically recognize human and mammalian FGF receptor types (Pirvola et al., 1995; Presta et al., 1995; Takahashi et al., 1995; Galvin et al., 1996; Konishi et al., 1996). Then, slides were rinsed three times and incubated for 30 min with biotinylated antimouse or antirabbit immunoglobulin (IgG; 1:200; Vector Laboratories, Burlingame, CA). Subsequently, the tyramide signal amplification system was applied according to the manufacturer instructions (TSA, NEN Research Products, Boston, MA).

Two different controls were used. First, negative controls were incubated with nonimmune sera. Second, each antibody was preincubated with the appropriate peptide or receptor fragment (provided by Santa Cruz Biotechnology), and the usual immunocytochemical staining was performed. Preincubation abolished specific staining with the exception of FGFR-2 antibody, which sometimes cross reacted with an unidentified molecule.

For double staining, slides were incubated for 30 min with polyclonal rabbit antibodies against dystrophin (1:100; P6; kindly provided by Dr. Sheratt, London) or monoclonal antibodies against fast (kindly provided by Dr. Fitzimons) and slow (Chemicon, Temecula, CA) neonatal myosin; myosin heavy chain embryonic (BF-G6) or adult (BA-D5), both kindly provided by Dr. Schiaffino, Padova; embryonic/fetal myosin (RNMY) or neonatal myosin (WB), both from Novocastra (Newcastle upon Tyne, United Kingdom), all 1:10. After washes, this step was followed by a 30 min incubation with antirabbit or antimouse TRITC-IgG (1:50; Jackson Immunoresearch, West Grove, PA). In some experiments, cell nuclei were counterstained by incubating the samples with bisbenzimidazole H 33258 fluorochrome ($2\ \mu\text{g}/\text{ml}$ in PBS; Sigma Chemical Co., St. Louis, MO) for 5 min at room temperature.

Myoblast Cultures

Human fetal leg muscle tissue was dissected out from 12-week-old fetuses and incubated in 0.2% sterile trypsin for 20 min at 37°C . After mechanical dissociation, cells were centrifuged at 2,000 rpm for 5 min and resuspended in Dulbecco's minimal essential medium plus 20% fetal calf serum (FCS). To eliminate fibroblast contamination, cells were plated on plastic dishes for 20 min; then, floating cells were seeded on collagen-coated dishes. After 5–7 days in vitro, FCS was reduced to 5% in order to induce myoblast differentiation. Myoblasts were identified by their positivity for myosin.

When they were needed, cultures were washed in PBS, and immunocytochemistry was performed as described above.

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