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Involvement of vessels and PDGFB in muscle splitting during chick limb development

Samuel Tozer¹, Marie-Ange Bonnin¹, Frédéric Relaix², Sandrine Di Savino¹, Pilar García-Villalba¹, Pascal Coumilleau¹ and Delphine Duprez^{1,*}

Muscle formation and vascular assembly during embryonic development are usually considered separately. In this paper, we investigate the relationship between the vasculature and muscles during limb bud development. We show that endothelial cells are detected in limb regions before muscle cells and can organize themselves in space in the absence of muscles. In chick limbs, endothelial cells are detected in the future zones of muscle cleavage, delineating the cleavage pattern of muscle masses. We therefore perturbed vascular assembly in chick limbs by overexpressing VEGFA and demonstrated that ectopic blood vessels inhibit muscle formation, while promoting connective tissue. Conversely, local inhibition of vessel formation using a soluble form of VEGFR1 leads to muscle fusion. The endogenous location of endothelial cells in the future muscle cleavage zones and the inverse correlation between blood vessels and muscle suggests that vessels are involved in the muscle splitting process. We also identify the secreted factor PDGFB (expressed in endothelial cells) as a putative molecular candidate mediating the muscle-inhibiting and connective tissue-promoting functions of blood vessels. Finally, we propose that PDGFB promotes the production of extracellular matrix and attracts connective tissue cells to the future splitting site, allowing separation of the muscle masses during the splitting process.

KEY WORDS: Chick, Limb, Muscle, Vessel, PDGF, VEGF, Collagen I, MyoD

INTRODUCTION

The mechanisms controlling the organization of muscles in space during vertebrate limb development are not fully understood. Myogenic cells of vertebrate limbs originate from the ventrolateral part of the somites (Chevallier et al., 1977; Christ et al., 1977; Schienda et al., 2006). Somite-derived muscle precursor cells undergo three main steps of spatial organization and concomitantly differentiate. (1) The migration process has been described in the chick wings as occurring between E2 (HH15) and E3 (HH20) (Chevallier et al., 1978; Solursh et al., 1987). (2) Once they have reached the limb mesenchyme, muscle precursor cells organize into dorsal and ventral masses (Schramm and Solursh, 1990). At this stage (E3/HH20), the muscle precursor cells activate the myogenic program by successively expressing the bHLH transcription factors MYF5, MYOD and myogenin, and then differentiate into myotubes. (3) Lastly, the dorsal and ventral muscle masses will progressively separate into the various muscle masses producing the individual muscles, a phenomenon called muscle splitting. In the chick wing forearm, this process has been described to occur in 48 hours, between E6/HH28 and E8/HH32 (Shellswell and Wolpert, 1977; Robson et al., 1994).

Classical experiments in avian embryos have shown that positional information for muscle patterning is carried by the limb mesenchyme and not by the myogenic cells (Chevallier et al., 1977; Christ et al., 1977; Lance-Jones, 1988; Hayashi and Ozawa, 1995; Kardon, 1998). The ligand HGF (hepatocyte growth factor) located in limb mesenchyme is able to direct the migration of the somite-derived cells expressing the C-MET receptor, a

transcriptional target of *Pax3* (Dietrich et al., 1999; Scaal et al., 1999; Relaix et al., 2003). SDF1/CXCR4 and ephrin-A5/EPHA4 also control the migration of muscle progenitor cells positively and negatively, respectively (Swartz et al., 2001; Vasyutina et al., 2005). Bone morphogenic proteins (BMPs) have been proposed to restrict the position of premuscle masses in chick limb buds (Amthor et al., 1998; Bonafede et al., 2006). Moreover, the molecular pathways involved in limb axis formation are involved in positioning the muscles in addition to positioning cartilage (Duprez, 2002). Sonic hedgehog (SHH), which is involved in anteroposterior axis formation, is able to transform anterior forearm muscles into muscles with a posterior identity (Duprez et al., 1999). The genetic proof that limb muscle cells can respond directly to SHH signaling (Ahn and Joyner, 2004) suggests that this muscle posteriorisation is not a consequence of cartilage respecification. *Lmx1b* expressed in the dorsal mesenchyme of the limb drives the dorsal muscle pattern (Riddle et al., 1995; Vogel et al., 1995; Chen et al., 1998). Recently, the transcription factor *Tcf4*, located in limb mesenchyme, has been proposed to establish a pre-pattern that will determine the site of myogenic differentiation (Kardon et al., 2003). However, the way this information is integrated by the muscle masses in order to split and form individual muscles is still poorly understood. Homeobox genes are candidates for involvement in limb muscle patterning. *Mox2* (*Meox2*)-homozygous mutant mice consistently display abnormal splitting of certain muscles or elimination of specific muscles in limbs (Mankoo et al., 1999). However, these mutants display an overall reduction of the muscle masses (Mankoo et al., 1999). The *Lbx1* homeobox gene can also be considered as a muscle-patterning gene, because *Lbx1* mutant mice display an absence of dorsal muscles in forelimbs, whereas the ventral muscles are unaffected (Schäfer and Braun, 1999; Gross et al., 2000; Brohmann et al., 2000). However, this gene is usually classified as involved in myoblast migration (Birchmeier and Brohmann, 2000). It is also worth noting that the *Hoxa11* and

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Hox13 homeobox genes have been described as being expressed in restricted domains of the muscle masses and in specific individual muscles in chick limbs, although their precise roles in muscle patterning are not clear (Yamamoto et al., 1998).

Other limb tissues have been studied as candidates for influencing muscle spatial organization. The tendons are good candidates to be involved in limb muscle patterning (Kardon, 1998; Edom-Vovard and Duprez, 2004). An influence from nerves has been eliminated (Schroeter and Tosney, 1991a), because the muscles split normally in the absence of innervation following neural tube ablation (Lance-Jones and Landmesser, 1980; Edom-Vovard et al., 2002). The involvement of blood vessels in muscle splitting has already been investigated by histological analysis, after hypervascularization or using ink injection (Schroeter and Tosney, 1991a; Flamme et al., 1995; Murray and Wilson, 1997). However, these studies did not provide evidence for a link between the vasculature and the process of muscle separation.

Analyzing new data led us to reinvestigate the influence of the vasculature in limb muscle patterning. Orthotopic somite transplantations from quail to chick have shown that somites provide endothelial cells to both the roof and sides of aorta, to cardinal veins, intersomitic vessels, kidney and limbs (Wilting et al., 1995; Pardanaud et al., 1996; Pouget et al., 2006). Owing to the fact that the limb buds appear after the primitive vascular network has formed in the trunk of the embryo, the limb vasculature can arise either from pre-existing vessels (i.e. by angiogenesis) or by coalescence of free (migrating) vascular endothelial progenitors (i.e. type II vasculogenesis). The contribution of each process in the early assembly of limb blood vessels is still not clear (Pardanaud et al., 1989; Feinberg and Noden, 1991; Brand-Saberi et al., 1995; Ambler et al., 2001; Vargesson, 2003). Subsequent vascular development involves several processes including vascular remodeling, mural cell recruitment and the establishment of artero-venous identity. Various signaling molecules are involved in these processes. The secreted glycoprotein VEGFA (vascular endothelial growth factor A) is a key molecule involved in various aspects of blood vessel development including vasculogenesis and angiogenesis, both physiological and pathological (Ferrara, 2000). Although the role of VEGFA in the formation of the general vasculature is well studied, its particular role in the establishment of limb vasculature is less well documented. Based on the capacity of VEGFA to activate endothelial cell migration, proliferation and survival in various systems and species (Cleaver and Krieg, 1998; Drake et al., 2000; Poole et al., 2001; Cho et al., 2002), VEGFA is probably one of the cues involved in the correct organization of the endothelial network in the limbs. However, recent studies highlight that the patterning of the vascular system is directed by attractive and repulsive neuronal guidance factors (Bates et al., 2003; Weinstein, 2005; Carmeliet, 2005). The PDGFB (platelet-derived growth factor B) is another secreted factor important for vessel formation (Betsholtz, 2004). PDGFB acts as a paracrine signal from endothelial cells to blood vessel mural cells expressing its receptor, PDGFR β . PDGFB and PDGFR β knockout mice display similar phenotypes, consistent with a PDGFB function in the recruitment, proliferation and migration of vessel mural cells (Hoch and Soriano, 2003; Betsholtz, 2004).

In the present paper, we analyze the influence of endothelial and muscle cells on each other during limb bud development. We provide evidence that vessels and PDGFB (located in endothelial cells) promote connective tissue formation at the expense of muscle.

MATERIALS AND METHODS

Chick and quail embryos

Fertilized chick eggs from commercial sources – JA 57 strain [Institut de Sélection Animale (ISA), Lyon, France] and White Leghorn (HAAS, Strasbourg) – and Japanese quail eggs (Chanteloup, France) were incubated at 37°C. Before E2, embryos were staged according to somite number. Young embryos (E3–E5) were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951), whereas old embryos (E5.5–E10) were staged according to days in ovo. To facilitate comparisons, the numbers of days of incubation are reported with the HH stages. The following day numbers and HH stages are equivalent: E5/HH26, E5.5/HH27, E6/HH28, E6.5/HH29, E7/HH30, E7.5/HH31, E8/HH32.

Pax3 mutant mice

Embryos from *Pax3*^{-/-} mutant (Relaix et al., 2003) and *Pax3*^{GFP/+} (Relaix et al., 2005) mice were collected after natural overnight matings. For staging, fertilization was considered to take place at 6 am.

Production and grafting of VEGF/RCAS-expressing or control RCAS-expressing cells

The chick *Vegfa* coding sequence (provided by Thierry Jaffredo, CNRS, Paris, France) was inserted in the sense orientation into the *Clal* site of the replication-competent retroviral vector RCASBP(A). VEGF/RCAS-expressing cells and RCAS-expressing cells were prepared for grafting as described by Edom-Vovard et al. (Edom-Vovard et al., 2002). Pellets of approximately 50 μ m in diameter were grafted into the right wings or wing presumptive regions of embryos at stages HH14 (E2) to HH22 (E4). At various times after grafting, embryos were harvested and processed for in situ hybridization to tissue sections. The left wing was used as an internal control. After grafting, the RCAS virus (and the inserted gene) will progressively spread to all limb tissues. Whereas grafting embryos at early stages of limb development (HH14, E2) leads to a general limb infection in 48 to 72 hours, grafting limbs at HH22 (E4) leads to more localized virus infection. Owing to a certain variability in the virus spread among embryos, the expression of the ectopic gene was systematically checked by in situ hybridization.

PDGFB and sFLT1 bead implantation

The rat PDGFB and human soluble VEGFR1 (sFLT1) recombinant proteins were obtained from RD Systems. Affigel Blue beads (Biorad) were washed in PBS and soaked in 500 ng/ μ l PDGFB for 1 hour or in 1 μ g/ μ l sFLT1 for 4 hours on ice. PDGFB or PBS beads were grafted into the right wings of normal embryos at stage HH19/20 (E3) to HH26 (E5). 24 to 72 hours after grafting, embryos were processed for in situ hybridization to whole-mount embryos or to tissue sections. sFLT1 or PBS beads were grafted into the right wings of embryos at about stage HH27/28 (E5.5/E6). Two days after grafting, manipulated wings were either injected with pure indian ink into the vessels of the allantoic, or processed for in situ hybridization to tissue sections.

Bromodeoxyuridine (BrdU) labeling in ovo

PDGFB or PBS beads were grafted into the right wings at stage HH26 (E5). Two days after grafting, 2 μ l of BrdU (Amersham) was directly injected into the circulation of the embryos. The embryos were fixed 2 hours later and then processed for wax sectioning.

In situ hybridization to whole-mounts and tissue sections

Normal or manipulated embryos were fixed in 4% (v/v) formaldehyde and processed for in situ hybridization to whole-mounts and wax tissue sections as previously described (Edom-Vovard et al., 2002). The digoxigenin-labeled mRNA probes were used as described: *Pax3*, *MyoD*, *Fgfr4* and mouse *MyoD* (Delfini and Duprez, 2004), quail *Vegfr2* (Eichmann et al., 1993), *Hif2 α* (Favier et al., 1999), *Pdgfb* (Horiuchi et al., 2002), *Pdgfr α* (Marcelle and Eichmann, 1992), *Tcf4* (Kardon et al., 2003). The mouse *Hif2 α* probe corresponds to a part of the mouse *Hif2 α* coding sequence (Ema et al., 1997). The probe for collagen I originates from the UMIST EST library (Boardman et al., 2002). Part of the coding sequence for chick *Pdgfr β* was isolated, using primer5' (5'-CGTCATCTCCCTGATCATCC-3') and primer3' (5'-TTGCTCATGTCCATGTAGCC-3'), from an RT-PCR-derived

cDNA library made from E6-E8 chick heart. To label nuclei, adjacent sections processed for in situ hybridization were incubated with Hoechst 33342 (Molecular Probes) for 15 minutes.

Immunohistochemistry

Differentiated muscle cells were detected on sections using the monoclonal antibody MF20 that recognizes sarcomeric myosin heavy chains (Developmental Studies Hybridoma Bank). The endothelial cells were visualized in quail embryos using the QH1 polyclonal antibody (Developmental Studies Hybridoma Bank). The endothelial cells in mouse embryos were recognized using the PECAM antibody, which recognizes the platelet/endothelial cell adhesion molecule 1, PECAM1 (Developmental Studies Hybridoma Bank). Fluorescent co-immunohistochemistry in mouse limbs was carried out according to Relaix et al. (Relaix et al., 2003) using the polyclonal antibody anti-GFP (Cell Signaling). Proliferating cells were detected using a monoclonal antibody against BrdU (Amersham).

RESULTS

Angiogenic cells are detected in limb somatopleure before myogenic cells

Classical surgical manipulations in avian embryos have shown that endothelial and muscle cells originate from somites (Wilting et al., 1995; Pardanaud et al., 1996; Kardon et al., 2002). Transplantation of *lacZ* transgenic mouse somites into wild-type mouse embryos enabled detection of *lacZ*-positive cells with an endothelial morphology in early limb buds, indicating that mouse endothelial cells also originate from somites (Beddington and Martin, 1989). Single quail into chick somite grafts do not lead to a strict correlation between the distribution of quail endothelial and muscle cells, indicating that these cell types migrate along different routes in the developing limb bud (Huang et al., 2003). However, in these studies, there was no indication of the timing of limb colonization. We therefore investigated the temporal relationship between muscle and endothelial progenitor cells in the presumptive wing regions. We used the tyrosine kinase receptor, *Vegfr2* as a marker of the somite-derived angioblasts (Shalaby et al., 1995) and compared its distribution with that of *Pax3*, a marker of muscle progenitor cells, by in situ hybridization on transverse adjacent sections of embryos at different stages at the brachial level (Fig. 1A-F). We found that *Vegfr2*-positive cells are clearly detected in the presumptive wing

regions, at 19-somite (Fig. 1A, arrowheads) and 25-somite (Fig. 1B, arrowheads) stages. At these stages, there was no *Pax3* expression in the wing somatopleure (Fig. 1D,E). At the 30-somite stage, *Pax3*-positive cells had started their migration to the chick wing bud and intermingled with the *Vegfr2*-positive cells (Fig. 1C,F). Using the QH1 antibody on quail embryos (Pardanaud et al., 1987), we found the same result: QH1-positive cells were observed in the wing somatopleure at the 22- and 24-somite stages, whereas *Pax3* expression was not detected (Fig. 1G,H). In conclusion, muscle progenitor cells and angioblasts do not colonize the chick wing somatopleure at the same time. We also analyzed whether a similar situation occurs in mice. The cell adhesion receptor, PECAM1, is a recognized marker for early vascular precursor cells in mice (Baldwin et al., 1994). We found that PECAM1 is expressed in the forelimb at E9.5, whereas PAX3-positive cells have just started to migrate into the limb (Fig. 2A). These results show that angioblasts colonize the limbs before muscle precursor cells, in chick and mouse embryos.

Vessel organization in the absence of muscle

In order to investigate the involvement of muscle cells in blood vessel formation, we took advantage of the existence of *Pax3*-deficient mice, in which no myogenic cell is detected in the limbs (Relaix et al., 2003). The presence of blood vessels in *Pax3* mutant mice has already been reported (De Angelis et al., 1999), but not the spatial organization of the vasculature. We analyzed the blood vessel pattern in the absence of muscle cells using two mouse vascular markers, PECAM1 and the bHLH-PAS transcription factor gene *Hif2α* (*Epas1* – Mouse Genome Informatics) (Peng et al., 2000). In control limbs, PECAM1- and *Hif2α*-positive cells were seen to surround the muscles (Fig. 2B,D). In *Pax3* mutant limbs, the vessels appeared to organize in a similar pattern, delineating the areas of the absent muscles (Fig. 2C,E). We conclude that the vasculature organizes itself correctly in the absence of muscles.

The vasculature delineates the cleavage pattern

Twelve main muscles can be identified in the forearm of the chick wing (Sullivan, 1962; Robson et al., 1994). We mainly focused on the ventral muscle mass that gives rise to ventral flexor muscles.

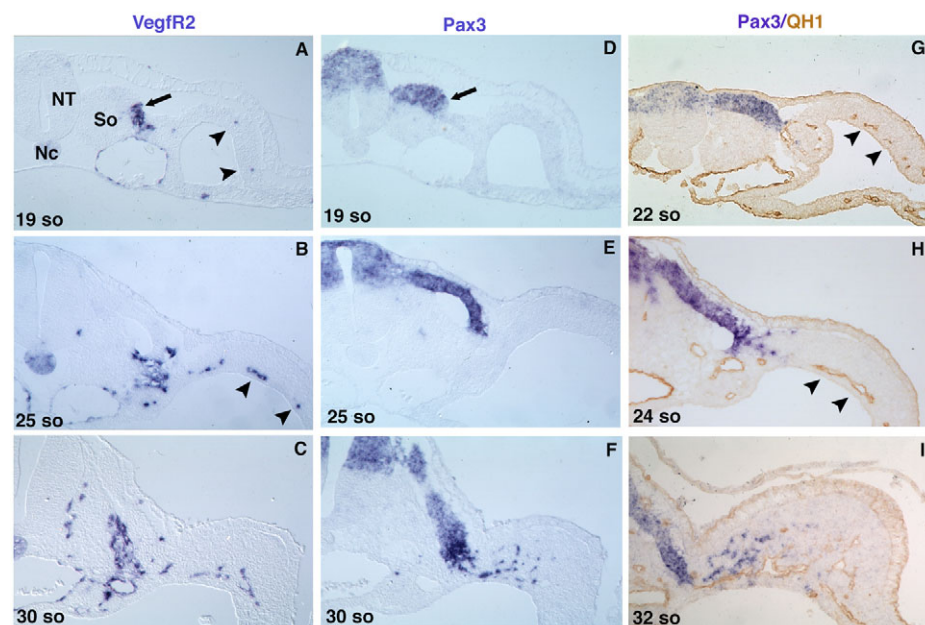


Fig. 1. Angiogenic cells are detected in wing somatopleure before muscle progenitor cells in avian embryos.

(A-F) Adjacent transverse sections of chick embryos, at the wing level, at 19 (A,D), 25 (B,E) and 30 (C,F) -somite stages were hybridized with *Vegfr2* (A-C) and *Pax3* (D-F) probes. (G-I) Transverse sections of quail embryos, at the wing level, at 22 (G), 24 (H) and 32 (I) -somite stages were hybridized with *Pax3* probe (blue) and then incubated with QH1 antibody (brown). Arrowheads point to endothelial progenitor cells visualized with *Vegfr2* probe (A,B) or QH1 antibody (G,H). Arrows indicate the overlapping domain of expression of *Vegfr2* (A) and *Pax3* (D) in somites. NT, neural tube; Nc, notochord; So, somites.

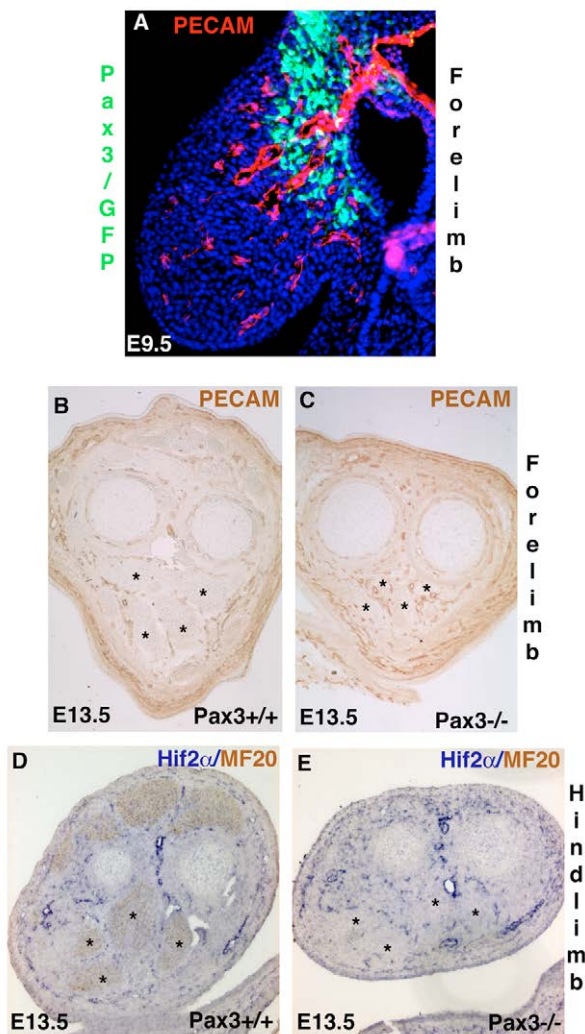


Fig. 2. The vascular network organizes itself independently of muscle. (A) Transverse sections of E9.5 forelimbs of *Pax3^{GFP/+}* mouse embryos were incubated with the PECAM antibody (red) to visualize vascular cells. GFP (green) reveals the *Pax3*-expressing cells. DAPI staining (blue) visualizes the nuclei. (B,C) E13.5 forelimbs from *Pax3^{+/+}* and *Pax3^{-/-}* mutant mice were cut transversally and then incubated with the PECAM antibody. (D,E) E13.5 hindlimbs of *Pax3^{+/+}* and *Pax3^{-/-}* mutant mice were cut transversally and hybridized with the *Hif2 α* probe and then incubated with the MF20 antibody. The asterisks show ventral muscles in control limbs (B,D) and the equivalent sites corresponding to the absent muscles in *Pax3^{-/-}* limbs (C,E). Both muscles and muscle-less sites are surrounded by the vasculature.

Based on serial cross-sections of wings at different stages, the successive separations of the ventral muscle mass can be schematized (see Fig. S1 in the supplementary material). Before the beginning of the splitting process, the vessels composed of endothelial cells surround the dorsal and the ventral muscle masses. When the splitting process is finished, the vessels surround the individual muscles in E10 wings (see Fig. S1 in the supplementary material).

At E5/HH26 and E5.5/HH27, based on myosin expression, there was no obvious sign of separation of the ventral muscle mass (Fig. 3A,B,D,E). However, at these stages, we detected endothelial cells, visualized using a *Vegfr2* probe, crossing the ventral muscle mass at

the precise site of the future cleavage zone (Fig. 3A,B,D,E). This cleavage zone was visualized at E6/HH28, separating the posterior and anterior muscle masses (Fig. 3C,F). This stereotyped organization of *Vegfr2*-positive cells crossing the ventral muscle mass was consistently observed in the wings of chick embryos at E5/HH26 and E5.5/HH27. We also observed QH1-positive cells at the future site of separation of the two parts of the flexor carpi ulnari in quail embryos (see Fig. S2 in the supplementary material). This stereotyped location of endothelial cells at the future sites of splitting in the ventral mass was observed over a certain length of the limb. By counting the sections displaying the endothelial marker *Hif2 α* crossing the ventral muscle mass, we estimate this organization to extend over 300 μ m along the proximal-distal axis (Fig. 3G-K). Half a day later, at E6/HH28, this cleavage zone had become obvious, containing numerous *Hif2 α* -positive cells and residual sparse MF20-positive cells (Fig. 3L). We also observed endothelial cells anteriorly, delineating the future separation of the central and the proximal anterior masses (Fig. 3L, arrow). In conclusion, the location of endothelial cells delineates the cleavage sites of muscle, before the effective separation of the muscle masses. This result suggests a link between vessel assembly and the splitting process.

Ectopic blood vessels inhibit muscle formation

In order to establish whether the vasculature influences muscle organization, we decided to modify vessel assembly. Ectopic VEGFA induces angiogenesis in a variety of in vivo models (Leung et al., 1989; Flamme et al., 1995; Wilting et al., 1996; Yin and Pacifici, 2001). We modified the expression of *Vegfa* using the avian RCAS retrovirus system (Fig. 4). Pellets of VEGF/RCAS-expressing cells were grafted dorsally into E4/HH22 wing buds (Fig. 4A), before the muscle splitting process started. The embryos were fixed once the splitting had finished, at E8/HH32. Ectopic VEGFA (Fig. 4B) led to a dramatic increase of blood vessels, visualized by the expression of *Hif2 α* , as compared with the left control wing (Fig. 4C,D). In the dorsal regions displaying an excess of blood vessels, we observed a reduction in muscle size and even a loss of certain muscles compared with the normal muscle pattern of the left wing (Fig. 4E,F). The muscles were visualized by *Fgfr4* expression, which labels muscle progenitor cells, and myosin expression, which labels muscle-differentiated cells (Edom-Vovard et al., 2001). Higher magnifications of a dorsal muscle (arrowed in Fig. 4C-F) show that the remaining muscles contain fewer myogenic cells than the corresponding control muscles (Fig. 4G-J). Grafting VEGF/RCAS earlier, at E2/HH14, into the presumptive wing regions led to a general and dramatic ectopic expression of *Vegfa* 5 days after grafting, and to an accompanying increase in blood vessels. The remaining muscles were severely reduced compared with those in the control limb (see Fig. S3A-E in the supplementary material). In order to determine whether this negative effect on muscles by vessels was stage-specific, we analyzed muscle formation at various stages after VEGF misexpression. Analysis of E2 VEGF grafts at various stages before E6 showed that muscle masses were never hypervascularized before E5, despite ectopic *Vegfa* expression (data not shown), suggesting that repulsive cues in muscle masses restrict angiogenesis at this stage. Ectopic vessels started to invade muscle masses from E5.5, indicating that muscle masses are permissive to vessel progression at this stage. However, E5.5 VEGF-infected limbs did not show any muscle modification (in terms of shape of muscle masses, density of myogenic cells) compared with the control limb (see Fig. S3F-K in the supplementary material), suggesting that later muscle alteration is a secondary effect to ectopic vessels and not a direct response of

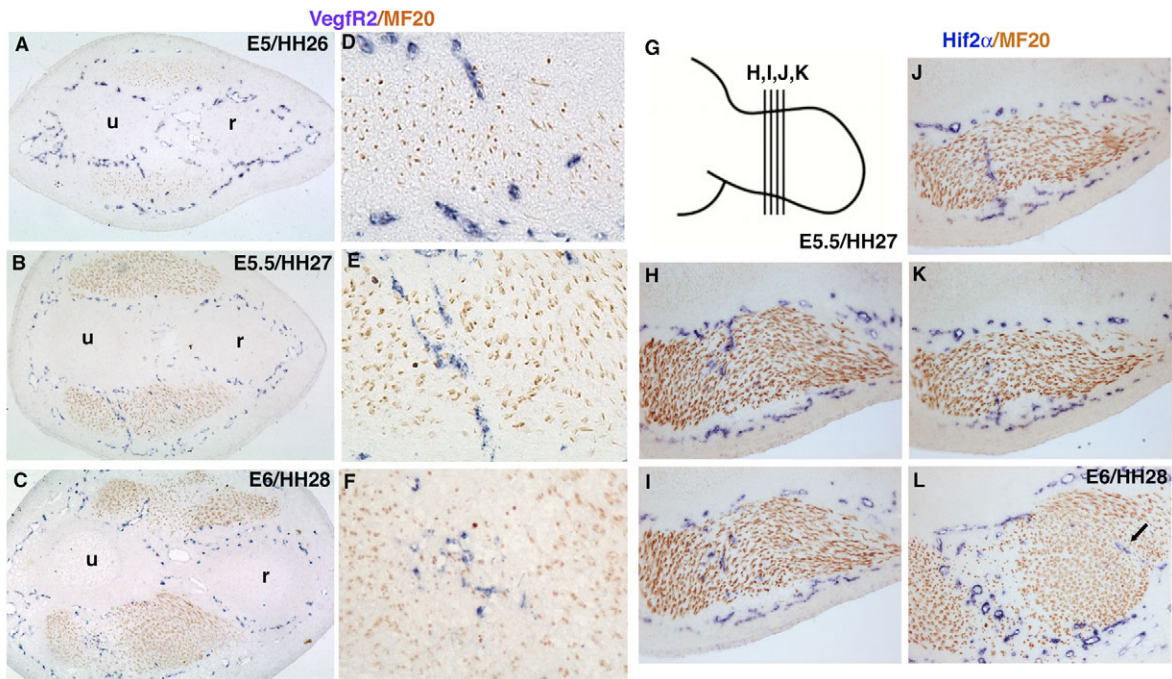


Fig. 3. The location of endothelial cells delineates the future cleavage zones of muscle masses. (A-F) Transverse sections of wings from E5/HH26 (A,D), E5.5/HH27 (B,E) and E6/HH28 (C,F) chick embryos were hybridized with the *Vegfr2* probe (blue) and then incubated with the MF20 antibody (brown). (D,E) High magnification of the future cleavage zone of the ventral masses from A,B. (F) High magnification of the cleavage zone of the ventral masses from C. (G-K) Wings from E5.5/HH27 chick embryos were cut transversely from proximal to distal areas along the forearm elements and then hybridized with the *Hif2α* probe (blue) followed by immunohistochemistry using the MF20 antibody (brown). The presence of *Hif2α*-positive cells at the future splitting site of the ventral muscle mass was estimated to extend over 300 μ m in length (shown are example images running from proximal to distal). (L) At E6/HH28, the ventral muscle mass was well separated into the posterior and anterior masses. The arrow indicates the *Hif2α*-positive cells delineating a future cleavage zone in the anterior part of the central muscle mass – cleavage that will produce the central and proximal anterior masses.

muscle cells to VEGF. Conversely, in E4-grafted wing, the absence of any shape malformation of the ventral muscles at E8, despite displaying ectopic vessels (Fig. 4C-F), is consistent with the possibility that the virus reached those ventral muscles too late to have an effect. Altogether, these results show that the presence of anarchic ectopic blood vessels has a negative influence on muscle formation at the time of muscle splitting.

In order to determine whether ectopic vessels could influence connective tissue formation, we analyzed the expression of the connective tissue marker collagen I. Collagen I is a major component of the extra-cellular matrix (ECM) of connective tissues. In addition to being located in all chick limb connective tissues, collagen I expression is enhanced in membranes surrounding muscles (Shellswell et al., 1980). We observed that collagen I expression is also enhanced in the recently cleaved sites of muscle masses and its presence can be correlated with that of vessels at these sites (Fig. 5A,B). Moreover, a net increase in collagen I expression was observed in hypervascularized muscles following VEGF misexpression, as compared with control muscles (Fig. 5C-H). These results show that ectopic vessels induced by VEGF misexpression promote connective tissue ECM production, while inhibiting muscle formation.

Local block of vessel formation leads to muscle fusion

We next aimed to block vessel formation during the time of muscle splitting in order to analyze muscle organization in the absence of vessels. We used the soluble form of VEGFR1 (FLT1), referred to

as sFLT1, which has been shown to bind VEGF with high affinity and to reduce angiogenesis in vivo (Drake et al., 2000; Bates et al., 2003). Application of sFLT1 beads to the dorsal aspect of HH28/E6 chick wings led to consistent local inhibition of vessel formation 2 days after grafting, whereas PBS beads did not affect vessel organization (Fig. 6A-D; $n=22$ sFLT1 beads; $n=14$ PBS beads). Analysis of *MyoD* expression showed reproducible muscle fusion in the dorsal regions of the sFLT1 grafted wings (Fig. 6E-H), whereas PBS beads did not alter muscle organization (data not shown). The fusion between the two muscles was observed along the entire length of two muscles (see Fig. S4 in the supplementary material). This experiment shows that the local absence of vessels prevents muscle splitting.

The vessel experiments highlight an inverse correlation between vessels and muscle. Hypervascularization inhibits muscle formation, whereas local hypovascularization leads to muscle fusion. The endogenous location of vessels in the future muscle cleavage zones together with the vessel experiments suggest an involvement of blood vessels in limb muscle splitting.

PDGFB reproduces the effect of blood vessels on muscle and connective tissue

We next tried to determine which molecular factors located in the vascular network could account for this negative effect of vessels on muscle. PDGFB, secreted by endothelial cells, is a putative candidate. During mouse development, PDGFB has been described as being located in endothelial cells, whereas its receptor PDGFR β is expressed in vascular smooth muscle cells (Lindahl et al., 1997).

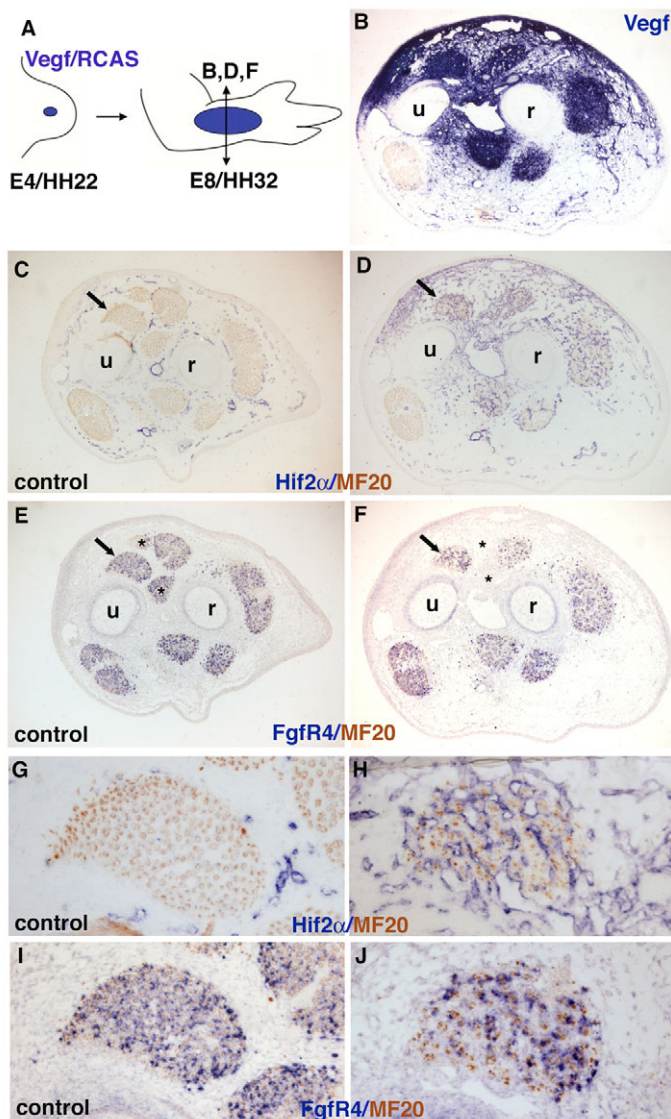


Fig. 4. Ectopic blood vessels inhibit muscle formation.

(A) VEGF/RCAS-expressing cells were grafted to the dorsal aspect of E4/HH22 chick wings and the embryos were fixed at E8/HH32. Consecutive sections of the manipulated wing (B, D, F, H, J) and of the control left wing (C, E, G, I) were cut at the same proximodistal level in order to allow comparison. Sections were hybridized with *Vegfa* (B), *Hif2α* (C, D, G, H) and *Fgfr4* (E, F, I, J) probes (blue), and then incubated with the MF20 antibody (brown) that recognizes myosins. (B) *Vegfa* transcripts show the extent of the infection in dorsal regions of the wings. Ectopic VEGFA leads to a dramatic increase in the density of blood vessels, visualized by the expression of *Hif2α* (D), compared with the normal vascular network of the control wing (C). In the dorsal regions displaying an excess of blood vessels, we observed a reduction of muscle size and even a loss of certain muscles (F), as compared with the normal muscle pattern of the left control wing (E). Asterisks label the control muscles (E) and their putative locations in the VEGF-treated limbs (F). (G–J) High magnifications of the ANC (Anconeus) muscle (arrowed in C–F) located in dorsal and posterior regions of the control and manipulated limbs, showing that the hypervascularized muscles contain fewer myogenic cells (J) than the corresponding control muscles (I). For all sections, the top is dorsal and left is posterior. u, ulna; r, radius.

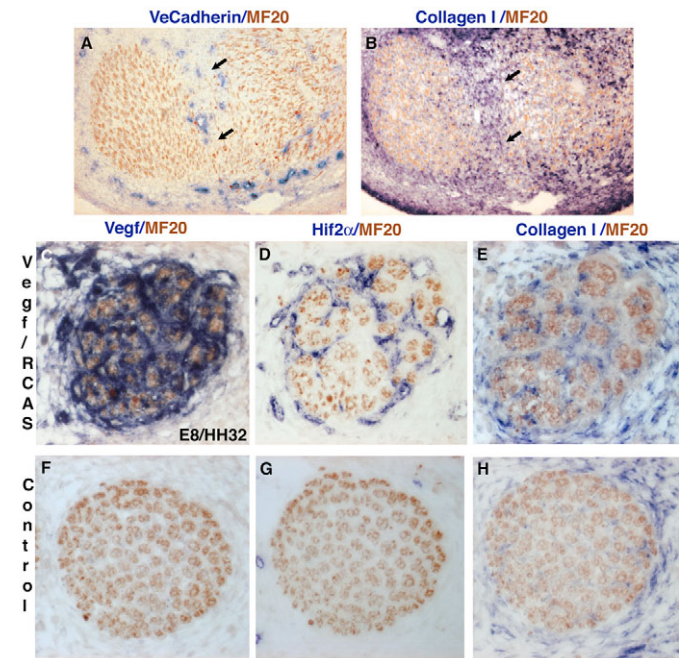


Fig. 5. Collagen I is associated with normal and ectopic vasculature in limb muscles.

Transverse and consecutive sections of wings from E6.5/HH29 chick embryos were hybridized with the VE-cadherin (A) and collagen I (B) probes followed by immunohistochemistry with the MF20 antibody. collagen I transcripts are detected with endothelial cells in the recently cleaved sites of the ventral muscle masses. (C–H) VEGF/RCAS-expressing cells were grafted to the dorsal aspect of E4/HH22 wings and the embryos were fixed at E8/HH32. Consecutive sections of VEGF-infected right limbs (C–E) and control left wings (F–H) were hybridized with the *Vegf* (C, F), *Hif2α* (D, G) and collagen I (E, H) probes (blue) then incubated with the MF20 antibody (brown). High magnifications are shown of the same dorsal muscle in experimental wings (C–E) and in control wings (F–H) at the same level. Ectopic hypervascularization (C, D) leads to an obvious increase in collagen I expression in muscles (E) compared with control muscles (H).

During chick limb development, we indeed observed *Pdgfrβ* transcripts in endothelial cells (Fig. 7A) and *Pdgfrβ* transcripts in smooth muscle cells (data not shown), similar to the mouse situation. We also observed an additional and unexpected site of *Pdgfrβ* expression in chick limb muscle masses (Fig. 7B, C), indicating that muscle cells could also respond to PDGFB signaling during muscle splitting. In order to investigate a possible role for PDGFB in muscle cleavage, we applied beads soaked in recombinant PDGFB to limb buds at E5 and analyzed the consequences for muscle development. Ectopic PDGFB inhibited the expression of the muscle marker, *MyoD* (Fig. 7D, E), around the beads, 2 days after grafting, whereas PBS beads did not impair *MyoD* expression (Fig. 7F). Interestingly, PDGFB bead application in the chick limb did not have any effect on muscle markers before E5 ($n=20$; data not shown), showing that the PDGFB effect is stage-specific. Application of PDGFB beads also inhibited the expression of its receptor, *Pdgfrβ* in muscle masses (Fig. 7G–I).

We next determined whether PDGFB could mimic the vessel effect on connective tissue. PDGFB application led to a clear upregulation of the expression of the muscle connective tissue marker, collagen I around the beads, in the region negative for

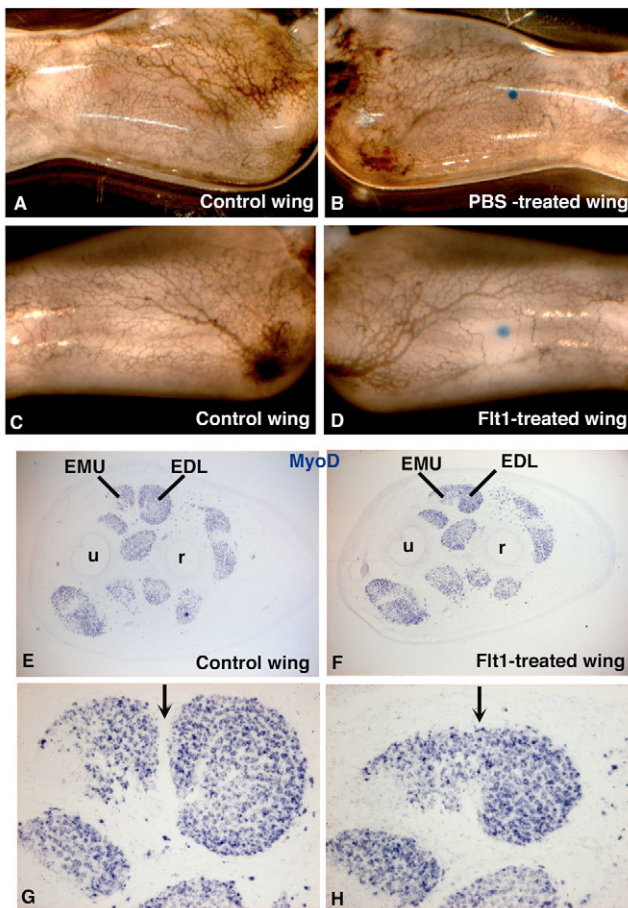


Fig. 6. Blocking vessel formation leads to muscle fusion.

(A-D) Dorsal views of wings from E8 chick embryos, which have undergone ink injection before fixation in order to visualize vessel organization. PBS bead implantation at E6 does not modify vessel assembly (B) as compared with the non-grafted left wing (A). sFLT1 bead implantation locally inhibits vessel formation (D) compared with the control left wing (C). (E-H) Transverse sections of sFLT1-treated right wings (F,H) and control left wings (E,G) were hybridized with the *MyoD* probe in order to visualize muscle organization. (G,H) High magnification of E,F, respectively. The EMU and EDC, two dorsal muscles, are cleaved in control wings, whereas they are fused in sFLT1-treated wings. EMU, extensor metacarpi ulnaris; EDC, extensor digitorum communis; u, ulna; r, radius.

muscle markers (Fig. 8A,B). We also analyzed the expression of two other connective tissue markers, *Tcf4* and *Pdgfra*, following PDGFB bead implantation (Fig. 8C-F). *Tcf4*, a transcription factor linked to the Wnt signaling pathway, provides a pre-pattern for vertebrate limb muscle patterning (Kardon et al., 2003) (Fig. 8C). *Pdgfra* transcripts have been described as being located in chick limb connective tissue (Ataliotis, 2000). We observed that *Pdgfra* transcripts display an expression pattern similar to that of collagen I, in general and muscle connective tissues, in chick limbs (data not shown). *Pdgfra* expression was also enhanced in the future regions of muscle cleavage before the effective separation of muscles (Fig. 8E). PDGFB application also led to an upregulation of the expression *Tcf4* and *Pdgfra* around the beads (Fig. 8C-F). Application of PDGFB beads did not induce ectopic expression of the tendon marker, scleraxis, 2 days after grafting (data not shown),

excluding a tendon identity for the tissue surrounding PDGFB beads. Application of PDGFB beads did not modify the expression of endothelial markers [HIF2 α , VE-cadherin (also known as cadherin 5)] or that of smooth muscle cell marker (SMA), indicating that PDGF-induced connective tissue is not highly vascularized and does not contain smooth muscle cells (data not shown).

Altogether, these results show that PDGFB promotes the expression of genes specific to muscle connective tissue, in addition to preventing muscle formation. Thus, PDGFB mimics the muscle-inhibiting and connective tissue-promoting function of blood vessels. The fact that the only source of PDGFB is endothelial cells makes PDGFB an obvious candidate for mediating the vessel effect on muscle and connective tissue.

PDGFB acts on connective tissue cells before it acts on myogenic cells

The bead experiments showed that PDGFB acts on two unrelated embryological cell types: connective tissue and myogenic cells. Both cell types are able to respond to PDGF signal because they express PDGF receptors, PDGFR α (connective tissue) and PDGFR β (muscle). Analysis of Hoechst-labeled nuclei showed that cell density was clearly enhanced 2 days after bead implantation (Fig. 9A-D). However, analysis of BrdU incorporation showed that PDGFB application did not modify the cell proliferation around the beads, 24 (data not shown) and 48 hours after grafting (Fig. 9E-G). This implies that PDGFB increased cell density around the bead by attracting cells. Given the net increase of connective tissue marker expression (and the absence of muscle marker) around the PDGFB beads (Fig. 7, Fig. 8, Fig. 9D,G), we concluded that PDGFB attracted connective tissue cells around the beads. We next tried to define on which cell type PDGFB acts first. By fixing embryos at various times after PDGFB bead implantation, we observed that PDGFB activated the expression of collagen I as soon as 9.5 hours after grafting, whereas the downregulation of *MyoD* expression was only observed 24 hours after grafting (Fig. 10A-F). This shows that PDGFB acts on connective tissue cells first. In order to estimate the contribution of gene transcription and cell migration, we analyzed Hoechst-labeled nuclei behavior at various times after PDGFB bead implantation. We did not observe any obvious sign of increase in cell density around the beads 9.5, 12, 16 (data not shown) and 24 hours (Fig. 10G,H) after PDGFB implantation, compared with the obvious cell accumulation 48 hours after bead implantation (Fig. 9A-D). However, we cannot exclude the existence of cell movement without modifying cell density. The modification of gene transcription (upregulation of collagen I and downregulation of *MyoD* expression) was observed before the cell accumulation around PDGFB beads.

DISCUSSION

Angiogenic cells behave independently of myogenic cells in chick and mouse limbs

In this paper, we show that angiogenic cells are clearly detected before muscle progenitor cells in presumptive limb bud regions, indicating that the angioblasts colonize the limb bud independently of muscle cells. Moreover, the fact that the vasculature is present and organizes properly in the absence of muscle in *Pax3* mutant embryos confirms the complete independence of vascular cells with respect to muscle cells, in the developing limbs. Different sets of experiments clearly indicate that endothelial and myogenic cells originate from a common progenitor (De Angelis, 1999; Kardon et al., 2002). Lineage tracing experiments in chick hindlimbs demonstrated that the common progenitor exists in the thirtyfirst somite (lumbar somite) at the 36-somite stage (Kardon et al., 2002).

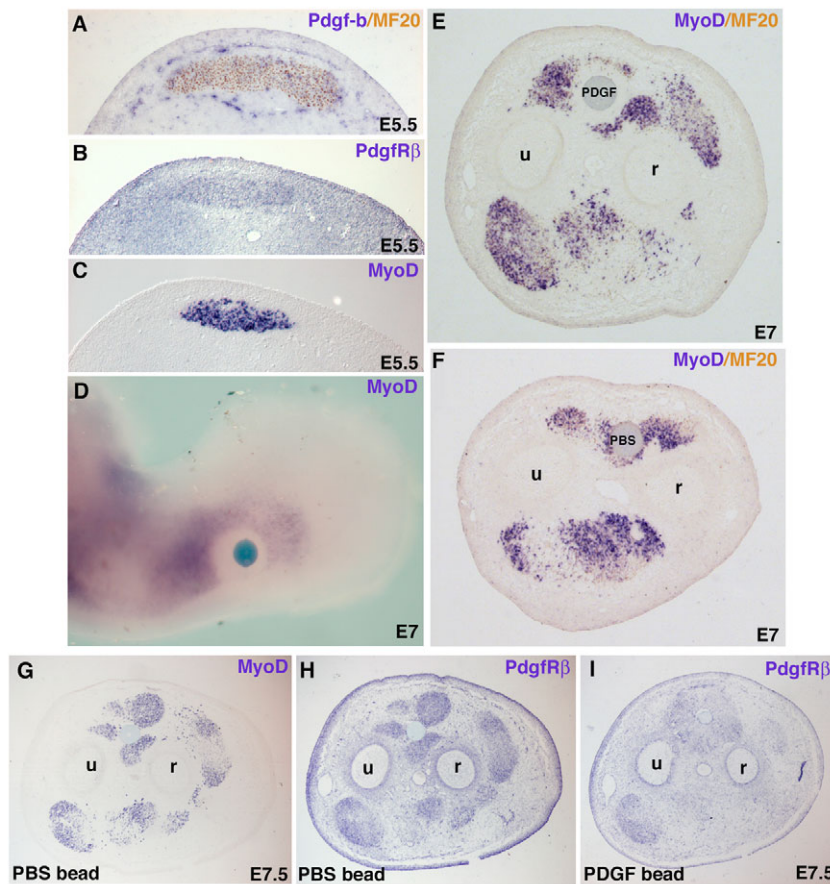


Fig. 7. PDGFB inhibits the expression of muscle markers. (A) Transverse sections of wings from E5.5/HH27 chick embryos were hybridized with the *Pdgfrβ* probe followed by immunohistochemistry with the MF20 antibody. (B,C) Transverse and consecutive sections of wings from E5.5/HH27 chick embryos were hybridized with the *Pdgfrβ* (B) and *MyoD* probe (C). (D-I) PDGFB or PBS beads were implanted into the dorsal regions of E5/HH26 wings and the embryos fixed 2 days or 2.5 days later, at E7/HH30 or E7.5/HH31. (D) Whole-mount preparations of PDGFB-implanted wings hybridized with *MyoD* show inhibition of *MyoD* expression around the bead. Sections of the PDGFB (E,I) or PBS (F,G,H) -treated wings were hybridized with the *MyoD* (E-G) or the *Pdgfrβ* (H,I) probes. The expression of *MyoD* (E) and *Pdgfrβ* (I) was inhibited around PDGFB beads, whereas PBS beads did not have any effect on *MyoD* (F,G) or *Pdgfrβ* (H) expression. The PBS bead (H) is shown at a more proximal level than the PDGFB bead (I), explaining the difference of muscle pattern in ventral muscles between limbs. The two anterior and ventral muscles visualized in the control PBS limbs (H) are only observed in proximal region of the forearm. For all the sections (A-C,E-I), top is dorsal and left is posterior; u, ulna; r, radius.

Consistent with this, there is an overlapping expression domain of *Pax3* and *Vegfr2* in the dorsolateral compartment of the chick brachial epithelial somites (Fig. 1A,D, arrowed). Our marker analysis shows that angioblasts behave differently to muscle precursor cells outside the somites in chick and mouse limbs. Whether angiogenic cells have an influence on the migration of the myogenic cells in the limb, as suggested by previous electron microscopy studies (Solursh et al., 1987), remains to be determined.

The vascular network influences the muscle splitting process

The vasculature is usually seen as a supplier of essential metabolic nutrients and oxygen to differentiating tissue, including muscle (Caplan and Koutropas, 1973). It has also been reported that endothelial cells promote liver and pancreatic organogenesis prior to blood vessel function (Matsumoto et al., 2001; Lammert et al., 2001). Here we provide evidence that the vascular system plays an additional role: directing a developmental patterning process. We have shown that the vasculature delineates the future cleavage zones in the ventral muscle mass. In addition, the vessel experiments show an inverse correlation between vessels and muscles. An increase of vessels leads to an inhibition of muscle formation, whereas blocking vessel formation leads to muscle fusion. These results highlight a potential role for the vascular network in muscle splitting. Moreover, we have shown that ectopic vessels promote collagen I expression, which is also increased at the splitting sites in developing embryos. Thus, we propose that vessels are involved in setting up a boundary of ECM-rich connective tissue between two dividing muscles. Interestingly, there is evidence in chick limbs indicating that blood

vessels also dictate cartilage patterning, as the vascular regression in limb presumptive cartilage regions is a required condition for the initiation of correct mesenchymal condensation and subsequent chondrogenesis (Yin and Pacifici, 2001).

From classical embryological experiments, we know that the positional information for muscle patterning resides in non-somitic cells (Chevallier et al., 1977; Christ et al., 1977; Lance-Jones, 1988; Kardon, 1998). Moreover, muscle fibers know their orientation before any splitting event occurs (Kardon, 1998). We therefore hypothesize that signals in the limb mesenchyme direct the spatial organization of the vasculature, which in turn influences muscle cleavage. The vasculature would be a relay system from limb mesenchymal cells to myogenic cells that would set up boundaries between muscles, secondarily to muscle fiber orientation. Other limb tissues, such as tendons (Kardon, 1998; Edom-Vovard and Duprez, 2004) and connective tissue (Kardon et al., 2003), are also involved in muscle patterning. The connection between tendons, vessels and connective tissue is an important issue to be addressed.

However, the involvement of the vasculature in muscle splitting does not resolve the problem of muscle patterning, as the mechanisms directing the stereotyped organization of the vasculature in the embryonic limb are largely unknown. It is accepted that the position of endothelial cells is regulated by their adhesive interactions with the ECM, probably through integrin interactions, leading to the establishment of the embryonic vascular network (Weinstein, 1999). Recently, guidance proteins involved in axon outgrowth, such as semaphorins or ephrins and their associated Eph receptors, have been shown to control vascular morphogenesis in the embryo (Bates et al., 2003; Weinstein, 2005; Carmeliet, 2005).

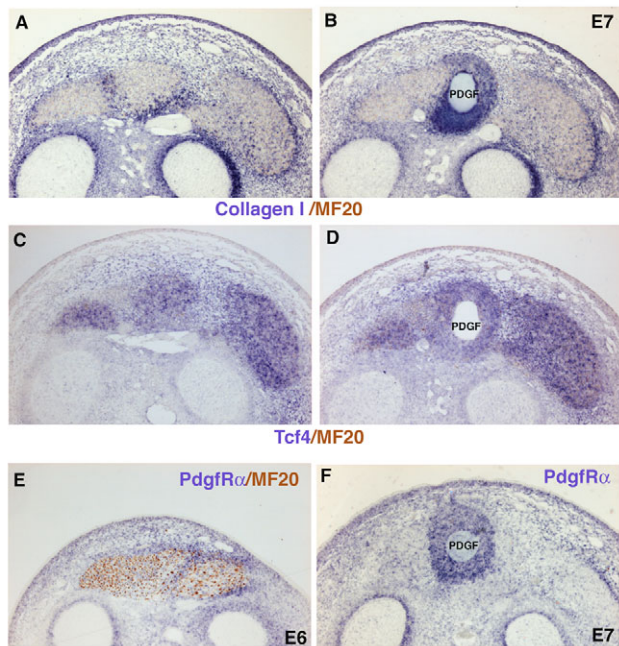


Fig. 8. PDGFB activates the expression of connective tissue markers. PDGFB or PBS beads were implanted into the dorsal regions of E5/HH26 chick wings and the embryos were fixed 2 days later, at E7/HH30. Consecutive sections of the PDGFB-treated wings (**B,D**) and of the corresponding control left wings (**A,C**) were hybridized with probes for the muscle connective tissue markers collagen I (**A,B**) and *Tcf4* (**C,D**) and then incubated with the MF20 antibody. (**E**) Transverse section of an E6 wing showing the endogenous expression of *Pdgfrα* in muscle connective tissue. *Pdgfrα* transcripts (blue) are detected in the dorsal muscle mass (brown) in a future site of cleavage. (**F**) PDGFB-treated wings were hybridized with the *Pdgfrα* probe.

There are also arguments indicating that the sensory nerves influence vascular remodeling and determine the pattern of arterial differentiation in the skin (Mukouyama et al., 2002). However, there is no such evidence in limbs. Our results indicate that the organization of the early vasculature is very stereotyped in avian limbs and reproducible among embryos, suggesting that specific rules govern this organization, although they remain to be determined. Interestingly, observations point to a role for Wnt signaling in vessel development (Goodwin and D'Amore, 2002). TCF4 has been shown to induce endothelial cell migration via the transcriptional activation of IL8 (Levy et al., 2002). Although the connection remains to be established, TCF4 providing a pre-pattern for limb muscle cells (Kardon et al., 2003) could also be involved in limb muscle splitting by inducing the correct positioning of endothelial cells.

Role of PDGF signaling in muscle splitting

We observed that PDGFB bead application mimics the vessel effect on muscle and connective tissue. Since PDGFB endogenous expression in the limb is restricted to endothelial cells at the time of muscle splitting, we propose that the PDGFB is a candidate for mediating the vessel effect on muscle and connective tissue. Since the PDGF receptors, *Pdgfrβ* and *Pdgfrα*, are both expressed at a suitable time in muscle masses and muscle connective tissue, respectively, the inhibitory effect of PDGFB on muscle formation could be a consequence of the combined responses of connective

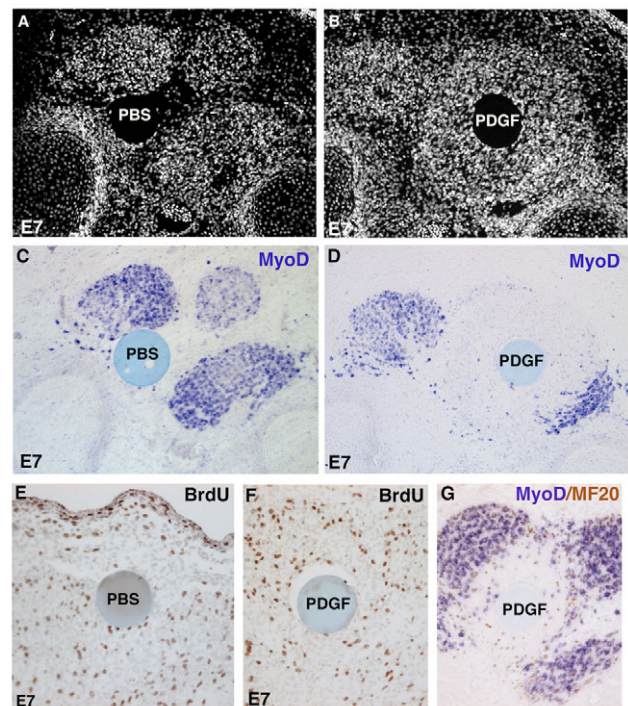


Fig. 9. PDGFB increases cell density without modifying cell proliferation. Cell density around PBS (**A**) and PDGFB (**B**) beads were visualized with Hoechst-labeled nuclei at E7, 2 days after grafting into chick wings. (**C,D**) Adjacent sections to those shown in **A,B**, hybridized with the *MyoD* probe. There is an increase in cell density homogeneously around PDGFB beads (**B**), where *MyoD* expression is inhibited (**D**), whereas PBS beads do not induce any cell accumulation (**A**) and do not modify muscle organization (**C**). It should be noted that cell density is higher in muscles compared with limb connective tissue at this stage (**A-D**). Transverse sections from PBS (**E**) or PDGFB (**F,G**)-treated wings incubated with the anti-BrdU antibody show that application of PDGFB does not modify cell proliferation around the bead. (**G**) The adjacent section to that shown in **F**, hybridized with the *MyoD* probe and then incubated with the MF20 antibody.

tissue (through *Pdgfrα*) and muscle (through *Pdgfrβ*). Our results show that the first event after PDGFB bead application is an increase in expression of the connective tissue marker collagen I. Levels of collagen I have been shown to be modified by PDGFB in human skin and rat tendon models (Nesbit et al., 2001; Wang et al., 2004). The analysis of the timing of transcription modification (in situ hybridization) versus cell density (Hoechst) after PDGFB application in chick limb (Figs 9, 10) suggests that the increase of ECM (collagen I) allows the migration of muscle connective tissue cells toward the source of PDGFB. Interestingly, PDGFB has been shown to drive dermal fibroblast migration on type I collagen matrix (Li et al., 2004).

The PDGF effect on *MyoD* expression occurs after the increase in collagen I expression (Fig. 10). One interesting question is whether PDGFB acts directly on muscle cells, possibly by inhibiting *MyoD* expression, or indirectly by recruiting connective tissue cells around the beads and excluding myogenic cells. There are several arguments indicating that myogenic cells can directly respond to PDGF signaling: (1) the presence of *Pdgfrβ* transcripts in chick muscle masses and muscles; (2) PDGF activity in undifferentiated myoblasts from various muscle cell lines (Jin et al., 1990; Yablonka-

Reuveni et al., 1990; Fiaschi et al., 2003); (3) the skeletal muscle phenotype observed in *Pdgfr β* mouse chimaeras (Crosby et al., 1998); and (4) the identification of various muscle markers as PDGFB transcriptional targets by microarray-coupled gene-trap mutagenesis (Chen et al., 2004). Although these arguments are consistent with the notion that myogenic cells can respond to PDGF signaling it is not clear whether, in our experiments, PDGF action on muscle is direct or indirect. A negative effect of PDGFB on muscle marker expression in chick limb is nevertheless supported by previous in vitro studies, in which PDGFB (and not PDGF-A) has been shown to specifically inhibit muscle terminal differentiation in various skeletal muscle cell lines (Yablonka-Reuveni et al., 1990; Jin et al., 1990; Jin et al., 1991; Jin et al., 1993; Yablonka-Reuveni and Seifert, 1993; Fiaschi et al., 2003). Moreover, abnormalities in

skeletal muscles have been noted, although not characterized, in *Pdgfr β* mutant mice (Lindhahl et al., 1997; Betsholtz et al., 2001). However, analysis of skeletal muscles in E13.5 and E14.5 *Pdgfr β* mutant mice did not show consistent modification of the limb muscle pattern (data not shown); indicating a possible redundancy with another PDGF (Bergsten et al., 2001). Although PDGFR α signaling is mainly associated with PDGF-A in epithelial-mesenchymal interactions, we found that chick limb muscle connective tissue cells are also responsive to PDGFB produced by endothelial cells. PDGFB can also activate PDGFR α signaling in eye, lung and skin, in mouse transgenic models (Betsholtz, 2004; Tallquist and Kazlauskas, 2004). Interestingly, defects in myotome patterning, including fusion of myotomes, have been observed in *Pdgfra* mutant mouse embryos (Soriano, 1997; Tallquist et al., 2000).

One attractive hypothesis is that PDGFB (produced by endothelial cells crossing the muscle masses) will increase the production of ECM (collagen I), which in turn will promote connective tissue cell migration and accumulation to the future site of cleavage. The progressive accumulation of connective tissue cells at the future splitting sites could exclude myogenic cells and thus allow muscle cleavage. We cannot exclude an additional and direct effect of

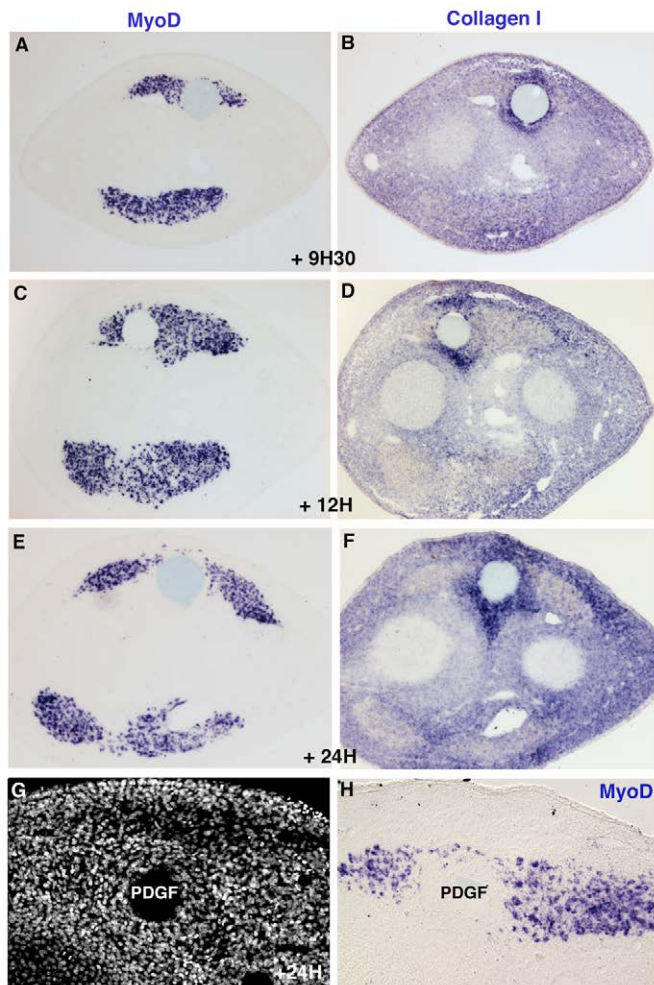


Fig. 10. PDGFB acts on connective tissue cells before it acts on myogenic cells. PDGFB beads were implanted into the dorsal regions of E5/HH26 chick wings and the embryos were fixed at various times after grafting. Consecutive sections of the PDGFB-treated wings, 9.5 (A,B), 12 (C,D) and 24 (E,F) hours after grafting were hybridized with the *MyoD* (A,C,E) and collagen I (B,D,F) probes. As soon as 9.5 hours, an increase in collagen I expression was observed (B), whereas no obvious effect on *MyoD* expression was observed (A). An effect on *MyoD* expression (loss of *MyoD* expression around the bead) was observed 24 hours after grafting. (E). (G) Analysis of cell density, visualized with Hoechst-labeled nuclei, 24 hours after PDGFB bead implantation. (H) An adjacent section to G was hybridized with the *MyoD* probe.

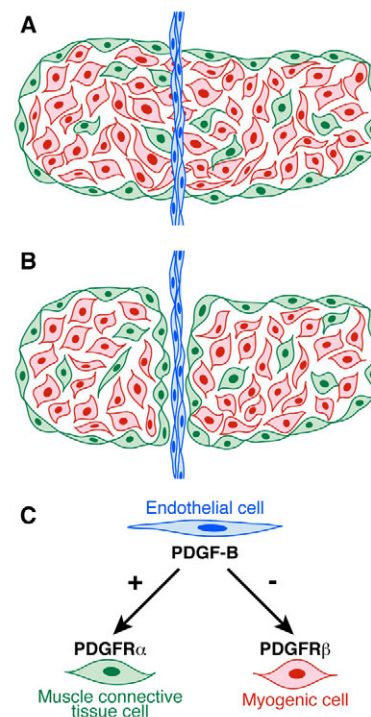


Fig. 11. Model for the effect of the vasculature on muscle mass separation. (A) The endothelial cells (blue) delineate the future cleavage site in the muscle mass, which is composed of myogenic cells (red) and muscle connective tissue cells (green). (B) At a later stage, the muscle masses are separated. (C) The PDGFB secreted by the endothelial cells acts in a paracrine manner on muscle connective tissue cells, which express PDGFR α . In response to PDGFB, connective tissue cells (expressing PDGFR α) increase the secretion of extracellular matrix by producing collagen I. This promotes the accumulation of connective tissue cells and the formation of a new muscle membrane, allowing muscle mass separation. Concomitantly, muscle differentiation will be inhibited as a consequence of the accumulation of muscle connective tissue and/or directly by PDGFB acting on muscle cells, which express PDGFR β .

PDGFB on *MyoD* expression. Gradual diminution in the number of the myogenic cells in the cleavage zone has been observed using electron microscopy (Schroeter and Tosney, 1991b). The residual myotubes in the cleavage zones are then thought to be selectively removed by phagocytic cells via an unknown mechanism (Schroeter and Tosney, 1991b). However, we did not detect any significant increase in apoptosis at the site of cleavage (data not shown). Our PDGFB experiments provide a molecular mechanism whereby PDGFB produced by the vessels can increase muscle connective tissue locally and exclude muscle cells, leading ultimately to muscle mass separation (Fig. 11).

In conclusion, our results highlight an unexpected potential role for vessels in the cleavage of muscle masses. The involvement of PDGFB/PDGFR signaling in the communication between endothelial and muscle cells, via connective tissue cells, provides a molecular mechanism underlying muscle splitting.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/14/2579/DC1>

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