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Signals regulating muscle formation in the limb during embryonic development

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ABSTRACT Classically, somites have been the preparation of choice for the study of muscle development, while the limb bud is the preferred model of axis formation. Nevertheless, the limb bud offers some experimental advantages for muscle studies. This review describes the successive events involved in limb muscle formation during embryonic development, the properties of the key marker molecules and resumes our current knowledge of the signalling pathways involved.

KEY WORDS: *Limb, muscle, shh, Fgf, Wnt, Bmp*

Introduction

In vertebrates, all the myogenic cells that form the striated skeletal muscles of the limb and trunk originate from the somites. The medial halves of the somites give rise to back and intercostal muscles -the epaxial musculature-, while cells derived from lateral somites will produce the muscles of the limbs and of the body wall, forming the hypaxial musculature (Christ *et al.*, 1977; Chevallier *et al.*, 1977; Ordahl and Le Douarin, 1992). Medial and lateral somitic cells originate from 2 distinct territories in the rostral part of the primitive streak, at the time of gastrulation (Selleck and Stern, 1991; Freitas *et al.*, 2001). Di-I and chick-quail grafting experiments show that these two lineages do not intermingle (Selleck and Stern, 1991; Ordahl and Le Douarin, 1992; Freitas *et al.*, 2001). However, these two regions retain a kind of plasticity, since the replacements, at the limb level, of a chick lateral half-somite by a quail medial half-somite (and vice and versa) leads to normal epaxial and hypaxial muscles (Ordahl and Le Douarin, 1992).

Quite a lot of effort has been made to understand the extrinsic signals regulating epaxial muscle formation in somites (see Boricky and Emerson, 2000). However, less is known about hypaxial muscle development. One advantage of the limb model for studying the successive steps of muscle formation is that the myogenic cells forming the skeletal muscles of the limb have a different mesodermal origin from those of other limb tissues (cartilage and connective tissues), which come from the lateral plate (Christ *et al.*, 1977, Chevallier *et al.*, 1977). This distinct mesodermal origin, which can be surgically manipulated in avian embryos using quail/chick chimeras, provides an opportunity to trace muscle cells with a marker other than differentiation markers and to study the interactions between muscle and other limb tissues. This review will focus on chick limb muscle formation but will refer to the mouse limb for genetic aspects and to other species when appropriate.

Steps of Chick Limb Muscle Formation *In Vivo*

Myogenesis involves activation of the myogenic program, cellular proliferation and differentiation. The mechanisms underlying the balance between proliferation and muscle terminal differentiation have been intensively studied *in vitro* using muscle cell lines. *In vivo*, the myogenesis steps occur in parallel to spatial arangement so we have to take into account the positioning of muscle cells in space and time.

The first spatial arrangement occurring during limb muscle development is the migration of the somitic muscle precursor cells to the limb bud, which includes cell delamination from the lateral part of the dermomyotome and cell migration. Cells from somites 16 to 21 (Chevallier *et al.,* 1977; Christ *et al.,* 1977; Zhi *et al.*, 1996) and somites 26 to 33 (Lance-Jones, 1988a) migrate to the wing and leg buds, respectively. Based on histological examinations on chick embryos or quail-chick chimeras, the migration of the somitic cells to the forelimb bud occurs in 24 hours between E2 (Hamburger and Hamilton, 1951, HH15) and E3 (HH19) (Chevallier, 1978; Solursh *et al.*, 1987). From E3.5 (HH21), soon after their migration, avian somitic muscle cells aggregate into dorsal and

Abbreviations used in this paper: BMP, bone morphogenetic protein; fgf, fibroblast growth factor; MRF, myogenic regulatory factor; shh, sonic hedgehog; Wnt, wingless int.

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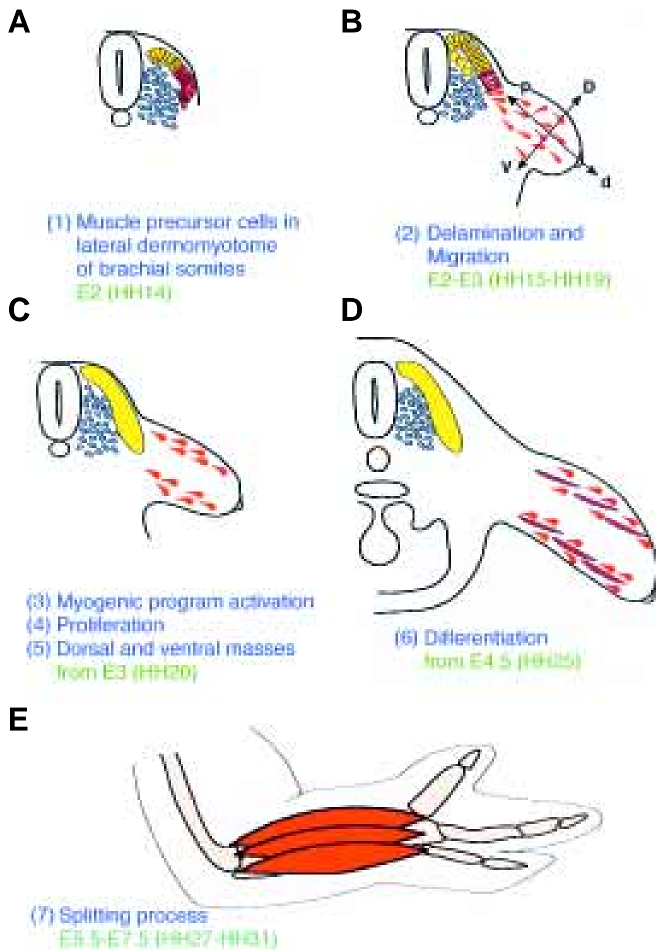

Fig. 1. Events defining muscle formation in the embryonic chick wing. *Somitic muscle cells* **(A)** *migrate into the wing somatopleure between E2 and E3* **(B)** *and then segregate into dorsal and ventral muscle masses* **(C,D)** *which undergo cleavages from E5.5 to give the final muscle pattern at E7.5* **(E)**. *The myogenic program is initiated from E3, concomitant with a proliferation step. The first signs of terminal differentiation are detectable from E4.5, from which time on the proliferation and terminal differentiation proceed in parallel during embryonic development. Between E2 and E6, the staging according Hamilton and Hamburger (1951) is more precise than the day staging; the corresponding stages in HH are thus given in brackets.*

ventral masses on both sides of the prechondrogenic core (Chevallier, 1978; Schramm and Solursh, 1990). Once the dorsal and ventral masses have formed, there is a period of about two days during which little spatial arrangement of the muscle cells occurs. These dorsal and ventral masses then split progressively to form the individual muscles. The splitting process takes place between E5.5 and E7.5 in the forearm (Robson *et al.*, 1994) and between E5 and E7 in the leg (Pautou *et al.*, 1982). Twelve and fifteen main muscles can be identified respectively in the forearm of the wing (Robson *et al.*, 1994; Zhi *et al.*, 1996; Duprez *et al.*, 1999a) and in the shank of the leg (Pautou *et al.*, 1982) in chick embryos.

The activation of the myogenic program is not marked by any manifestation other than the expression of the MRFs (Myogenic Regulatory Factors). Based on MRF expression, the activation of the myogenic program occurs at E3 (HH20) in the chick wing (Delfini *et al.*, 2000). Activation of the myogenic program occurs just after the migration and before the segregation into dorsal and ventral masses. It is quite difficult to determine precisely the onset of proliferation, since there is no specific marker for this step (see below); it probably begins as soon as the migration is over. The first sign of terminal muscle differentiation is observed with the appearance of the first polynucleated cells, the myotubes, at E4.5 (HH25) in the chick wing. From this moment on, postmitotic myotubes coexist with proliferating myoblasts, enabling the constant growth of muscles during embryonic development.

In summary, muscle formation in the embryonic limb involves the successive events schematised in Fig. 1: (1) formation of the muscle precursor cells in the lateral dermomyotome, (2) delamination and migration of these precursor cells to the limbs (3) activation of the myogenic program (4) proliferation (5) formation of the dorsal and ventral muscle masses (6) muscle differentiation and (7) muscle splitting.

Vertebrate Myogenic Cells do not contain any Positional Information for Early Muscle Patterning

Surgical experiments in the chick embryo have shown that somitic limb muscle progenitor cells do not contain any positional information. Somitic cell transplantations along the medio-lateral and dorso-ventral axis do not generate any limb muscle perturbation (Aoyama and Asamoto, 1988; Ordahl and Le Douarin, 1992). Heterotopic transplantations of somites in the limb regions result in muscle patterns corresponding to the limb segment (wing or leg) irrespective of the origin of the grafted somite (Chevallier *et al.*, 1977; Chevallier, 1979; Lance-Jones, 1988a). The inversion of the limb somites along the anterior-posterior (a-p) axis leads to axial malformations but to normal limbs (Aoyama *et al.*, 1988; Lance-Jones, 1988b). In contrast, surgical manipulations of the limb lateral plate (somatopleure) generate muscle changes. The inversion along the a-p axis of the lateral plate of the presumptive limb, before somitic cell invasion, leads to limbs where the outgrowth is directed towards anterior regions, showing that the a-p polarity is inverted (Michaud *et al.*, 1997). In addition, transplantation of limb somatopleure in the interlimb region is able to initiate the migration of somitic cells from the flank; showing that the limb bud mesenchyme controls and directs the migration of the somitic muscle cells to the limb (Hayashi and Ozawa, 1995). All these experiments in the chick embryo show that the tissues of somatopleural origin are responsible for the spatial organisation of the somitic muscle cells.

However, even if the somitic muscle cells do not contain any positional information, they have to possess the correct receptors and signalling pathways in order to respond to the environmental cues of the limb mesenchyme. In addition, the existence should be noted of a chick mutant, the *crooked neck dwarf* (*cn/cn*), which displays a late defect in muscle patterning and where the defect is intrinsic to somitic cells. In this mutant the splitting arrangement of muscles is normal up to E7.5 days, from that time the muscles coalesce and fuse, resulting in an unorganised muscular tissue surrounding bones (Mauger *et al.*, 1983). The introduction of normal somitic myogenic cells from a quail embryo into a mutant limb led to a normally patterned musculature (Mauger *et al.*, 1983). This shows that somitic muscle cells contain some kind of information required to maintain the correct muscle patterning, late in development.

Involvement of Muscle Connective Tissue in Muscle Patterning

The limb somatopleural mesoderm contains the information for muscle patterning. However, it is not clear which region or tissue of this mesoderm supplies the muscle positional information during development. Muscle masses and later muscles are composed of myogenic cells originating from somites and non-myogenic cells originating from lateral plate (Fig. 2). Non-somitic cells inside and surrounding muscles are usually gathered together under the term "muscle connective tissue". This dual origin of muscle tissue has raised the question of the role of the non-somitic cells in the development of the limb muscle pattern (Chevallier and Kieny, 1982). Tendons, which link muscle to cartilage also originate from lateral plate as the connective tissues and cartilage (Chevallier *et al.*, 1977; Christ *et al.*, 1977). Tendons have been studied as one candidate tissue for being involved in muscle regionalisation during development (Kieny and Chevallier, 1979; Kardon, 1998). Confocal analysis of tendon developement using tenascin as a marker show that tendons develop close to myogenic cells, from E5 (Kardon, 1998). However, due to the lack of a specific earlier tendon marker, it is not clear where tendon progenitor cells are located before E5. The bHLH transcription factor, *scleraxis* has been recently identified as a general tendon marker, once they are recognisible (Schweitzer *et al.*, 2001). Its early expression is strikingly correlated with muscle gene expression at E4 (see Fig. 4 in Schweitzer *et al.*, 2001). If we assume that *scleraxis* really marks the tendon progenitor cells, which is not yet proved, somitic muscle cells and tendon progenitor cells would be intimatly intermingled during their early development. This proximity would allow early cellular interactions between these two embryologically different cell lineages, which are maybe responsible for early muscle patterning.

Muscle Markers associated with Limb Developmental Stages

Migration

A number of genes, mainly homeobox genes, have been shown to be expressed in lateral somites and migrating somitic cells. The transcription factors *Pax3* (Paired Box3), *Lbx1* (Ladybird homeobox1) and the receptor *cMet* are the main genes whose functions have attracted most attention (for recent review and references, see Birchmeier and Brohmann, 2000). Briefly, *Pax3*, *cMet* and *Lbx1* are believed to be respectively and successively involved in (*i*) the establishment of the muscle precursor pool in the lateral dermomyotome, (*ii*) the delamination and (*iii*) the migration process. Migrating cells also express the transcription factor *Msx1*, whose expression is thought to prevent muscle differentiation during the migration step (Bendall *et al.*, 1999, Houzelstein *et al.*, 1999). The homeobox *Mox2* gene is also detected in lateral dermomyotome and migrating myoblasts and mice homozygous for the *Mox2* gene exhibit an absence of specific limb muscles (Mankoo *et al.*, 1999). Another homeobox gene, *Hoxa11* is located in migrating muscle cells, although its function in limb muscles is not clear (Yamamoto *et al.*, 1998).

Activation of the Myogenic Program

The myogenic program is triggered by the expression of the MRFs, a family of bHLH transcription factors, including *Myf5*, *MyoD*, myogenin and MRF4. Ectopic expression of MRFs is able to convert several non-muscle cell types into skeletal muscle in tissue culture. Conversely, knockout of these genes in mice leads to various muscle defects (see Arnold and Braun, 2000, Tajbakhsh and Buckingham, 2000). Moreover, cells deprived of *Myf5* or *MyoD* assume a non-muscle fate (Tajbakhsh *et al.,* 1996; Kablar *et al.*, 1999).

Lateral somitic cells designed to migrate to the limbs do not express any MRFs (Bober *et al.*, 1994; Tajbakhsh and Buckingham, 1994). In the chick embryo, low levels of *Myf5* transcripts are nevertheless detected in the entire dermomyotome (Hirsinger *et al.*, 2001; Kiefer and Hauschka, 2001). However, it is not clear whether the future migrating cells in the lateral dermomyotome express *Myf5*. In addition, normal migration occurs in the absence of *Myf5* (Tajbakhsh and Buckingham, 1994), indicating that the first activation of MRFs occurs in limb muscle cells after their migration. *Myf5* is the first MRF whose transcripts are detected in the limbs, followed by *MyoD* and myogenin in chick (Delfini *et al.*, 2000; Fig. 3) and mouse (Ontell *et al.*, 1995) embryos. MRF4

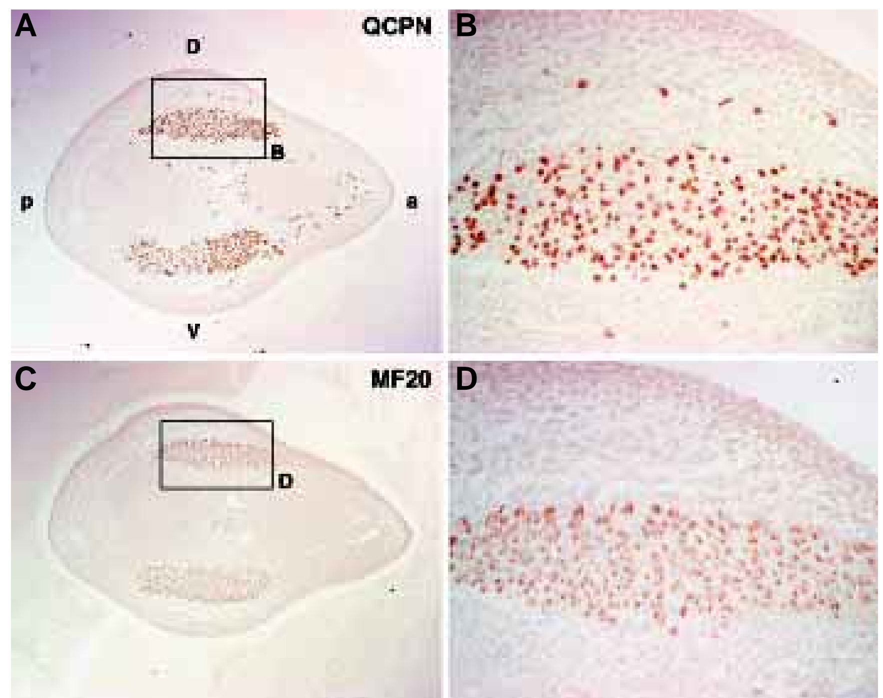

Fig. 2. Limb muscle tissues are composed of cells of double mesodermal origin. *Adjacent transverse sections of a chimeric wing from an E6 chick embryo, where the brachial somites have been replaced by their quail equivalents at E2, incubated with the QCPN antibody specifically recognising the quail cells* **(A,B)** *or incubated with the MF20 antibody recognising myosin* **(C,D)**. *(B,D) show higher magnifications of the dorsal muscle masses of (A,C), showing the mixture of quail and chick cells (B) and of the MF20$^+$ and MF20$^-$ cells (D) inside the muscle masses. Note the QCPN-positive (A,B) and MF20-negative (C,D) cells outside the muscle masses that correspond to endothelial cells.*

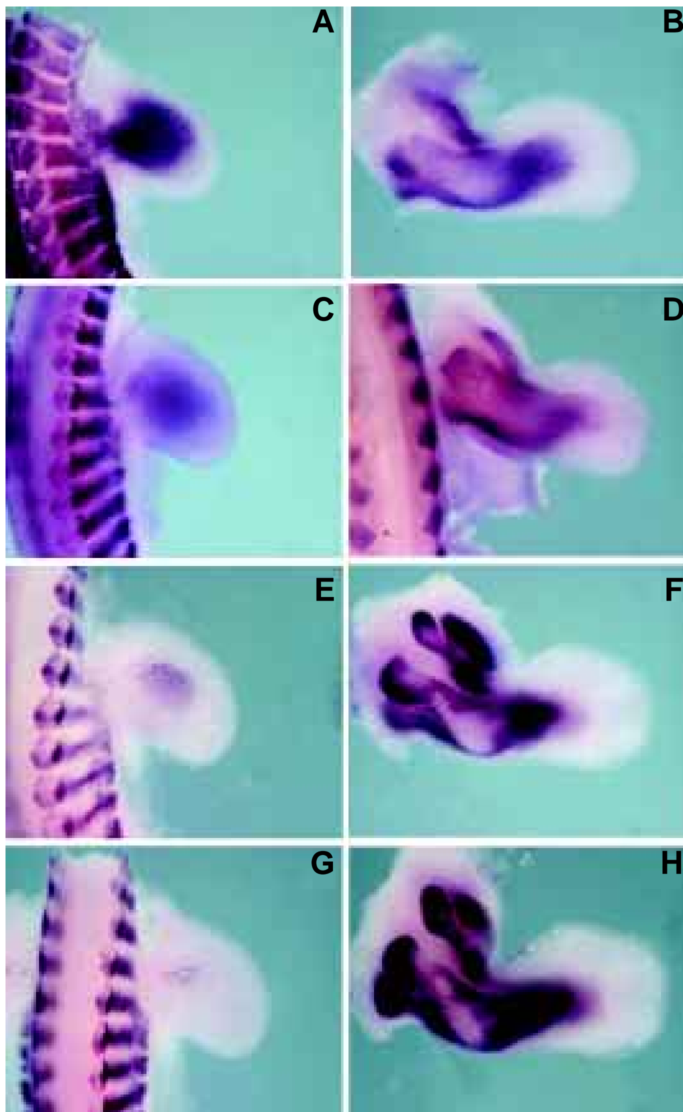

Fig. 3. Location of different muscle factors in the chick wing before (E4, left column) and after (E5, right column) the beginning of muscle terminal differentiation. *Dorsal views of wings from E4 (HH 23) (A,C,E,G) and E5 (HH 26/27) (B,D,F,H) chick embryos hybridised with the* Pax3 **(A,B)**, Myf5 **(C,D)**, MyoD **(E,F)** *and* myogenin **(G,H)** *probes. At stage HH23,* Pax3 *(A) and* Myf5 *(C) display very similar expression domains, while* MyoD *(E) and* myogenin *(G) are just beginning to be expressed. From E5, all genes are detected in muscle masses with equivalent domains (B,D,F,H). The expression of* Pax3 *and* Myf5 *diminishes while that of* MyoD *and* myogenin *increases, during limb development.*

transcripts are only detected in differentiated muscle fibres from D16 in mouse limb (Ontell *et al*., 1995). The endogenous expression of MRF4 has not been described in the chick limb. This sequential activation during limb development reflects the hierarchy among MRF family members defined by gene targeting. *Myf5* lies in a genetic pathway upstream of the three other MRFs (Rudnicki *et al*., 1993; Tajbakhsh *et al*., 1997; Valdez *et al*., 2000). The presence of *Myf5* in the absence of the 3 other MRFs is enough to drive normal myoblast specification in presumptive muscle regions, but not differentiation (Valdez *et al*., 2000). Double mutant mice have highlighted compensatory functions between *Myf5* and *MyoD* (Rudnicki *et al*., 1993). *Myogenin*, have a role in activating muscle cell terminal differentiation. The analysis of MFR4 gene disruption do not reveal any consistent phenotype, probably due to the proximity of the *Myf5* gene (see Arnold and Braun, 2000).

Progress has been made in understanding the molecular cascade triggering the initiation of the MRF expression in muscle cells. One candidate gene involved in *MyoD* activation is *Pax3*. Retroviral forced expression of *Pax3* in somite explants is able to induce ectopic *MyoD* expression (Maroto *et al*., 1997; Heanue *et al*., 1999). Low ectopic activation of *MyoD* can also be obtained in the chick limb, but to a lesser extent compared to somitic explants, probably due to environmental inhibiting influences (Bendall *et al*., 1999). In addition *Myf5* and *Pax3* double mutant mice show a defect in *MyoD* activation in the somite, while the single mutant mice do not, indicating that in the absence of *Myf5*, *Pax3* can activate *MyoD* (Tajbackhsh *et al*., 1997). *Pax3* is obviously not the only molecule able to activate *MyoD*, since concomitant misexpression of the couple *Dach2/Eya2* or *Eya2/Six1* is able to activate *MyoD* transcription in somite explants (Heanue *et al*., 1999).

Proliferation

Since only a limited number of cells migrates to the limb, a proliferation step is necessary to attain the numbers of cells required to form the muscle masses. There is no universally-accepted marker for the myoblasts in proliferation. *Pax3* transcripts are detected in proliferating myoblasts in the chick limb (Amthor *et al*., 1998), so it could be considered as a marker of proliferating myoblast. This would be consistent with its earlier role in the establishment of the precursor pool in the lateral dermomyotome (Borycki *et al*., 1999a). Moreover, the anarchic myoblast proliferation in alveolar rhabdomyosarcomas is correlated with the presence of the fusion protein *PAX3-FKHR*. In this fusion protein, the DNA binding domain of *Pax3* fuses with the trans-activation domain of a forkhead gene, *FKHR*, increasing artificially the transcriptional activity of *Pax3* (Khan *et al*., 1999).

The bHLH transcription factor, *Paraxis* displays an expression profile similar to that of *Pax3*, located in proliferative myoblasts and decreasing during development (Delfini and Duprez, 2000). Its role in myoblast proliferation is not established although it should be noted that the *Paraxis* mutant mice display hypotrophic muscles in the limb in addition to an inability to form epithelial somites (Burgess *et al*., 1996).

The Fibroblast growth factor receptor 4 (*FgfR4* also called *Frek*) could also fullfill the conditions to be a marker of the proliferating myoblasts. *Frek* transcripts are detected in the avian limb muscle cells from E3 (HH20), after their migration and then in proliferating mononucleated cells surrounding the myotubes (Marcelle *et al*., 1995; Edom-Vovard *et al*., 2001a; Fig. 4). In addition, *Frek* expression has been associated with myoblast proliferation in primary muscle cell culture (Halevy *et al*., 1994). In contrast to *Pax3* (or *Paraxis*), whose expression decreases during development, *Frek* transcripts are detected in muscles throughout embryonic development (Marcelle *et al*., 1995).

Differentiation

Skeletal muscle differentiation is initiated when proliferating myoblasts withdraw from the cell cycle and subsequently synthesise muscle-specific proteins. The classical markers of terminal muscle

differentiation, among many, are myosin and actin. From the onset of differentiation, a balance between proliferation and differentiation is established. Post-mitotic myoblasts fuse to form multinucleated fibres. The fusion process involves several adhesion molecules, including neuronal and vascular cell adhesion molecules (N- and V-CAM), cadherins, integrins and members of ADAM (containing a desintegrin and metalloprotease domain) family. Although the fusion process has been explored in cell culture and has been the subject of a recent explosion of interest in Drosophila (see Baylies and Michelson, 2001), there is little information available on how fusion occurs *in vivo* in vertebrates. Skeletal muscle fibres are formed by two successive waves of fusion, which have been referred to as primary and secondary myogenesis. The primary fibres have been suggested to act as a scaffold for the secondary muscle fibres. Primary and secondary muscle fibres also vary in their metabolism and speed of contraction, leading to the main categories of fibre types: slow and fast. In birds and mammals, each muscle displays a specific pattern of slow and fast fibre distribution. This specific fibre type pattern seems to be intrinsic to myoblasts (Nikovists *et al.*, 2001), but can be modulated by innervation, hormones and growth factors (see Blagden and Hughes, 1999)

In Vivo Signals involved in Limb Muscle Formation

The signals regulating the progression through the above-described developmental steps occurring during limb muscle formation remain poorly characterised. There is quite a lot of effort concentrated on the migration step (see Birchmeier and Brohmann, 2000). The defect of migration observed in the c-met homozygous mutant mouse has allowed the identification of a paracrine signalling system driving the migration of the somitic cells to the limb bud (Bladt *et al.*, 1995). The ligand HGF (Hepatocyte Growth Factor) is indeed located in the limb bud mesenchyme (Scaal *et al.*, 1999; Dietrich *et al.*, 1999) and is able to direct the migration of the somitic muscle cells expressing the c-met receptor (Heymann *et al.*, 1996; Brand-Saberi *et al.*, 1996; Scaal *et al.*, 1999). The migration is also under negative influence, since the ephrin-A5 located in distal region of the limb is able to restrict the migration of somitic cells (expressing the EphA4 receptor) to proximal limb regions (Swartz *et al.*, 2001). However, the signals and the mechanisms responsible for the aggregation of somitic cells as dorsal and ventral masses and then regulating the successive cleavages of these two masses into individual muscles are far from being understood. Moreover, the *in vivo* signals activating the myogenic program through the induction of MRF expression, regulating proliferation and differentiation in the limb are also not known. Notch signalling could be involved in maintaining the myoblasts in waiting for their differentiation. Ectopic activation of the Notch pathway via an overexpression of *Delta1* inhibits *MyoD* expression and consequently muscle terminal differentiation in chick limb (Delfini *et al.*, 2000) and somites (Hirsinger *et al.*, 2001). This result is consistent with previous studies on muscle cell lines and with the endogenous location of the receptor (*Notch1*, Fig. 3A) and ligands *(Delta1* and *Serrate2*), which have exclusive locations, outside and inside muscle fibres, respectively, during limb development (Delfini *et al.*, 2000). However, Delta1-activated-Notch does not affect proliferation or *Pax3* or *Myf5* expression (Delfini *et al.*, 2000; Hirsinger *et al.*, 2001).

Quite strikingly, the growth factors (and the associated transduction pathways) that have attracted most attention in somite myogenesis (see Borycki and Emerson, 2000) are the same molecules as are involved in limb bud initiation, growth and limb axis formation. These include Shh, (Sonic hedgehog), Fgfs (Fibroblast growth factors), Wnts (Wingless-ints) and Bmps (Bone morphogenetic proteins). These factors, able to initiate a new limb or to specify the limb axis (see Tickle, 2000), are obviously able to trigger molecular cascades in limb mesenchyme that can modify muscle formation. In a similar vein, the genes involved in limb indentity (forelimb versus hindlinb), such as Tbx4/5 and Pitx1 (see Tickle, 2000) also interfere with muscle formation.

Sonic Hegdehog

Ectopic application of Shh in E4 muscle masses chick limbs (after the initiation of the myogenic program) using viruses (Duprez *et al.*, 1998) or beads (Amthor *et al.*, 1998) leads to a clear muscle hypertrophy three days after grafting. Loss of Shh activity in mouse leads to the expected inverse phenotype, i.e a severe deficiency of limb muscle (Krüger *et al.*, 2001). The interpretation of the myogenic phenotype following Shh misexpression is complicated by the involvement of Shh in a-p axis formation of the limb. Indeed, application of a local source of Shh in the anterior limb region at E3

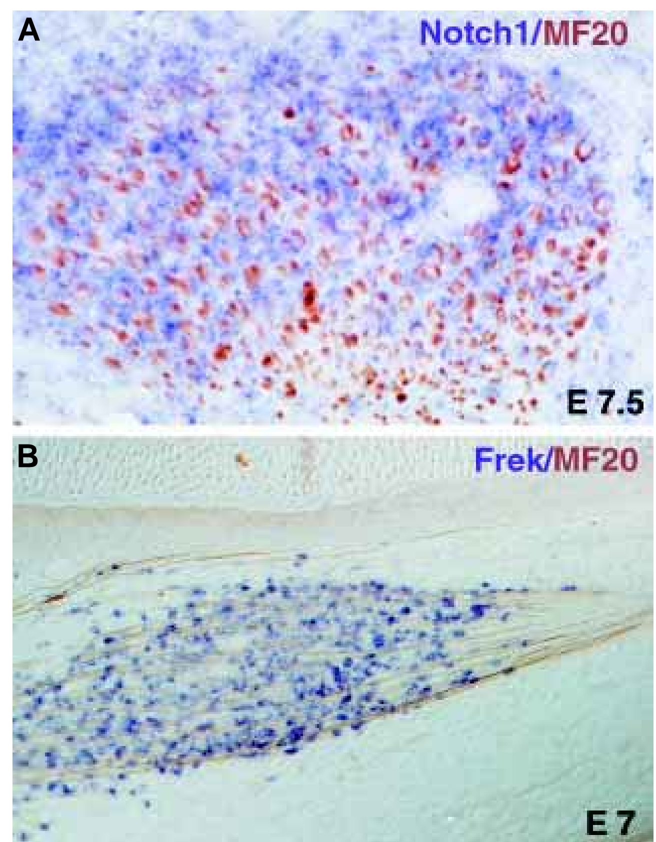

Fig. 4. Examples of location of signalling molecules related to muscles. (A) *Notch transcripts (blue) are located outside the muscle fibres visualised by the MF20 antibody (brown) on transverse sections of E7.5 wing.* **(B)** *Longitudinal sections of E7 wing hybridised with the* Frek *probe (blue) and incubated with the MF20 antibody (brown) show the expression of* Frek *transcripts along the muscle fibres.*

causes the development of a mirror-image pattern of all limb tissues (Riddle *et al.*, 1993), thus *a fortiori* of muscle (Duprez *et al.*, 1999a). This involves the creation of additional muscles around the extra digits and posteriorisation of the anterior muscles in the forearm (Duprez *et al.*, 1999a). The absence of Shh activity in the mouse generates numerous molecular defects linked with limb axis formation, resulting in severe skeletal defects (Chiang *et al.*, 1996) that make difficult the analysis of the nature of muscle deficiency. In the chick limb, the position and timing of the Shh-grafts can be adjusted to obtain enhanced myogenesis in the absence of any skeletal defects (Duprez *et al.*, 1998; Amthor *et al.*, 1998); however, we cannot exclude that the limb mesenchymal cells retain the ability to respond to Shh, since, at the time of the graft, all mesenchymal cells express the receptor *Patched1* (Duprez *et al.*, 1998). In other words, it is not clear whether Shh has an effect on the myogenesis steps independently of its muscle patterning effect. Nevertheless, it is clear that Shh is not involved in the migration and the initiation of the myogenic program through MRF activation since these two steps occur in the limbs of the *Shh* mutant (Krüger *et al.*, 2001).

In the limb, *Shh* is not expressed close to cells of the muscle lineage since *Shh* transcripts are restricted to the posterior mesenchyme of the limb in the ZPA (Zone of Polarising Activity) (Riddle *et al.*, 1993). There is evidence for the presence of a gradient of a freely diffusible form of Shh (cholesterol modified) across the a-p axis of the limb (Zeng *et al.*, 2001; Lewis *et al.*, 2001). However, no specific relationship between limb muscle lineage and Shh signalling components has been highlighted to date. It is therefore not clear whether Shh located in the ZPA acts directly on the muscle cell lineage or indirectly through the limb mesenchyme. It should be noted that no effect of Shh on muscle cell lines has been reported to date. The situation might be different in the somites. The proximity of the Shh source (the notochord and ventral neural tube) and the presence of Shh signalling components in the muscle progenitor cells in the somites (see Boricky and Emerson, 2000) favour a direct effect of Shh. However, the precise developmental step(s) at which Shh acts on somite myogenesis is(are) not clear since effects have been reported on several different stages: survival and/or extension of muscle lineage (Teillet *et al.*, 1998; Marcelle *et al.*, 1999, Krüger *et al.*, 2001), myogenic activation (Boricky *et al.*, 1999b) and differentiation enhancement (Amthor *et al.*, 1999) in somitic muscle progenitors.

In zebrafish somites, there is a well demonstrated effect of Shh on slow fibre formation (Blagden *et al.*, 1997). There is no such evidence for an equivalent role for Shh in the limb (or even in the somite) in chick and mouse embryos. Here again ectopic localised Shh application in the anterior limb is able to re-specify the fibre type distribution of each muscle fibre, along the a-p axis (Duprez *et al.*, 1999a). However, ectopic expression of *Shh* in limb muscle masses at E4 does not induce a general differentiation of slow fibres (Delphine Duprez, unpublished).

Fibroblast Growth Factors

Fibroblast growth factors (Fgfs) comprise a family of at least 20 polypeptides that mediate various biological responses through four high-affinity structurally related receptor tyrosine kinases, FgfR 1 to 4 (see Martin, 1998). In the limb, the Fgf signal is involved in limb initiation, ridge formation and also directs limb outgrowth along the proximo-distal axis (see Tickle, 2000). Fgfs are very potent inhibitors of myoblast differentiation in cell culture through activation of myoblast proliferation (see Olson, 1992). In relation with their effect on limb outgrowth, Fgf2, 4 and 8 located in the ridge maintain the underlying mesenchyme in an undifferentiated state and therefore create an environment preventing muscle differentiation in the distal limb (Robson and Hughes, 1996). The involvement of Fgf signalling in muscle development in an *in vivo* context was first identified by Itoh and coll. (1996). They generated various constructs of Fgf signalling components using a defective virus that they injected in the somites and observed consequences in limb muscles. Blockade of Fgf signalling inhibits somitic cell migration to the limb bud, highlighting a role for Fgf signalling in myoblast migration. They also showed that somitic cells infected with a constitutively active form of FgfR1 migrate to the limb but remain as unfused myoblasts, indicating that the down-regulation of Fgf signalling in muscle cells is necessary *in vivo* for muscle terminal differentiation (Itoh *et al.*, 1996). *FgfR1* transcripts display an ubiquitous expression in limb mesenchyme and are located in muscles, both inside and outside fibres (Edom-Vovard *et al.*, 2001a), indicating that all limb cells can respond to Fgf signals. Consistent with this ubiquitous expression, block of Fgf signalling in all cell types, by injecting a truncated form of *FgfR1* in RCAS in the limb, after the migration process, results in loss of skeletal muscle mass and connective tissue (Flanagan *et al.*, 2000). This is consistent with a role of Fgf in maintaining a pool of muscle and fibroblast cells. *FgfR4/Frek* transcripts present a more restricted expression pattern, since they are located in the cells surrounding muscle fibres (Marcelle *et al.*,1995; Fig. 3B). However, *FgfR4* mutation in mice is not informative, since the homozygous *FgfR4* null mutation exhibits no overt muscle abnormalities (Weinstein *et al.*, 1998).

Retroviral application of ectopic *Fgf4* (Edom-Vovard *et al.*, 2001a) or *Fgf5* (Clase *et al.*, 2000) in the limb from E4 (after the steps of migration and the activation of myogenic program) down-regulates *MyoD* and myosin expression. However, *Pax3* is also down-regulated in presence of ectopic *Fgf4* (Edom-Vovard *et al.*, 2001a). By following cells of somitic origin, using the quail marker, a clear decrease in muscle cell number was observed following Fgf4 application; a decrease not due to apoptosis (Edom-Vovard *et al.*, 2001a). The prevention of somitic muscle cell proliferation by Fgf4 is somewhat unexpected given the *in vitro* studies that tend to show a mitogenic effect of Fgfs on muscle cell lines. *In vivo*, this inhibition of cell proliferation is specific to somitic muscle cells, since Fgf exposure induces a clear expansion of the connective tissue (Clase *et al.*, 2000; Edom-Vovard *et al.*, 2002). In the chick limb, *Fgf4* transcripts are restricted to the extremities of muscle fibres close to the myotendinous junction (Edom-Vovard *et al.*, 2001a, 2002) from E6, while *Fgf8* is detected in tendons (Edom-Vovard *et al.*, 2001b) from E7. It is not clear whether misexpression of Fgfs mimics the endogenous Fgf4 in muscle, Fgf8 in tendons or another Fgf. However, there is evidence that fusion occurs preferentially at the extremities of the muscle fibres during secondary myogenesis (Zang and McLennan, 1995), *i.e.* at the time and place of *Fgf4* expression. Exit from the cell cycle is a prerequisite for myoblast fusion. By down-regulating *Frek* expression, *Fgf4* located at the extremities of the fibre could inhibit proliferation of the surrounding myoblasts and allow their incorporation into fibres (Edom-Vovard *et al.*, 2001a). Consistent with this idea, *Frek* transcripts are not detected near the myotendinous junction (Fig. 3B) where *Fgf4* transcripts are present (Edom-Vovard *et al.*, 2001a). Thus in one sense, Fgf4 could be involved in the growth of

muscle fibres. Alternatively but not exclusively to the previous hypothesis, ectopic Fgf could mimic Fgf4 in muscle and/or Fgf8 in tendons in expanding the connective tissue (Edom-Vovard *et al.,* 2002).

Fgf6 is another Fgf whose expression is restricted to the muscle lineage. It appears to be involved in post-natal muscle development, since its inactivation in mice leads to a severe defect in muscle regeneration, presumably via a lack of activation or proliferation of satellite cells (Floss *et al.*, 1997). The expression of Fgfs 2, 5, 7 and 9 have also been described as being related with muscles, however their cellular locations and their roles remain to be clarified.

Wnt Signalling

The involvement in myogenesis of Wnt signalling has been extensively studied in somites (see Boricki and Emerson, 2000). From the somite work, Wnts are usually considered as positive regulators of myogenesis. In mouse somite explants, *Myf5* and *MyoD* are differentially activated by Wnt1 and Wnt7a, respectively (Tajbakhsh *et al.*, 1998). However, in the limb it is not clear whether a differential activation of *Myf5* and *MyoD* exists and whether the same Wnts play this role. The most obvious involvement of Wnt signalling in limb muscle formation is related to its role in specifying dorso-ventral limb axis. Rotation of limb bud ectoderm along the d-v axis induces a corresponding reversal in muscle and skeletal patterns, showing that the dorsal non-AER ectoderm is involved in the dorsal specification of the limb (see Tickle, 2000). *Wnt7a* is located in dorsal ectoderm in chick and mouse and is therefore a good candidate for mediating the dorsalising effect of the ectoderm. Moreover, the loss of *Wnt7a* function in homozygous mutant mice results in transformation of dorsal limb structures to a more ventral phenotype (Parr and MacMahon, 1995). Wnt7a mediates its effect through the homeobox gene *Lmx1* located in the dorsal mesenchyme and whose expression is under the control of Wnt7a. The ectopic expression of *Lmx1* in the ventral compartment leads to a nice double dorsal limb pattern (muscle, tendon and ectoderm) (Riddle *et al.*, 1995, Vogel *et al.*, 1995). The converse experiment in mice, i.e. the invalidation of the *Lmx1* gene, gives a perfect double ventral limb pattern clearly visualised with the muscle pattern (Chen *et al.*, 1998). Thus, Wnt7a via *Lmx1* specifies the dorsal muscle pattern. There are probably many other Wnts and Frizzleds (their putative receptors) in the limb at various stages of development. However, the precise relationship between their sites of expression and cells of muscle lineage has not been described yet. Wnt signalling is modulated by antagonists called Sfrps (secreted-frizzled-related proteins). *Sfrp3/Frzb1* is detected in limb cartilage region (Duprez *et al.*, 1999b) from E4, and may buffer the Wnt myogenic effect in cartilage regions. However, misexpression of *Frzb1* using RCAS in the chick limb does not lead to any muscle phenotype (D. Duprez, unpublished). In contrast, transplacental delivery of *Frzb1* to mouse embryos inhibits skeletal myogenesis in somites (Borello *et al.*, 1999). However, *Frzb1*-injected embryos exhibit a general growth retardation (Borello *et al.*, 1999), making it difficult to evaluate the specific effect of *Frzb1* on myogenesis. *Sfrp2* transcripts have been described as flanking the muscle regions in the limb and somites (Terry *et al.*, 2000; Ladher *et al.*, 2000). Based on this expression pattern, one can speculate that *Sfrp2* could be a good candidate for limiting the positive myogenic effect of Wnts at muscle surfaces. Interestingly, in somites, *Sfrp2* expression is up-regulated by Shh (Lee *et al.*, 2000).

Bone Morphogenetic Proteins

Bmps (Bone morphogenetic proteins) constitute a subgroup of the TGFβ (Transforming growth Factors β) super family, whose members act through a heteromeric complex of serine/threonine kinase receptors. Bmps were identified by their abilities to induce cartilage formation when implanted at intramuscular sites in adult rats (Wozney *et al.*, 1988). In the limbs, Bmps (and associated signalling components) are located in the areas of cartilage condensation, surrounding cartilage and the joints (Macias *et al.*, 1997; Zou *et al.*, 1997; Pizette and Niswander, 2000). Activation of Bmp signalling via retroviral systems using either ligands (Duprez *et al.*, 1996a) or receptors (Zou *et al.*, 1997) or via beads as Bmp carriers (Macias *et al.*, 1997) in the embryonic chick limbs leads to a dramatic increase of cartilage formation at the expense of muscle. It is still not clear whether ectopic Bmps can convert muscle cells into cartilage or whether there is a proliferation of cartilage cells, or both. The idea of recruitment has already been suggested *in vitro*, where Bmp2 is able to convert C2C12 myoblasts into the osteoblast lineage (Katagari *et al.*, 1994).

Bmp bead application in dorsal muscle masses of E4 chick limbs inhibits the expression of muscle markers *Pax3* and *MyoD* in 27 hours (Amthor *et al.*, 1998). An ectopic source of Bmps also down-regulates *Pax3* and *MyoD* in somites (Pourquie *et al.*, 1996; Amthor *et al.*, 1999; Reshef *et al.*, 1998). Given all these experiments, Bmps are usually considered as inhibitors of muscle development. Nevertheless, the inhibition of muscle markers after ectopic Bmp application in the limb might simply be the consequence of the loss of muscle cells, since Bmps are known to be potent apoptotic signals for the limb mesenchyme (Yokouchi *et al.*, 1996; Macias *et al.*, 1997; Amthor *et al.*, 1998). Notably, the cell death following Bmp bead application is specifically detected in the undifferentiated mesenchyme and not in the ectoderm or differentiating chondrogenic cells (Macias *et al.*, 1997). Moreover, using quail-chick chimeras to follow muscle cells, specific loss of muscle cells has been observed in micromass cultures under Bmp exposure (Duprez *et al.*, 1996b). Consistent with the idea of Bmps inducing a loss of muscle cells, endogenous expression patterns of Bmp2, 4 and 7 (anterior and posterior mesenchyme, apical ectodermal ridge) in the early chick limb are disjoint from muscle lineage (Amthor *et al.*, 1998). In particular, the Bmp4 expression domain from E3 to E4 is complementary to that of *Pax3* (Amthor *et al.*, 1998). However, Bmps can also activate myogenesis, since application of low levels of Bmps (Bmps 2, 4 and 7) in the wing mesenchyme at E4 up-regulates *Pax3* and then *MyoD* expression (Amthor *et al.*, 1998). The same situation occurs in the somite, where application of low levels of Bmp beads to E4 embryos increases *Pax3* and *MyoD* expression in dermomyotome and myotome, respectively (Amthor *et al.*, 1999). The level of Bmp activity can be modulated by its antagonists, which bind Bmps and prevent them from interacting with their receptors. The Bmp antagonist noggin, although presenting a complex expression pattern, is located close to the *MyoD*-positive cells in limb (Amthor *et al.*, 1998) and somite (Capdevilla and Jonhson, 1998). The absence of noggin activity in the mouse results in an excess of Bmp activity and consequently increases cartilage formation (McMahon *et al.*, 1998; Brunet *et al.*, 1998) and decreases the expression of muscle markers, at least in somites (McMahon *et al.*, 1998). Conversely, ectopic application of noggin-expressing cells, buffering Bmp activity, induces a clear up-regulation of *MyoD* expression in somites (Hirsinger *et al.*, 1997; Marcelle *et al.*, 1997). However,

in the limb, retroviral ectopic expression of *noggin* does not up-regulate muscle markers as might have expected, but down-regulates *Pax3* and MF20 expression at E5, in addition to blocking chondrocyte differentiation (Pizette and Niswander, 2000). The situation in the limb might be complicated by the effect on cartilage and/or the transcriptional relationships between ligands, receptors and antagonists. For instance, Bmp4 has been shown to induce the expression of its own antagonist *noggin* and receptor *BmpR1b* (Merino *et al.*, 1998; Amthor *et al.*, 1999). In addition, the potential involvement of other Bmp antagonists, such as Follistatin (Amthor *et al.*, 1996) and gremlin (Merino *et al.*, 1999) that are detected in muscles at different stages during limb development could further complicate the *in vivo* scheme.

In summary, high Bmp signalling surrounding the *Pax3*-positive domain in the early mesenchyme and in the cartilage-forming regions could be involved in positioning the muscle cells at the right place, while low Bmp signalling could have a positive role in myogenesis.

Other Signalling Molecules

Other sub-groups of the Tgfβ super family are thought to drive muscle formation. Tgfβs themselves have dual roles in muscle terminal differentiation (activation or inhibition), depending on the culture conditions, but do not affect proliferation (see Olson, 1992; Cusella-De Angelis *et al.*, 1994). The inhibition of muscle differentiation by Tgfβs is mediated via repression of MyoD activity and involves physical interactions between a component of the Tgfβ signalling pathway (smad3) and the bHLH domain of MyoD (Liu *et al.*, 2001). However, the positive and negative myogenic effects of Tgfβ have not been confirmed by functional data *in vivo.* A member of another sub-group of the Tgfβ superfamily, Gdf8 (Growth and differentiation factor-8, also called myostatin) is a potent negative regulator of muscle growth in mouse embryos (McPherron *et al.*, 1997). Surprisingly, on the basis on *in situ* hybridisation and northern analysis, Gdf8 transcripts are restricted to developing skeletal muscles (McPherron *et al.*, 1997). It will be of a particular interest to determine whether Gdf8 cellular location in muscles is of somitic or of somatopleural origin.

Igfs (Insulin growth factors) form another family affecting muscle formation. Igfs are thought to promote myogenesis, since the Igf-I mutant mice present a muscle hypoplasia (Powel-Braxton *et al.*, 1993) and gain of function of Igf-I using retroviruses in the chick limb leads to enlarged muscles due to increased myoblast proliferation (Mitchell *et al.*, 2002). Egf (Epidermal growth factor) and Tgfα located in muscle cells during mouse development (Yamane *et al.*, 1997; Dealy *et al.*, 1998) are part of another signalling pathway whose involvement in muscle formation should be explored.

Conclusion

The analysis of the role of signalling molecules (and associated transduction signals) in limb myogenesis is complicated by the existence of numerous ligands, receptors and antagonists, giving a large range of possibilities of regulation. This plethora of molecules is expressed in the limb with different but overlapping patterns, which implies possible redundancies. In addition, these signalling molecules are often involved in early steps of development. Subsequently, loss of function of these molecules can result either in no apparent phenotypic alterations of limb muscle development because of redundancy or in early lethality. This could be overcome either by inactivating several genes or by using conditional mutants. A meticulous (and consequently fastidious) cartography of the tissue but also cellular expression of the members of the different families (at the transcripts but also protein level) will be a necessary step for the choice of the genes to inactivate and the choice of the cre/lox promotors and also for correct interpretation of the phenotypes of loss and gain of function. In addition an investment in biochemistry to better understand the binding specificities of the numerous ligands with their receptors and antagonists would be necessary in order to discard possibilities of regulation. The problem of concentration has to be kept in mind, since different levels of molecules such as Bmps elicit opposite effects on muscle. Lastly, there is a tight cooperation between the different signalling molecules, well highlighted for the limb axis formation (Tickle, 2000), and which probably also occurs in muscle formation. Thus, in addition to transcriptional relationships between components of different families, it will be of importance to dissect the downstream cascades (post-receptor) and target genes of each signalling pathways in order to determine the interactions between the different signalling pathways, related with limb muscle formation.

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