Interactions between FGF18 and retinoic acid regulate differentiation of chick embryo limb myoblasts

Gi Fay Mok [1], Ryan Cardenas, Helen Anderton, Keith H.S. Campbell, Dylan Sweetman *

Division of Animal Sciences, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Sutton Bonington LE12 5RD, UK

ABSTRACT

During limb development *Pax3* positive myoblasts delaminate from the hypaxial dermomyotome of limb level somites and migrate into the limb bud where they form the dorsal and ventral muscle masses. Only then do they begin to differentiate and express markers of myogenic commitment and determination such as *Myf5* and *MyoD*. However the signals regulating this process remain poorly characterised. We show that FGF18, which is expressed in the distal mesenchyme of the limb bud, induces premature expression of both *Myf5* and *MyoD* and that blocking FGF signalling also inhibits endogenous *MyoD* expression. This expression is mediated by ERK MAP kinase but not PI3K signalling. We also show that retinoic acid (RA) can inhibit the myogenic activity of FGF18 and that blocking RA signalling allows premature induction of *MyoD* by FGF18 at HH19. We propose a model where interactions between FGF18 in the distal limb and retinoic acid in the proximal limb regulate the timing of myogenic gene expression during limb bud development.

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Introduction

Amniote limb muscles are derived from myoblasts that originate in somites and migrate into developing limb buds (Chevallier et al., 1977; Christ and Brand-Saberi, 2002). Delamination and migration of these cells from the ventro-lateral lip of the hypaxial dermomyotome is regulated by Hepatocyte Growth Factor/Scatter Factor (HGF/SF) (Dietrich et al., 1999; Scaal et al., 1999) and requires the paired box transcription factor *Pax3* (Franz et al., 1993). Once in the limb myoblasts migrate to form the dorsal and ventral muscle masses. Only then do they begin to express the Myogenic Regulatory Factors (MRFs), basic helix loop helix transcription factors comprising *Myf5, MyoD, myogenin* and *MRF4* which, ultimately, leads to differentiation of mature, functional myotubes (Buckingham et al., 2003; Mok and Sweetman, 2011).

Myogenesis has been extensively studied during embryo development and provides an excellent paradigm to understand how inductive signals regulate differentiation. Much of this work has focused on somites and extensive work has shown that interactions between Wnt, Shh and BMP signalling in both chicken and mouse embryos are critical for myogenesis (Borycki et al., 1999, 1998; Hirsinger et al., 1997; Munsterberg et al., 1995; Munsterberg and Lassar, 1995; Pourquie et al., 1996; Tajbakhsh et al., 1998). However

it is clear that the signalling events that control myogenesis in developing limbs are distinct from those in somites. It has been suggested that limb myoblasts differentiate via a default pathway once they escape inhibitory BMPs (Amthor et al., 1998). Nevertheless other signals are involved in limb myogenesis (Christ and Brand-Saberi, 2002; Duprez, 2002; Venters et al., 2004) including fibroblast growth factors (FGFs) (Edom-Vovard et al., 2001; Marics et al., 2002) and retinoic acid (RA) (Reijntjes et al., 2010) both of which have been reported to have inductive and repressive roles depending on concentration and cellular context. Recently Shh has also been shown to have an important role in the initiation of *Myf5* and *MyoD* expression in limb myoblasts as well as their subsequent migration (Anderson et al., 2012; Hu et al., 2012).

Several lines of evidence suggest that FGF receptors play important roles in limb myogenesis (Flanagan-Steet et al., 2000; Lagha et al., 2008; Marcelle et al., 1995; Marics et al., 2002). Grafting of FGF soaked beads has been shown to negatively regulate muscle cell differentiation in somites (Sweetman et al., 2006) while retroviral FGF4 mediated expression can inhibit myogenesis in limbs (Edom-Vovard et al., 2001). However it has not been clearly established which of the FGF ligands are responsible for this activity in vivo and is further complicated by the ability of FGFs to induce their own negative regulators, resulting in complex feedback loops (Eblaghie et al., 2003; Smith et al., 2005). Although ectopic FGF4 can inhibit limb bud muscle gene expression (Edom-Vovard et al., 2001) it is normally expressed in the apical ectodermal ridge (AER) which is probably too far from the developing myoblasts to play a role (Christen and Slack, 1999) while FGF10, which is expressed in the limb bud mesenchyme, is not able to induce

myogenic gene expression in vivo (Ward et al., 2003). Other FGFs expressed in the limb include FGF8 and FGF2 which, like FGF4, are expressed in the AER, and FGF12 and FGF13 which are intracellular FGFs and do not signal via tyrosine kinase receptors (Karabagli et al., 2002). At least two FGF receptors, FGFR1 and FGFR4, are expressed in areas of the limb where myoblasts are located (Marcelle et al., 1995; Sheeba et al., 2010) and loss of function of either of these receptors disrupts limb muscle formation (Flanagan-Steet et al., 2000; Itoh et al., 1996; Marics et al., 2002).

We wished to determine which FGF is likely to regulate limb bud myogenic gene expression in vivo. We identified FGF18 as a candidate because it is expressed in the limb bud mesenchyme (Maruoka et al., 1998; Ohuchi et al., 2000) and can signal through FGFR4 which is known to play a role in myogenesis (Kwiatkowski et al., 2008; Marics et al., 2002; Zhang et al., 2006). FGF18 has been shown to regulate chondrocyte proliferation and hence bone growth in the developing limb (Liu et al., 2007) as well as hair follicle growth (Kawano et al., 2005; Leishman et al., 2013) and lung development (Elluru et al., 2009; Usui et al., 2004) but has not previously been implicated in myogenesis.

Interactions between retinoic acid and FGF signals have also been proposed to pattern the proximal–distal axis of the developing chick limb (Cooper et al., 2011; Mercader et al., 2000; Roselló-Díez et al., 2011, 2014). While this idea remains controversial (Cunningham et al., 2013) we hypothesised that interactions between these signals could provide a mechanism for controlling the timing of myoblast commitment and differentiation in the developing chicken limb. In this context it is worth noting that FGF and RA signalling pathways are known to interact during axis extension (del Corral et al., 2003) and that FGF18 expression is also regulated by RA signalling in both the trunk (Zhao and Duester, 2009) and the digits (Zhao et al., 2010).

Our data suggest that FGF18 from the distal limb bud regulates the timing of expression of the myogenic markers *Myf5* and *MyoD* through the ERK MAP kinase signalling pathway and that this is antagonised by high levels of retinoic acid in the proximal limb. We propose that interactions between these signalling pathways control the timing of progression of myoblasts from proliferative precursors to committed myocytes.

Materials and methods

Probes and in situ hybridisation

Dig-11-UTP (Roche) labelled antisense RNA probes were generated from full length cDNAs for *Myf5* and *MyoD* cloned into pGEM (Promega) (Sweetman et al., 2008) were linearised with SacII and transcribed with SP6 RNA polymerase. *Myogenin* probes were generated from pBS-SK-Mgn linearised with SalI and transcribed with T7 RNA polymerase. Full length FGF18 was cloned from whole D5 embryo cDNA into pGEM using the following primers: FGF18F: ATGTATT-CACTGCTCTCC, FGF18-HA-R (also includes sequence for c terminal HA tag): TAAGCGTAATCTGGAACATCGTATGGGTAACTGGGGTTGGTGGG-TCG. PCR was performed with Phusion DNA polymerase (NEB), A-tailed and cloned into pGEM-T Easy. For Dig labeled probe transcription plasmid DNA was linearised with SacII and transcribed with SP6 RNA polymerase. MRF probes were as described in Sweetman et al. (2008) and Pax3 probes as in Abu-Elmagd et al. (2010). In situ hybridisation was as described in Smith et al. (2005).

Immunostaining

Embryos were harvested and dissected in cold PBS and fixed in 4% paraformaldehyde at 4 °C overnight. Embryos were washed in PBS for 30 min at room temperature and then washed in 0.2%

Triton X-100 at 4 °C overnight. Embryos were washed in PBS for 30 min at room temperature and then incubated in PBS with Mouse-anti p44/42 MAPK (Erk1/2) (Cell Signalling Technology, cat no 9102,) diluted 1:50 in PBS at 4 °C for 72 h in darkness. Embryos were then washed in PBS and incubated in PBS with Alexa Fluor 488 goat anti-mouse IgG2b (Invitrogen) diluted 1:200 at 4 °C overnight in the dark. Embryos were fixed and imaged on a Leica MZ10F steromicroscope.

FGF and pharmacological inhibitor beads

Heparin beads (Sigma H-5263) were soaked for 1 h at room temperature in recombinant human FGF18, FGF4 or FGF10 (Peprotech) at 0.5 mg/ml reconstituted in PBS with 0.1% BSA before grafting into limb buds. Control beads were soaked in PBS with 0.1% BSA for 1 h. AG 1-X2 beads (BioRad) were incubated in either 4 mg/ml all-trans retinoic acid (Sigma) or the following pharmacological inhibitors: 10 mM U0126 (Merck), 10 mM SB203580 (Tocris bioscience), 2 mg/ml BMS493 (Tocris), 10 mM LY294002 (Calbiochem), 10 mM SU5402 (Calbiochem) or 10 mM FIIN 1 hydrochloride (Tocris bioscience) all dissolved in DMSO. Control beads were soaked in DMSO. Beads were soaked in inhibitors for at least 1 h at room temperature in the dark, washed briefly in 2% phenol red then rinsed in PBS prior to grafting.

Chick embryo manipulations

Fertile chicken White Leghorn eggs were obtained from Henry Stewart & Co Ltd (Norfolk, UK) and incubated at 37.5 °C until the desired Hamburger & Hamilton stage was reached. Manipulated embryos were visualised by injection of Windsor & Newton Black India Ink diluted 1:500 in PBS under the embryo. Beads were grafted into slits cut into limb buds with a sharpened tungsten wire needle. Eggs were re-sealed with sellotape and incubated at 37.5 °C for either 1, 6 or 24 h.

Results

FGF18 expression in developing limb mesenchyme is consistent with a role in myogenic gene induction

As FGF18 expression has been described in developing chicken and mouse embryo limbs (Maruoka et al., 1998; Ohuchi et al., 2000) we first confirmed that it is expressed in chick embryos with a spatio-temporal pattern consistent with a role in myogenesis. We cloned a full length FGF18 cDNA for in situ hybridisation and examined its expression from HH stage 18 to HH stage 26, the period when myoblasts migrate into and differentiate in limb buds. At HH18 FGF18 is expressed in the tailbud, pharyngeal arches, nasal placode and isthmus, consistent with previous reports (Fig. 1A) (Ohuchi et al., 2000). We first detected FGF18 in limb buds at HH20 where it is expressed in the distal mesenchyme of both fore- and hindlimbs (Fig. 1B). Expression of FGF18 is maintained in the distal mesenchyme from stages HH22 to HH26 (Fig. 1C, D and E) and at HH26 is also detected in the condensing cartilage of the hind limb (Fig. 1E). Sections through limb buds at HH22 show expression of FGF18 distal to the dorsal and ventral muscle masses where Pax3 expressing myoblasts are beginning to differentiate and express markers such as *Myf5* and *MyoD* (Fig. 1F–I).

Grafting FGF18 beads into developing limbs induces premature expression of Myf5, MyoD and myogenin

To test directly if FGF18 can induce expression of muscle markers we grafted beads soaked in recombinant FGF18 protein
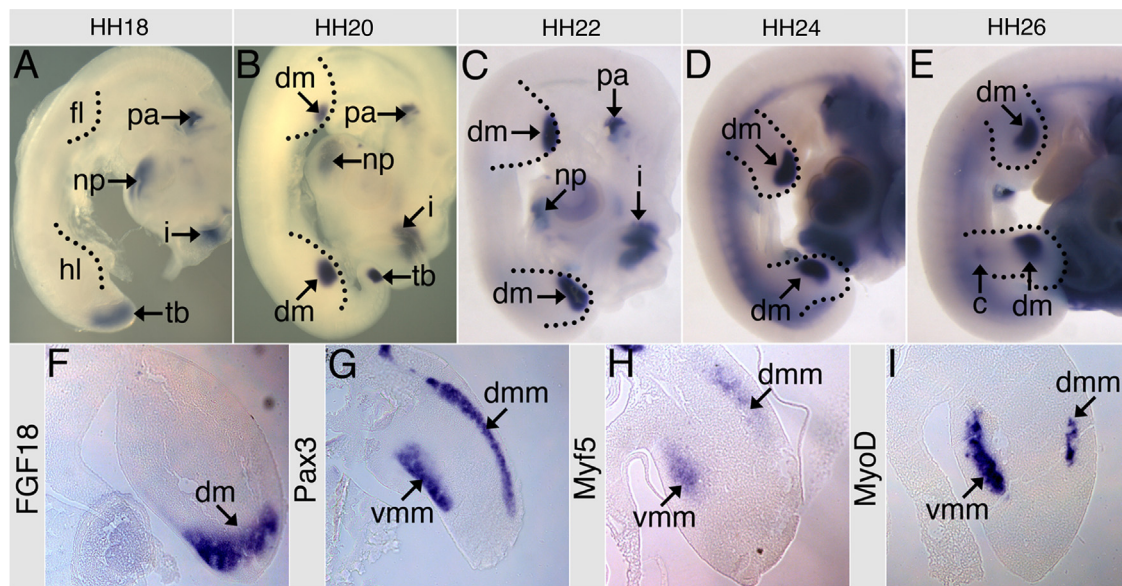
Fig. 1. Expression of FGF18 during limb development is consistent with a role in myogenic differentiation. (A) At HH18 FGF is expressed in the isthmus, pharyngeal arches, nasal placode and tailbud. (B) FGF18 is first detected in limbs at HH20 where is expressed in the distal mesenchyme of the limb bud. (C) and (D) FGF18 expression is maintained in the distal mesenchyme of the limb bud at HH22 and HH24 and (E) at HH26 is also detected in the condensing cartilage of the zeugopod. (F) Section through developing forelimb at HH22 showing expression of FGF18 in the distal mesenchyme adjacent to the dorsal and ventral muscle masses shown be expression of *Pax3* (G), *Myf5* (H) and *MyoD* (I). fl, forelimb; hl—hindlimb; pa—pharyngeal arches; np—nasal placode; tb—tailbud; i—isthmus; dm—distal mesenchyme; dmm—dorsal muscle mass; vmm—ventral muscle mass; c-condensing cartilage.

into developing limbs at stages between HH19 and HH21 and analysed effects on myogenesis by in situ hybridisation with specific markers for different stages of muscle development. Ectopic expression of *Myf5* in migratory myoblasts was detected after six hours incubation with FGF18 at HH19 (6/6 embryos, Fig. 2A, B and M) and HH21 (7/7 embryos, Fig. 2G, H and N). *MyoD* is upregulated by FGF18 after six hours at HH stage 21 (12/12 embryos, Fig. 2I and J) but not at HH stage 19 (12/12 embryos, Fig. 2C and D). To assess the effects on later markers of myogenesis we also examined *myogenin* expression after FGF18 bead grafts at HH21. Six hours after grafting we did not detect ectopic *myogenin* expression (4/4 embryos, Fig. 2E and F) but did after 24 h incubation with FGF18 beads (5/6 embryos, Fig. 2K and L).

As *Pax3* is expressed in proliferative myoblasts and down-regulated as differentiation proceeds we also examined the effects of FGF18 on this gene. Although we did not observe large scale changes in expression in wholemount embryos with FGF18 beads grafted at HH21 (4/4 embryos) (Fig. 2O) sections through these limbs did show localised downregulation of *Pax3* immediately adjacent to the FGF18 bead (Fig. 2P).

We also tested the ability of other FGFs to induce ectopic *MyoD* expression. FGF4 was able to induce *MyoD* expression (9/9 embryos, Fig. 2Q) while FGF10 was not (7/7 embryos, Fig. 2R).

Embryos grafted with control beads soaked in 0.1% BSA did not show ectopic expression of *Myf5* at HH19 (4/4 embryos, Fig. 1S and T) or HH21 (5/5 embryos Fig. 2U and V). We also did not detect ectopic *MyoD* expression following 0.1% BSA bead grafts at HH19 (5/5 embryos, data not shown) or after grafting at HH21 (6/6 embryos, Fig. 1W and X).

FGF18 dependant MyoD expression requires ERK phosphorylation

To identify the signal transduction pathway responsible for ectopic *MyoD* expression following FGF18 bead application we used a phospho specific antibody staining to detect activiation of ERK MAP kinase. Within 1 h of bead grafting we detected high levels of phospho-ERK in the mesenchyme surrounding the bead (3/3 embryos, Fig. 3A–C). Beads soaked in FGF10 (4/4 embryos,

Fig. 3D–F), which is also expressed in limb bud mesenchyme but has different receptor specificity (Zhang et al., 2006), or control beads soaked in 0.1% BSA (4/4 embryos, Fig. 3G–I) did not induce ERK phosphorylation.

To confirm that these results were specific to FGF receptor activation and not off target effects from FGF beads we co-grafted FGF18 beads with beads soaked in an inhibitor of all four FGFRs, FIIN 1 hydrochloride, which was able to block FGF18 induced *MyoD* expression (18/19 embryos, Fig. 4A). We also tested SU5402, another FGFR inhibitor which blocks signalling from FGFR1 and FGFR3, but this did not prevent FGF18 induced *MyoD* expression (8/10 embryos, Fig. 4D).

As MEK is upstream of ERK and is responsible for its phosphorylation we tested if its activity was required for ectopic *MyoD* expression induced by FGF18 by grafting FGF18 and beads soaked in the MEK inhibitor U0126 adjacent to each other in developing limb buds for 6 h. In these embryos U0126 beads blocked FGF18 induced expression of *MyoD* (12/13 embryos, Fig. 4B). To confirm the specificity of MEK in blocking FGF18 induced *MyoD* expression we also co-grafted FGF18 and beads soaked in the PI3K inhibitor LY294002. In these embryos FGF18 was still able to induce *MyoD* expression in the presence of LY294002 (8/11 embryos, Fig. 4C). Control beads soaked in DMSO did not affect *MyoD* induction by FGF18 (6/6 embryos, Fig. 4E).

To test if inhibition of MEK could also block endogenous expression of *MyoD* we grafted U0126 beads into embryos at HH23, the point at which MyoD expression is first detected in limb buds by in situ hybridisation. U0126 grafted limbs had reduced MyoD expression when compared to contralateral unmanipulated limbs (8/10 embryos, Fig. 4F and G) while DMSO control beads did not (10/10 embryos, Fig. 4H and I).

We then grafted beads soaked in either FIIN 1 hydrochloride, U0126 or DMSO into limb buds at HH21, harvested them after 24 h and examined *MyoD* expression by comparing operated limb buds to contralateral controls. FIIN 1 hydrochloride beads abrogated MyoD expression, particularly in the dorsal muscle mass (9/11 embryos, Fig. 4J and K) while U0126 beads did not affect *MyoD* expression in the majority of cases (17/24 embryos, Fig. 4L and M)
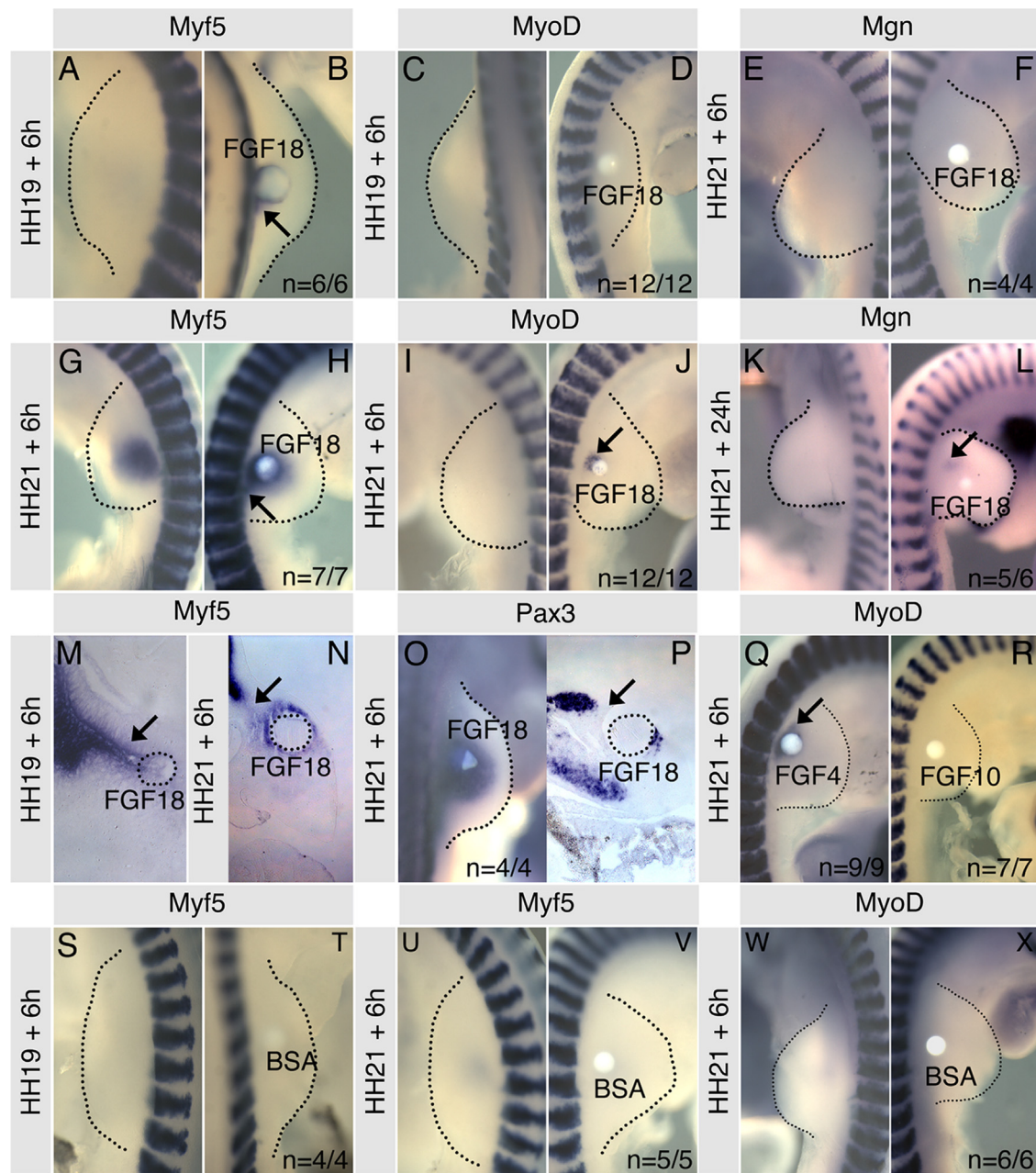
Fig. 2. FGF18 induces MRF expression in limb buds in vivo. FGF18 beads grafted into developing limbs *in ovo* at HH19 induce *Myf5* expression (B) but not *MyoD* expression (D) after 6 h incubation. Unmanipulated contralateral limbs from the same embryos are shown in (A) and (C). FGF18 beads grafted into developing limbs *in ovo* at HH21 induce both *Myf5* expression (H) and *MyoD* (J) expression after 6 h incubation. Unmanipulated contralateral limbs from the same embryos are shown in (G) and (I). FGF18 beads grafted into developing limbs *in ovo* at HH21 do not induce *myogenin* after 6 h incubation (F) but do after 24 h (L). Unmanipulated contralateral limbs from the same embryos are shown in (E) and (K). Transverse sections of *Myf5* stained embryos grafted with FGF18 beads at HH19 (M) and HH21 (N) (positions of beads shown by dotted circles) show expression of *Myf5* in migrating myoblasts from hypaxial somites. FGF18 beads grafted into developing limbs *in ovo* at HH21 does not obviously alter *Pax3* expression in wholemount in situ hybridisation (O) but in transverse sections localised downregulation of *Pax3* is seen close to the FGF18 bead (P). Beads soaked in FGF4 induce *MyoD* expression when grafted at HH21 (Q) but FGF10 soaked beads do not (R). Beads soaked in 0.1% BSA do not induce *Myf5* expression when grafted at HH19 (T). BSA beads grafted at HH21 do not induce *Myf5* (V) or *MyoD* (X) expression. Unmanipulated contralateral limbs from the same embryos are shown in (S), (U) and (W). Dotted lines show outlines of limb buds. Arrows indicate ectopic MRF expression (B, H, J, L, M, N, Q) or loss of *Pax3* expression (N)

although localised MyoD downregulation was observed in some cases (7/24 embryos). Control embryos with DMSO beads had normal MyoD expression in most cases (15/19 embryos, Fig. 4N and O).

Retinoic acid prevents FGF18 induced MyoD expression while RA inhibitors potentiate it

Retinoic acid is known to have both positive and negative effects on limb muscle differentiation depending on concentration (Reijntjes et al., 2010) and it has also been suggested that interactions between retinoic acid and FGF signalling can influence proximal–distal limb patterning (Cooper et al., 2011; Mercader et al., 2000; Roselló-Díez et al., 2011). Therefore we tested if RA signalling could affect the ability of FGF18 to induce myogenic gene expression. All trans retinoic acid (ATRA) soaked beads were grafted into forelimbs at HH21 and embryos harvested after 24 h. *MyoD* expression was reduced in these forelimbs compared to contralateral limbs (9/9 embryos, Fig. 5A and B), consistent with previous reports (Reijntjes et al., 2010). We then tested the ability
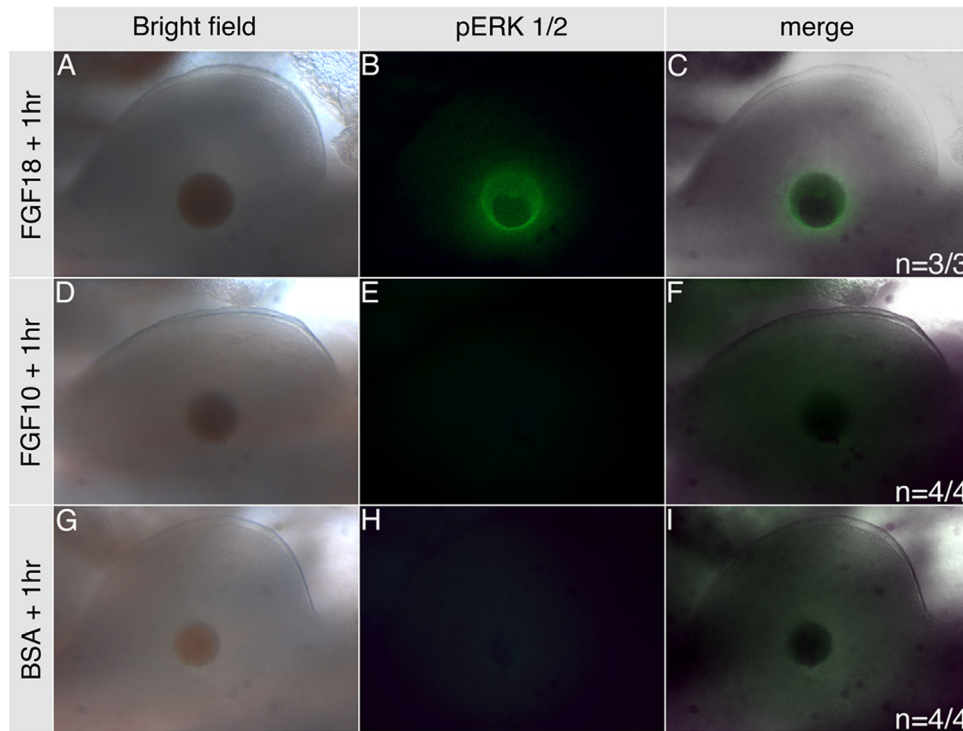
Fig. 3. FGF18 beads induce ERK phosphorylation. FGF18 (A)–(C) beads, grafted into HH21 limb buds and incubated for 1 h induce ERK phosphorylation. FGF10 (D)–(F) or 0.1% BSA (G)–(I) beads do not induce ERK phosphorylation. (A), (D) and (G) brightfield images, (B), (E) and (F) immunostaining with phospho-ERK specific antibody, (C), (F), (I) merged images.

of ATRA to block FGF18 induced *MyoD* expression directly by grafting FGF18 soaked beads adjacent to ATRA soaked beads into HH21 limb buds. The majority of these embryos did not show ectopic *MyoD* expression (19/25 embryos, Fig. 5C and D). Control embryos grafted with FGF18 and beads soaked in DMSO at HH21 showed the expected induction of *MyoD* (6/6 embryos, Fig. 4D). We grafted beads soaked in BMS493, a retinoic acid antagonist, into HH19 embryos along with FGF18 soaked beads. In these embryos we saw ectopic expression of *MyoD* (8/9 embryos, Fig. 5E and F) in contrast to embryos grafted with FGF18 alone at HH19 which do not express ectopic *MyoD* (Fig. 2D). Beads grafted into HH19 forelimbs soaked in BMS493 did not induce *MyoD* expression after 6 h (10/10 embryos, Fig. 5G) and neither did control beads soaked in DMSO (6/6 embryos, Fig. 5H).

Discussion

Our results show that FGF18 in limb buds can induce expression of the key regulators of myogenesis, *Myf5* and *MyoD* within 6 h of bead grafting, and that this is mediated via ERK MAP kinase signalling. We also demonstrate differing temporal responses in that *Myf5* is induced in both early (HH19) and later (HH21) limb buds while *MyoD* expression is only induced at later stages (HH21+). Finally we show that ectopically applied retinoic acid can inhibit the ability of FGF18 to induce *MyoD* while a retinoic acid antagonist, BMS493, can potentiate it in early limb buds. We propose that interactions between these two signals regulate the timing of onset of *Myf5* and *MyoD* expression in limb myoblasts (Fig. 6A and B).

FGF18 induces myogenic gene expression

Signals inducing myogenic expression have been extensively studied in somites and both *Myf5* and *MyoD* are known to be induced by Wnt and Shh signalling in the epaxial myotome (Borycki et al., 1998; Munsterberg et al., 1995; Tajbakhsh et al., 1998) and by Wnt and BMP in the hypaxial myotome (Dietrich et al., 1998; Geetha-Loganathan et al., 2005; Marcelle et al., 1997). In developing limbs the inductive signals are much less well characterised although several factors have been shown to inhibit early myogenesis including HGF/SF (Scaal et al., 1999), retinoic acid (Reijntjes et al., 2010), BMPs (Amthor et al., 1998) Shh (Duprez et al., 1998) and Notch (Delfini et al., 2000; Mayeuf-Louchart et al., 2014) while other factors known to induce early myogenic gene expression in somites, such as Wnts and Shh, appear to regulate later stages of limb muscle development (Anakwe et al., 2003; Anderson et al., 2012; Hu et al., 2012). As a result it has been proposed that limb bud myoblasts undergo differentiation as a result of the withdrawal of inhibitors of differentiation rather than in response to inductive signals (Amthor et al., 1998).

A strong candidate for an inducer of myogenic genes in developing limbs is FGF signalling through FGFR4. FGFR4 is required during myogenic differentiation of C2C12 cells (Kwiatkowski et al., 2008), is expressed in myoblasts as they migrate into the limb (Sheeba et al., 2010) and a dominant negative form of this receptor can inhibit *Myf5*, *MyoD* and MHC expression in limb myoblasts during development (Marics et al., 2002). Our data are consistent with a role for FGFR4 as both FGF18 induced and endogenous *MyoD* expression was blocked by a pan-FGFR inhibitor (FIIN 1 hydrochloride) (Zhou et al., 2010) but not SU5402 which is known to block FGFR1 and 3 (Grand et al., 2004; Mohammadi et al., 1997) but has not been shown to directly affect FGFR4 activity.

However, it has not been established which of the FGFs mediates this activity. Our data show that FGF4 or FGF8 soaked beads can induce *MyoD* in developing limbs in the same way as FGF18 (Fig. 2 and data not shown) but these FGFs are normally restricted to the apical ectodermal ridge, some distance from the differentiating myoblasts. Although FGFs can act over several cell diameters (Christen and Slack, 1999) the AER is probably too far from the myoblasts to be the source of an inductive signal for these
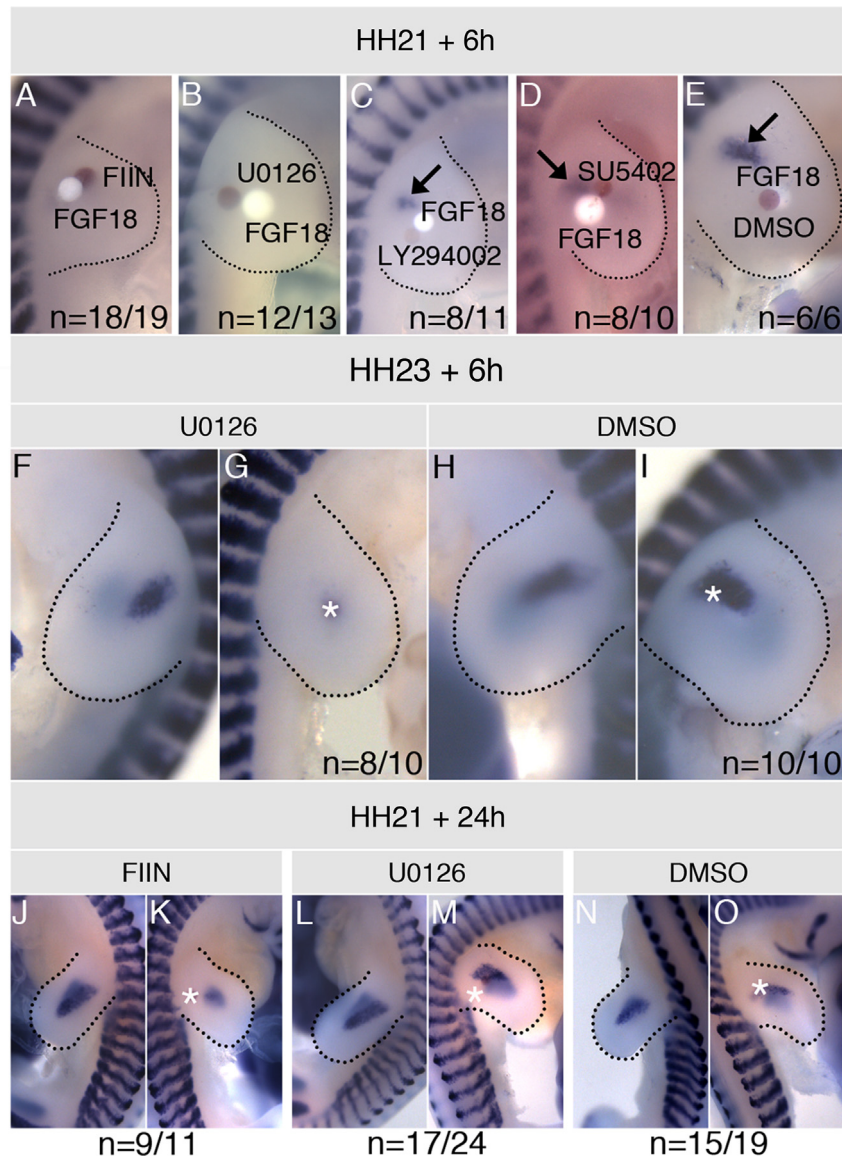
Fig. 4. Inhibition of FGF receptors and MEK blocks FGF18 induced ectopic *MyoD* expression in forelimbs but MEK inhibition does not affect long term induction of endogenous *MyoD*. FIIN hydrochloride (A) or U0126 (B) beads grafted adjacent to FGF18 beads at HH21 block induction of ectopic *MyoD* while beads soaked in LY294002 (C), SU5402 (D) or DMSO (E) do not. Embryos grafted with U0126 beads at HH23 and harvested after 6 h show reduced MyoD expression (G) compared to unoperated contralateral limbs (F) while control beads soaked in DMSO do not (H) and (I). Embryos grafted with FIIN 1 hydorochloride beads at HH21 and harvested after 24 h at HH26 show reduced *MyoD* expression (K) compared to contralateral control limbs (J). Beads soaked in U0126 (L) and (M) or DMSO (N) and (O) do not affect *MyoD* expression. Asterisks marks position of grafted bead. Arrows indicate ectopic *MyoD* expression.

cells and ERK phosphorylation in response to ridge FGFs does not seem to extend into the myogenic regions of the limb bud (Corson et al., 2003). In contrast to our data, previous reports have shown that FGF4 in limb buds can lead to loss of myogenic gene expression (Edom-Vovard et al., 2001). We believe that this can be reconciled with our observations as the manipulations we performed were different in two important respects; our observations were carried out over very short time scales, making it likely that this conflicting data was uncovering a later function of FGF signalling, and we used bead grafts to deliver FGF while Edom-Vovard et al. used RCAS retroviral misexpression. This makes it possible that we delivered higher doses of FGF which, in vitro, can lead to a switch between induction and repression of myogenesis (Pizette et al., 1996). It is also possible that longer term exposure to FGF results in the upregulation of negative regulators of FGF signalling such as Sprouty or MAPK phosphatases (Eblaghie et al., 2003; Ozaki et al., 2001; Smith et al., 2005) which could also lead to the discrepancy between these results and those we observe.

Another potential candidate is FGF10 which is expressed in the limb bud mesenchyme (Ohuchi et al., 1997) but this has been shown not to induce myogenesis in vivo (Ward et al., 2003) and does not signal through FGFR4 (Zhang et al., 2006). Based on the combination of its expression in the limb bud mesenchyme (Fig. 1 and Ohuchi et al., 2000), and its ability to signal through FGFR4 (Ellsworth et al., 2002; Xu et al., 2000) we identified FGF18 as a candidate inducer of limb bud myogenesis although our data does not rule out contributions from other FGFs in inducing limb bud myogenesis.

One observation from our data is that myogenic induction in these manipulations was mostly observed proximal to the bead. Although this might seem to conflict with our model, which would predict that myoblasts in the proximal limb bud should be more resistant to myogenic induction than more distal cells, this can be explained by the position of the myoblasts within the limb bud at these stages. Using *Pax3* in situ hybridisation to label these migratory cells shows that the majority of myoblasts at HH 20/21
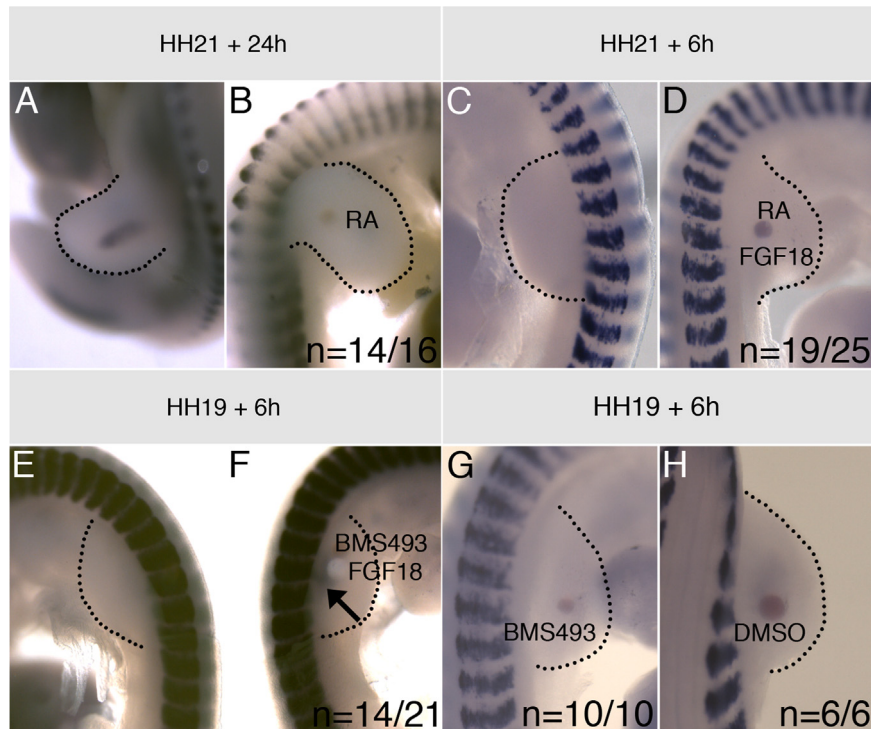
Fig. 5. RA signaling interacts with FGF18 induced *MyoD* expression. ATRA beads grafted into forelimbs at HH21 and incubated for 24 h (B) reduce endogenous *MyoD* expression compared to unmanipulated contralateral control limbs (A). Limbs grafted with both FGF18 and ATRA do not show ectopic *MyoD* expression (D). Unmanipulated control limbs from the same embryo also show no endogenous *MyoD* expression. Forelimbs at HH19 with both FGF18 and BMS493 beads grafted for 6 h show ectopic induction of *MyoD* (F) compared to contralateral control limbs (E) while HH19 limb grafted with BMS493 alone (G) or DMSO (H) do not show ectopic *MyoD*. Arrows indicate ectopic *MyoD* expression.
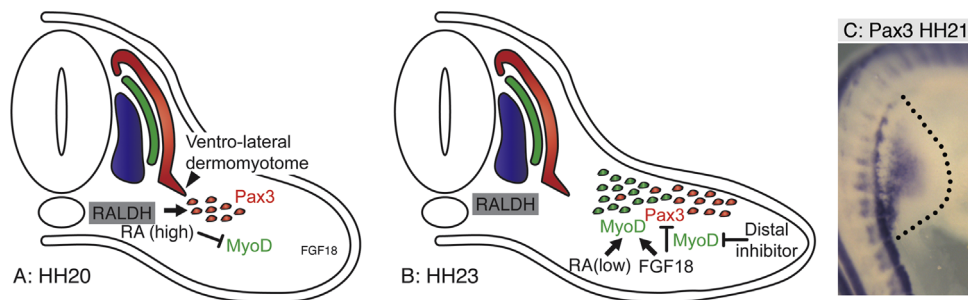

Fig. 6. Model of FGF and retinoic acid interactions regulating limb bud myogenesis. (A) At HH 20, prior to MyoD expression, myoblasts expressing *Pax3* are migrating from the ventro-lateral dermomyotome into the limb bud. RALDH in the flank generates retinoic acid in the proximal limb that represses *MyoD* expression. (B) At HH23 myoblasts have migrated further into the limb, away from the source of RA, and the FGF18 expression domain in the distal limb has expanded allowing downregulation of *Pax3* and induction of *MyoD* by FGF18 while inhibitory signals prevent premature MyoD expression in the distal limb bud. (C) In situ hybridization for *Pax3* at HH21 shows the position of migrating myoblasts in the proximal limb bud.

are found proximal to the position of grafted beads (Fig. 6C). Therefore it is no surprise that we detect the strongest response in this region of the limb. In addition myogenic cells introduced into the distal region of the limb bud show reduced myogenesis. Therefore, it is also likely that signals in the distal limb are also operating to supress muscle gene expression (Robson and Hughes, 1996). This could also explain why, in normal limb buds, myogenesis is seen first in the proximal then distal limb while our model, with proximal RA repressing and distal FGF18 inducing muscle gene expression, would predict the opposite. Combining our observations with the previous data showing that the distal limb can repress myogenesis resolves this conflict. There are many signalling molecules in the limb bud which have been shown to inhibit myogenic gene expression such as BMPs (Amthor et al., 1998), Notch (Delfini et al., 2000), HGF/SF (Scaal et al., 1999) and Shh (Duprez et al., 1998) and it is likely that interactions between RA, FGF18 and these repressive factors are also important for myoblast differentiation.

We also observe that in later manipulations loss of MyoD expression is seen in the dorsal but not the ventral muscle mass. Although it may be the case that the dorsal and ventral muscle masses are responding differently to the inhibitors we use, as they do to Shh signalling (Anderson et al., 2012; Hu et al., 2012), it is also possible that this merely reflects the dorsal position of the bead following grafting.

FGF18 induced MyoD expression requires ERK signalling

Our data show that FGF18 beads induce phosphorylation of ERK MAP Kinase within one hour and that blocking this with the MEK inhibitor U0126 prevents ectopic *MyoD* expression. Similarly we show that U0126 can inhibit the onset of endogenous MyoD expression. However blocking ERK signalling over longer time scales does not inhibit *MyoD* and after a 24 h incubation with U0126 beads manipulated limbs have similar *MyoD* expression to

contralateral controls. It is possible that in these embryos the inhibitor is no longer active after this period of time or that induction of *MyoD* by FGF18 is regulating the timing of onset of *MyoD* expression rather than being absolutely required for myogenesis. Interestingly in FGF18 null mice skeletal development is also delayed but not abrogated (Liu et al., 2007) suggesting that the regulation of timing of differentiation may be a conserved feature of FGF18 function although muscle defects in this mouse have not been reported. In contrast long term effects on myogenesis are seen following grafts of the irreversible FGFR inhibitor FIIN 1 hydrochloride. This could reflect different stability of these inhibitors in vivo or it is possible that there are multiple phases of FGF signalling that are differently affected by these drugs. In this scenario U0126 can block the initial induction of MyoD through the ERK pathway but later induction is driven by FGF signalling through an alternative signalling pathway.

Interactions between FGF18 and retinoic acid control timing of MyoD

One striking feature of our data are the different temporal responses of *Myf5* and *MyoD* to FGF18 beads. *Myf5* is upregulated in early limb bud stages while *MyoD* is only induced prematurely after HH21; however this can be overcome by co-grafting FGF18 beads adjacent to beads soaked in BMS493, an antagonist of retinoic acid signalling. This implies that retinoic acid, which is synthesised in the embryonic flank, prevents premature differentiation of myoblasts as they migrate into the limb. As the limb bud expands they move away from the RA producing flank and towards the distal limb which expresses FGF18 as well as retinoic acid catabolising genes such as CYP26B1 (Reijntjes et al., 2003). Therefore we propose a model where the timing of limb myoblast differentiation is controlled by these opposing activities with high levels of RA in the proximal limb bud maintaining a proliferative myoblast pool while FGF18 and lower levels of RA in the distal limb promote *MyoD* expression and differentiation (see Fig. 6). The possibility that high concentrations of RA inhibit myogenesis while lower ones promote it (Reijntjes et al., 2010) could also help explain the proximal–distal direction of myogenesis, especially when combined with a distal inhibitory signal. Interestingly, a similar two signal model of opposing RA and FGF gradients has been proposed to pattern the proximal–distal limb axis in chicken embryos (Cooper et al., 2011; Mercader et al., 2000; Roselló-Díez et al., 2011) although work in mice has challenged this view (Cunningham et al., 2013).

Differential responses of MRFs to FGF18

Although our data show that FGF18 can induce both *Myf5* and *MyoD* it is not clear if these are independently regulated or if *MyoD* is downstream of *Myf5* given that *Myf5* can induce *MyoD* expression in chicken (Delfini and Duprez, 2004; Sweetman et al., 2008) and mouse (Relaix et al., 2013) embryos. It is possible that the developmental delay before migrating myoblasts are competent to respond to FGF18 and upregulate *MyoD* is due to a requirement for *Myf5*. If this is the case this could explain why *Myf5* can be induced by FGF18 at HH19 but *MyoD* is not.

However while *Myf5* is expressed before *MyoD* in chick limbs there is only partial overlap of these two genes (Delfini et al., 2000). It is also clear from genetic ablation experiments in mice that there are *Myf5* independent muscle cell lineages (Gensch et al., 2008; Haldar et al., 2008) but not *MyoD* independent ones (Wood et al., 2013) while cell labelling and culture experiments have also suggested that at least two distinct populations of cells contribute to limb myogenesis (Kablar et al., 2003; Picard and Marcelle, 2013). It may be the case that the different temporal responses we observe in these assays are a result of distinct precursor populations of myoblasts in the developing limb. If so then we would expect to see *Myf5* negative cells in the limb which respond to FGF18 by expression of *MyoD*.

An alternative explanation is that the transcriptional regulation of MyoD is more sensitive to RA mediated repression than Myf5. In this case ERK activation at HH19 can induce Myf5 but in these cells MyoD expression is not induced because the influence of RA at this time is still too strong, possibly because of interactions between RA and ERK response elements in the *MyoD* regulatory regions. Distinguishing between these possibilities will provide important insights into the mechanisms of cell fate determination.

Author contributions

DS designed the original study, DS, KHSC, GFM, RC and HA contributed to experimental design, DS, GFM, RC and HA performed experiments and DS wrote the manuscript.

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