Journal of Animal and Veterinary Advances 2 (4): 196-201, 2003

FGF10 Stimulates Avian Myogenesis *In vitro* But Not *In vivo*

Peter Ward, Kari Clase and Kevin Hannon
Department of Basic Medical Sciences, School of Veterinary Medicine,
Purdue University, West Lafayette, Indiana 47907

Abstract: The functional properties of FGF10 during avian skeletal muscle development *in vitro* and *in vivo* are currently unknown. To elucidate such a function, the hindlimb skeletal muscle and primary muscle cultures of chick embryos were inoculated with high-titer RCAS-FGF10 retrovirus. Ectopic FGF10 stimulated a 4-fold increase in primary muscle cell proliferation and differentiation ($p<.05$), thereby significantly enhancing primary skeletal cell growth and development *in vitro*. In contrast, ectopic FGF10 expression had no significant effect on skeletal muscle development *in vivo*. These results suggest that FGF10 is not a primary growth factor involved in stimulation of the initial events of avian skeletal muscle development *in vivo*, and demonstrate the importance of confirming growth factor activity *in vitro* with its properties *in vivo*.

Key words: FGF10, limb, skeletal muscle and development

Introduction

Skeletal myogenesis *in vivo* occurs in several discrete phases including migration of myogenic precursor cells from the somite, commitment and proliferation of the myoblast population and fusion of these cells into the fully-differentiated myocyte *reviewed in* (Teran *et al.*, 1999). Each of these discrete phases is modulated by external inductive cues, such as peptide growth factors like the FGFs. Differentiation of the mononucleated myoblasts into the multinucleated myocyte structures *in vitro* is dramatically repressed by supplementation of culture media with FGF1, FGF2, and FGF4 (Gospodarowicz, 1974; Gospodarowicz, 1975; Hannon *et al.*, 1996; Linkhart, 1980; Linkhart *et al.*, 1981; Olwin and Rapraeger, 1992). While FGF2 acts as a mitogen for these cells, its ability to disrupt myocyte formation is separable from its ability to enhance cell proliferation (Seed and Hauschka, 1988). Interestingly, the capacity of these FGFs to inhibit morphological and biochemical differentiation *in vitro* has not always been duplicated *in vivo*. For example, somite muscle precursor cells infected with virus producing FGF1 or FGF4 differentiate into muscle fibers in the limb bud (Itoh *et al.*, 1996). In contrast, the hindlimbs of avian embryos overexpressing FGF5 or FGF4 were found to contain substantially fewer myofibers and myoblasts (Vovard *et al.*, 2001; Clase *et al.*, 2000). In addition, overexpression of a dominant repressive form of the high affinity receptor for FGF2, FGFR1, elicits an inhibitory effect on avian hind limb muscle formation (Steet *et al.*, 2000), suggesting that certain FGFs are actually required for normal skeletal muscle development *in vivo*. Therefore, it is essential that the effects of specific FGFs be examined in skeletal muscle *in vivo* to clarify their functional capabilities as mediators of avian skeletal muscle growth and development.

In this manuscript we report on the functional properties of FGF10 during avian skeletal muscle development *in vitro* and *in vivo*. FGF10 mRNA expression is upregulated in avian skeletal muscle following 2 and 11 days of stretch activation (Mitchell *et al.*, 1999). This suggests a growth stimulatory function for FGF10 during skeletal muscle development. To elucidate a function for FGF10 during avian skeletal muscle growth and development, the hindlimb skeletal muscle and primary muscle cultures of chick embryos were infected with high-titer RCAS-FGF10 retroviral inoculates. Ectopic FGF10 stimulated an increase in primary muscle cell proliferation and differentiation, thereby significantly enhancing skeletal muscle growth and development *in vitro*. In contrast, ectopic FGF10 expression had no significant effect on skeletal muscle development *in vivo*. These results suggest that FGF10 is not a primary growth factor for stimulation of the initial events of avian skeletal muscle development *in vivo*, and demonstrate the importance of confirming growth factor activity *in vitro* with its properties *in vivo*.

Materials and Methods

Retroviral Construction and Virus Production: An RCAS-FGF10 construct was generously supplied by Dr. S. Noji (Ohuchi *et al.*, 1997). Chicken

embryo fibroblasts (CEFs) were isolated from embryonic day 13 (ED13) embryos and cultured in low glucose Dulbecco's modified Eagle medium containing 15% chicken serum and 1% penicillin/streptomycin. Sub-confluent cultures were transfected with RCAS or RCAS-FGF10 proviral DNA and serially passaged twice. Retrovirions were collected from the culture supernatant and titered as described (Fekete and Cepko, 1993). Titers were estimated to be $2X10^8$ colony forming units (cfu)/mL.

Primary Cell Culture: For ED11 skeletal muscle cultures, hind limb skeletal muscle tissue was isolated from the hind limbs of ED11 chicken embryos, minced with curved scissors, triturated in DMEM + 15% chicken serum and incubated for 20 minutes at 37°C. Following incubation, dissociated tissue was passed through a 40 micron Falcon cell strainer and pre-plated for 20 minutes at 37°C to remove fibroblasts. Nonattached cells were harvested, counted by hemocytometer, and plated at a density of 3×10^5 cells per $22mm^2$ on gelatin-coated glass coverslips. Cells were cultured at 37C and 5% CO_2. Twelve hours post plating, cells were infected with 5 µl ($2x10^8$ cfu) of RCASBP(A) or RCAS-FGF10. Forty-eight hours post-infection, 10 µl of a 3mg/ml BrdU solution was added to cultures to achieve a final concentration of 3 µg/ml. Sixty hours post-infection, cells were fixed in methanol. Coverslips were incubated in 2N HCl for 30 minutes at 37°C, rinsed in 1XTBE, 1XPBS and then incubated in PBS + 5% goat serum (GS) for 1hour at 22°C. Cells were then sequentially incubated with: 1) anti-BrdU hybridoma supernatant (G3G4, Developmental Studies Hybridoma Bank) diluted 1:100 in PBS+5% GS; 2) FITC conjugated goat anti-mouse IgG1 (Fisher, Pittsburgh, PA) diluted 1:100 in PBS+5% GS; 3) a monoclonal anti-myosin antibody [MF20; Developmental Studies Hybridoma Bank, Iowa City, IA] diluted 1:10 in PBS+1% GS; 4) Texas Red conjugated goat anti-mouse Ig2b (Fisher) diluted 1:100 in PBS containing 1% GS; and finally 5) Hoechst 33342 dye (Sigma, St. Louis, MO) diluted 1:1000 in PBS. All incubations were for 1 hour at 22°C. To determine the extent of RCAS infection, additional coverslips of cells were fixed with 3.7% formaldehyde for 8 minutes at 22°C, blocked in PBS+1% GS + 0.1% Triton X-100 for 1 hour at 22°C, incubated with AMV-3C2 primary antibody [Developmental Studies Hybridoma Bank] which recognizes avian myoblastosis virus gag protein p19, diluted 1:1000 in PBS+1% GS for 1 hour at 22°C, and incubated with FITC conjugated goat anti-mouse IgG1 (Fisher) diluted 1:100 in PBS+1% GS for 1 hour at 22°C. To determine the percentage of primary culture that was myogenic, additional coverslips of cells were fixed with 3.7% formaldehyde for 8 minutes at 22°C, blocked in PBS+1% GS + 0.1% Triton X-100 for 1 hour at 22°C, incubated with an anti-Myf5 primary antibody [Courtesey of Dr. Steve Konieczny, Purdue University], diluted 1:250 in PBS+1% GS for 1 hour at 22°C, and incubated with FITC conjugated goat anti-rabbit IgG1 (Fisher) diluted 1:100 in PBS+1% GS for 1 hour at 22°C. Three representative fields/coverslip containing an average of 500 cells/field and 3 coverslips/treatment were photographed and scored. The number of total, BrdU-positive and myosin-positive cells were counted. The ED11 experiment was replicated 3 times. Statistical analysis was done by ANOVA-two factor with replication analysis supplied in Microsoft Excel.

Embryo Injections and Collections: Fertilized standard-specific pathogen-free (S-SPF) eggs were obtained from SPAFAS (Norwich, CT). Embryos were prepared and injected as described previously (Fekete and Cepko, 1993). Briefly, a window was created in the eggshell at Embryonic Day 4 (Hamburger and Hamilton stage 22) (Hamburger and Hamilton, 1951). Once exposed, the embryonic sack was perforated, and approximately $2X10^4$ cfu of RCAS-control or RCAS-FGF10 virus was injected into the developing right hind limb bud using glass micropipettes. Following the injection the eggshell was sealed with tape and the embryo returned to the incubator until harvest. Embryos were harvested 5 (HH stage 35), 6 (HH stage 36) or 7 (HH stage 37) days after injection. Six embryos from each treatment group were isolated from each time point. Embryos were decapitated and incubated in STRECK fixative (Streck Labs; Omaha, NE) overnight at room temperature followed by infiltration with 30% sucrose at 4°C prior to embedding in OCT (Miles) and storage at −80°C. Embryos were cryosectioned at a thickness of 14 µm.

Toluidine Blue-Fast Green: Tissue sections generated as described above were stained for 5 min. in 0.1% fast green FCF (Sigma) in 1% acetic acid, rinsed twice in 1% acetic acid, and then stained for 15 min in 0.04% toluidine blue O (Sigma) in 0.2 M acetate buffer. The sections were dehydrated through a graded ethanol series and clarified with mixed xylenes prior to mounting.

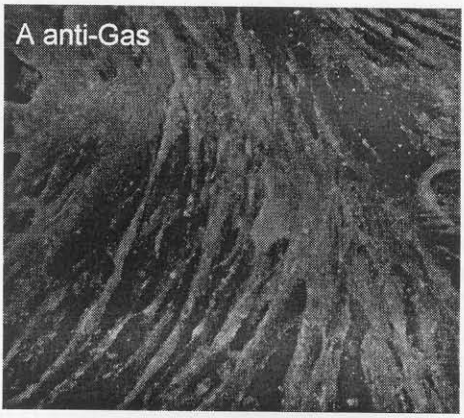
Immunohistochemistry: For immunohistochemical staining, slides were fixed in

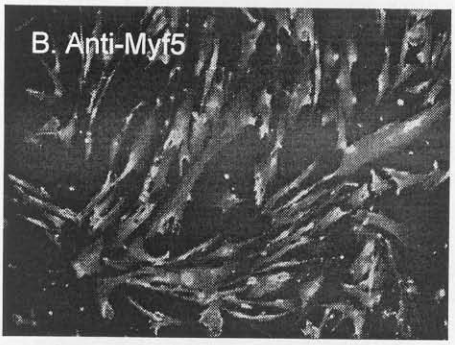
STF for five minutes followed by a rinse in water for five minutes. Slides were then incubated with a blocking solution [PBS (138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4·7H2O, 1.2 nM KH_2PO_4) + 5% horse serum] for 1 hour at room temperature. When using horseradish peroxidase conjugated avidin/biotin complex for 3,3'-diaminobenzidine (DAB) colorometric detection, hydrogen peroxide was added to the blocking solution at 0.3% (v/v). After blocking, slides were rinsed in PBS for five minutes prior to incubation with primary antibody diluted in PBS + 1% horse serum for 1 hour at room temperature. The following primary antibodies and dilutions were used: anti-viral gag protein (AMV-3C2; Developmental Studies Hybridoma Bank; 1:10 hybridoma culture supernatant) and anti-myosin heavy chain (MF20; Developmental Studies Hybridoma Bank; 1:10 hybridoma culture supernatant). Sections were rinsed twice in PBS and incubated with a biotinylated horse anti-mouse secondary antibody (Vector Laboratories, Inc.) diluted 1/400 in PBS + 1% horse serum for 1 hour at room temperature. Immunoreactivity was detected using the ABComplex and 3,3-diaminobenzidine substrate as per manufacturer's instructions (Vector Laboratories, Inc.).

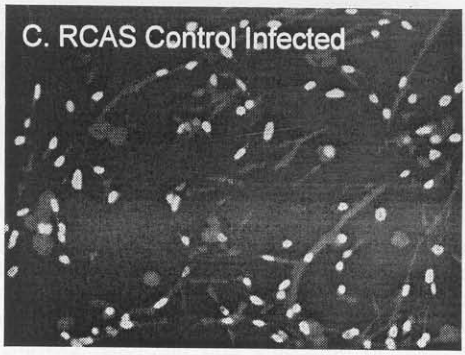
Data Analysis: Digital images were captured with a Leaf Microlumina camera mounted on an Olympus BX50 microscope. Sections stained with toluidine blue were scanned at 1.25X magnification and used to determine anatomically comparable regions within the uninjected contralateral control and injected limbs. Sections immunostained for *gag* protein were compared with toluidine blue slides to ensure that the regions of the limb being analyzed were infected with virus. The images were imported into Adobe Photoshop 4.0 and muscle area was determined by obtaining a total pixels number within the outlined area. Ten sections/embryo/collection time point were analyzed statistically by ANOVA as described above.

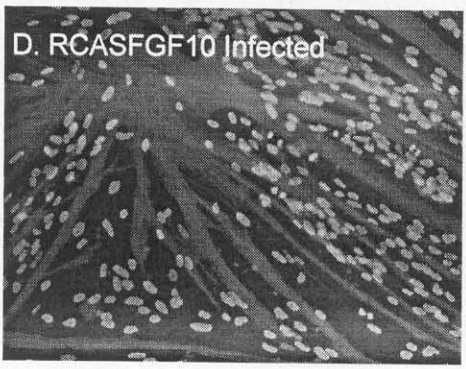
Results

In Vitro Infection and Proliferation: ED11 primary myoblast cultures were infected with an RCAS-FGF10 virus or an RCAS virus containing no insert. Complete viral infection was verified by fluorescent immunolocalization of the viral gag protein (Fig. 1.A). That the primary cultures were primarily myogenic was demonstrated by immunostaining for Myf5 (Fig. 1.B). Ectopic









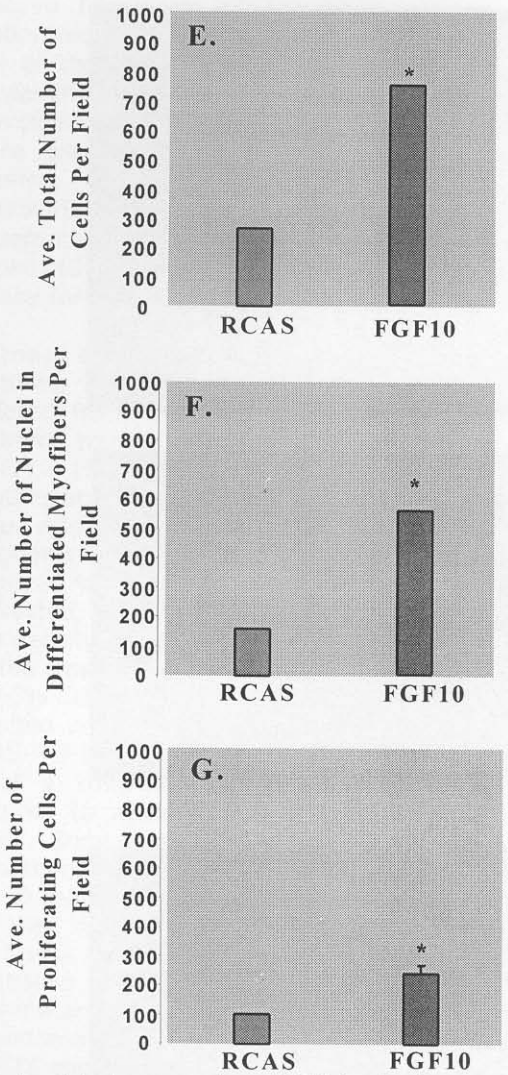


Fig. 1: Ectopic expression of FGF10 stimulates myoblast expansion and myofiber formation *in vitro*. ED11 primary myoblast cultures were infected with RCAS or RCAS-FGF10 viral constructs as described in Materials and Methods. Complete viral infection was verified by fluorescent immunolocalization of the viral gag protein (A; Green: Gap protein: Blue: Hoechst staine nuclei). That the primary cultures were primarily myogenic was demonstrate by immunostaining for Myf5 (B; Green nuclear staining). Ectopic expression of FGF10 resulted in a significant increase in total cell number as compared to the RCAS control group (C,D,E). Similarly, the infected myofibers showed an increase in fused nuclei, causing an increase in muscle fiber formation and size (C,D,F). Infected myoblasts also showed a significant increase in BrdU positive, prolife rating nuclei (C,DG). *indicates $p<0.05$; SEM indicated

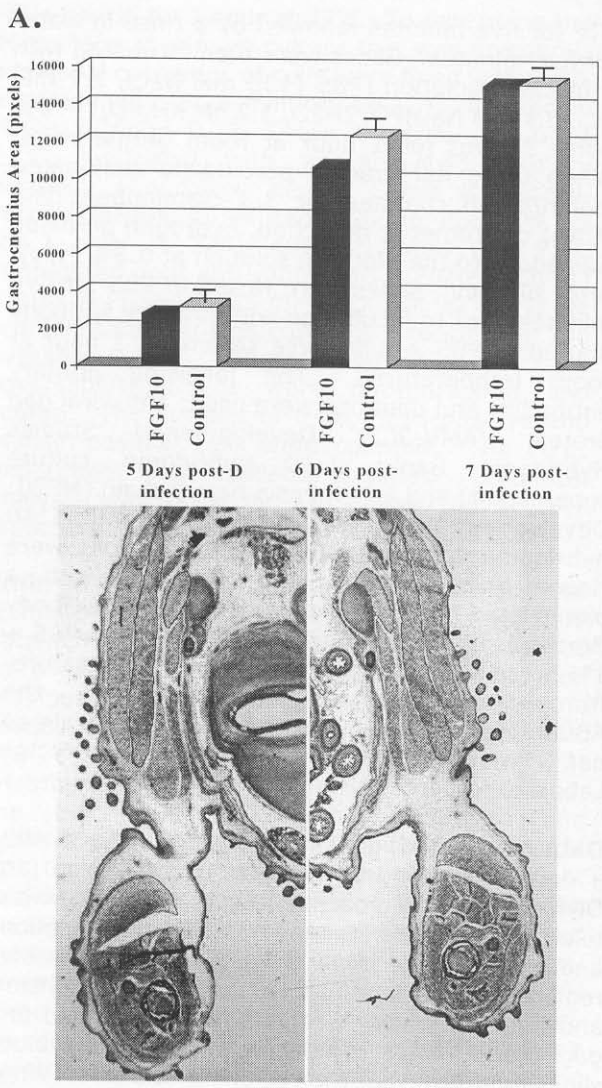


Fig. 2: Ectopic expression of FGF10 has no effect on muscle growth and development *in vivo*. Stage 22 embryos were injected with an RCAS-FGF10 virus in the right hind limb. Five 6 and 7 days post-infection, embryos were sectioned, stained and analyzed as describe in Materials and Methods. (A) There was no significant difference in the area of the gastrocnemius muscles between FGF10 infected and contralateral control limbs. (B;C) Representative exampleof section through the hind limb of an embryo 7 days post-infection. Gross examination of individual muscles (Red=Gastrocnemius; Orange =Iliotibialis; Deep blue = Biceps femoris, Green = Semitendinous; Sky Blue = Semimembranous; Purple = Femoritibialis externis; Yellow = Ischiofemoralis) show that muscles ectopically expressing FGF10 © are not different in size compared to contralatera) control muscles (B). Scale bars in B and C represent 500 µm; t=Tibiotarsus; f=Fibula; I=ischium; SEM indicated in (A).

expression of FGF10 resulted in a significant increase in total cell number as compared to the RCAS control group (Fig. 1C,D,E). Similarly, the infected myofibers showed an increase in fused nuclei, resulting in an increase in muscle fiber formation and size (Fig. 1C,D,F). Infected myoblasts also showed a significant increase in BrdU positive, proliferating nuclei (Fig. 1C,D,G). These results demonstrate that ectopic FGF10 stimulates an increase in muscle cell proliferation and differentiation to mature muscle fibers. While it is clear that there are downstream mediators of FGF10 function, our results clearly show that FGF10 initiates the increase in muscle mass in vitro. Our *in vitro* results also demonstrate that our FGF10 virus contains biologically active FGF10.

Viral Infection *In vivo*: Chicken embryos were injected with RCAS(A)-FGF10 at day 4 (stage 22) in the right hind limb as described in Materials and Methods. To track the progression of viral infection, serial sections through the embryos were made after harvest five to seven days post injection. Viral infection was visualized following immunolocalization for the viral protein *gag*. As previously described, the virus remains almost entirely localized to the right hind limb tissues for the first five days following infection before crossing over to the left limb by day seven post-infection (data not shown) (Clase *et al.*, 2000; Mitchell *et al.*, 2002).

FGF10 Has No Significant Effect On Skeletal Muscle Development *In vivo*: Five, 6 and 7 days post-infection, embryos were sectioned and stained. Muscles ectopically expressing FGF10 were not significantly different in size when compared to contralateral control muscles (Fig. 2). In addition, infection with the RCAS-control virus had no effect on muscle development (data not shown)(Clase *et al.*, 2000; Mitchell *et al.*, 2002.). Ectopic expression of FGF10 had no significant effect on overall limb morphogenesis (Fig. 2.B,C).

Discussion

In this manuscript, we examined the role of FGF10 during skeletal muscle development *in vitro* and *in vivo*. FGF10 mRNA expression is significantly increased in adult chicken anterior latissimus dorsi muscle undergoing stretch stimulated hypertrophy (Mitchell *et al.*, 1999). This observation suggests an important function for FGF10 during skeletal muscle growth and development. However, the role or roles of FGF10 during avian skeletal muscle development has not yet been well characterized. We demonstrated that ectopic FGF10 expression in myoblast cell lines *in vitro* results in a proliferative response coinciding with an increase in the number of nuclei contained within differentiated myofibers. In contrast, our results suggest that FGF10 ectopic expression in the developing avian hindlimb did not affect the formation or quantity of skeletal muscle. Evidence that biologically active FGF10 was expressed include : 1. The biological activity of FGF10 demonstrated in our cultured muscle cells; and 2. The FGF10 RCAS virus used in our studies was given to us by Dr. S. Noji, who used it to initiate limb bud outgrowth (Ohuchi *et al.*, 1997). FGF10 has been shown to be required during limb development as the causative agent of the progress zone during limb outgrowth (Ohuchi *et al.*, 1997). FGF10 protein is endogenously expressed in the progress zone of developing limbs (Tamura *et al.*, 1999). FGF10, FGF8 and FGFR2 create a positive feedback loop between the apical epidermal ridge and the progress zone (Ohuchi *et al.*, 1997). Therefore, while FGF10 has important functions during the initial events of limb development, our results suggest that FGF10 regulatory functions during the later stages of limb organogenesis are limited.

The ability of FGF10 to stimulate proliferation in cultured skeletal muscle is in agreement with observations that FGF1, FGF2, FGF4 and FGF6 stimulate proliferation in addition to inhibiting differentiation (Linkhart *et al.*, 1980; Linkhart *et al.*, 1981; Olwin and Rapraeger, 1992; Rando and Blau, 1994). Therefore, it was believed classically that FGFs were responsible for stimulating proliferation of myogenic precursor cells *in vivo*. While this may be true for some FGFs, it is becoming more clear that not all FGF-family members will have a proliferation stimulating / differentiation inhibiting function with regard to skeletal muscle development *in vivo*. For example, the hindlimbs of avian embryos overexpressing FGF5 or FGF4 were found to contain substantially fewer myofibers and myoblasts (Vovard *et al.*, 2001; Clase *et al.*, 2000). In contrast, somite muscle precursor cells infected with virus producing FGF1 or FGF4 differentiate into muscle fibers following arrival in the limb bud, suggesting that these FGFs do not inhibit differentiation *in vivo* as they do *in vitro* (Itoh *et al.*, 1996). In addition, skeletal muscle in mice with a null mutation for FGF6 develops normally (Fl,oss *et al.*, 1997). Interestingly, the FGF6 (-/-) mice show a severe retardation of muscle regeneration that is accompanied by extensive fibrosis. This suggests

that FGF6 is required for satellite cell activation and subsequent myofiber formation during regeneration, and does not appear to have an inhibitory effect on myogenic differentiation *in vivo*. Our results with FGF10 are distinct from either of these results, as they suggest that this FGF-family member had no significant effect on embryonic muscle development *in vivo*. It is possible that FGF10 could have an effect on myoblasts from muscle from different developmental stages, such as adult satellite cells. The definitive regulatory mechanisms of each individual FGF *in vivo* at individual developmental stages will probably be due to variations in FGF-ligand-receptor affinities and receptor(s) expression/availability within skeletal muscle, and remain to be elucidated.

In conclusion, our results demonstrate that FGF10 is not a candidate for stimulation of the initial events of avian skeletal muscle development *in vivo*. Our results also demonstrate the importance of confirming growth factor functions *in vitro* with their corresponding functions *in vivo*.

References

Fernandez-Teran, M., M. E. Piedra, M. A. Ros and J. F. Fallon, 1999. The recombinant limb as a model for the study of limb patterning, and its application to muscle development, Cell Tissue Res., 296, 121.

Gospodarowicz, D., 1974. Localisation of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth, Nature, 249: 123.

Gospodarowicz, D., J. Weseman and J. Moran, 1975. Presence in brain of a mitogenic agent promoting proliferation of myoblasts in low density culture, Nature, 256: 216.

Hannon, K., A. J. Kudla, M. J. McAvoy, K. L. Clase and B. B. Olwin, 1996. Differentially expressed fibroblast growth factors regulate skeletal muscle development through autocrine and paracrine mechanisms, Journal of Cell Biology, 132: 1151.

Linkhart, T. A., C. H. Clegg and S. D. Hauschka, 1980. Control of mouse myoblast commitment to terminal differentiation by mitogens, J. Supramolecular Structure, 14: 483.

Linkhart, T. A., C. H. Clegg and S. D. Hauschika, 1981. Myogenic differentiation in permanent clonal mouse myoblast cell lines: regulation by macromolecular growth factors in the culture medium, Developmental Biology, 86: 19.

Olwin, B. B. and A. Rapraeger, 1992. Repression of myogenic differentiation by aFGF, bFGF, and K-FGF is dependent on cellular heparan sulfate, J Cell Biol., 118: 631.

Seed, J. and S. D. Hauschka, 1988. Clonal analysis of vertebrate myogenesis. VIII. Fibroblasts growth factor (FGF)-dependent and FGF-independent muscle colony types during chick wing development, Developmental Biology, 128: 40.

Itoh, N., T. Mima and T. Mikawa, 1996. Loss of fibroblast growth factor receptors is necessary for terminal differentiation of embryonic limb muscle, Development, 122: 291.

Edom-Vovard, F., M. A. Bonnin and D. Duprez, 2001. Misexpression of Fgf-4 in the chick limb inhibits myogenesis by down-regulating Frek expression, Dev Biol., 233: 56.

Clase, K. L., P. J. Mitchell, P. J. Ward, C. M. Dorman, S. E. Johnson and K. Hannon, 2000. FGF5 stimulates expansion of connective tissue fibroblasts and inhibits skeletal muscle development in the limb, Dev Dyn, 219: 368.

Flanagan-Steet, H., K. Hannon, M. J. McAvoy, R. Hullinger and B. B. Olwin, 2000. Loss of FGF receptor 1 signaling reduces skeletal muscle mass and disrupts myofiber organization in the developing limb, Dev Biol., 218, 21.

Mitchell, P., T. Steenstrup and K. Hannon, 1999. Expression of fibroblast growth factor family during postnatal skeletal muscle hypertrophy, J. Appl Physiol., 86: 313.

Ohuchi, H., T. Nakagawa, A. Yamamoto, A. Araga, T. Ohata, Y. Ishimaru, H. Yoshioka, T. Kuwana, T. Nohno, M. Yamasaki, N. Itoh and S. Noji, 1997. The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor, Development, 124: 2235.

Fekete, D. and C. Cepko, 1993. Replication-competent retroviral vectors encoding alkaline phosphatase reveal spatial restriction of viral gene expression/transduction in the chick embryo, Molecular & Cellular Biology, 13: 2604.

Hamburger, V. and H. L. Hamilton, 1951. A series of normal stages in the development of the chick embryo, J. Morphol., 88: 49.

Mitchell, P. J., S. E. Johnson and K. Hannon, 2002. Insulin-like growth factor I stimulates myoblast expansion and myofiber development in the limb, Dev Dyn., 223: 12.

Yonei-Tamura, S., T. Endo, H. Yajima, H. Ohuchi, H. Ide and K. Tamura, 1999. FGF7 and FGF10 directly induce the apical ectodermal ridge in chick embryos, Dev. Biol., 211: 133.

Rando, T. A. and H. M. Blau, 1994. Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy, J. Cell Biology, 125: 1275.

Floss, T., H. H. Arnold and T. Braun, 1997. A role for FGF-6 in skeletal muscle regeneration, Genes Dev., 11: 2040.