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Local Extrinsic Signals Determine Muscle and Endothelial Cell Fate and Patterning in the Vertebrate Limb

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Summary

Both the muscle and endothelium of the vertebrate limb derive from somites. We have used replication-defective retroviral vectors to analyze the lineage relationships of these somite-derived cells in the chick. We find that myogenic precursors in the somites or proximal limb are not committed to forming slow or fast muscle fibers, particular anatomical muscles, or muscles within specific proximal/distal or dorsal/ventral limb regions. Somitic endothelial precursors are uncommitted to forming endothelium in particular proximal/distal or dorsal/ventral limb regions. Surprisingly, we also find that myogenic and endothelial cells are derived from a common somitic precursor. Thus, local extrinsic signals are critical for determining muscle and endothelial patterning as well as cell fate in the limb.

Introduction

Vertebrate limb muscle and endothelial cells both originate from somites (Chevallier et al., 1977; Christ et al., 1977; Pardanaud et al., 1996). Their precursors migrate into the developing limb bud where myogenic precursors differentiate and are patterned to form an elaborate array of muscles and, concurrently, endothelial precursors differentiate and assemble into a vascular network.

Somites mature along a caudal to cranial gradient, with the most newly formed somites designated as stage I (Ordahl, 1993). The somites become specialized to form a dorsal dermomyotome and ventral sclerotome. The lateral part of the dermomyotome gives rise to the body wall and limb muscles (reviewed in Borycki and Emerson, 2000; Ordahl et al., 2000). Beginning at somite stage III–IV (Ordahl et al., 2000; G.K., unpublished data), hind limb myogenic precursors migrate from the lateral edge of the dermomyotome of somites 26–33 (Lance-Jones, 1988). Cells enter the base of the limb, split around the developing chondrogenic cores, and occupy the dorsal and ventral sides of the limb bud while generally maintaining their relative anterior/posterior position (Hayashi and Ozawa, 1991).

Once in the hind limb, myogenic cells concomitantly form a pattern, consisting of 45 distinctive hind limb muscles (Baumel et al., 1993; Hudson et al., 1959), and differentiate into myotubes (Kardon, 1998). Each muscle is uniquely identifiable by its position within the limb, its size and shape, orientation of its fibers, and points of origin and insertion on bone, and characterized by a stereotyped composition of slow and fast myotubes. Slow and fast myotubes differ in their speed of contraction, and this is correlated with the presence of specific isoforms of myofibrillar contractile proteins, such as myosin heavy chain (MHC; reviewed in Kelly and Rubinstein, 1994). The early embryonic pattern of slow and fast myotubes develops as cells differentiate, is independent of innervation, and largely maintained during growth (Butler et al., 1982; Crow and Stockdale, 1986; Fredette and Landmesser, 1991; Page et al., 1992; Phillips and Bennett, 1984; Phillips et al., 1986).

The overall pattern of limb muscles is specified by the limb environment. Somites from any axial level can give rise to normal limb muscle and therefore are not prepatterned to form limb muscles (Chevallier et al., 1977; Jacob and Christ, 1980). Other experiments demonstrate that muscle is not patterned by the limb's nerves or vasculature (Caplan and Koutroupas, 1973; Shellswell and Wolpert, 1977), but is instead patterned by the surrounding limb mesoderm (Jacob and Christ, 1980). This patterning of limb muscle is ultimately closely linked to the early specification of the dorsal/ventral (D/V), proximal/distal (P/D), and anterior/posterior (A/P) axes of the limb (Geduspan and MacCabe, 1987; Akita, 1996; Robson et al., 1994; Duprez et al., 1999; Shellswell and Wolpert, 1977), and is basically complete by the time myotubes differentiate early in the limb (Kardon, 1998). However, when and where individual limb myogenic cells become patterned along the D/V, P/D, and A/P axes is not known. While it is likely that the overall muscle pattern is governed by extrinsic limb signals, there is considerable controversy over whether commitment of myogenic cells to a slow or fast fiber-type fate is an intrinsic property of the myogenic cells (Lance-Jones and Van Swearingen, 1995; Nikovits et al., 2001) or controlled by extrinsic factors within the limb (Duprez et al., 1999; Robson et al., 1994).

The limb vasculature is also derived, at least in part, from the somites (Pardanaud et al., 1996; Wilting et al., 1995). In particular, the dorsolateral region of the somites is an important source of limb endothelium (Eichmann et al., 1993; Wilting et al., 1995). These endothelial precursors migrate into the limb and then associate into vessels, largely by the process termed type II vasculogenesis (Ambler et al., 2001). Additional limb vasculature is formed by the processes of angiogenesis, whereby new vessels form as outgrowths from preexisting flank vessels (Ambler et al., 2001; Brand-Saberi et al., 1995). Little is known of when somitic cells become committed to an endothelial cell fate or patterned to form the endothelium.

Results

Lineage Analysis of Chick Hind Limb Somitic Derivatives Using CHAPOL

To follow the fate of clonally related somitic cells in the hind limb, lumbosacral somites were injected with a complex retroviral library, CHAPOL. CHAPOL is a library

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of replication-defective viruses, each encoding human alkaline phosphatase (AP) and a distinct 24 bp oligonucleotide insert (Golden et al., 1995). Histochemical staining for AP allowed virally infected cells to be identified in tissue section. Infected cells were manually harvested and used as a substrate for PCR amplification and subsequent sequencing. Each insert uniquely tagged members of a clone of cells.

Nine st 17–20 chick embryos were injected with CHAPOL virus into the lateral edge of somite 31 at somite stages I (n = 3), V (n = 2), and VII (n = 2) and into the proximal limb just lateral to somite 31 (n = 2). Embryos were harvested at st 32–33 (Hamburger and Hamilton, 1951) when the basic muscle pattern of primary myotubes in the hind limb has been established (Kardon, 1998) but before the formation of secondary myotubes (Fredette and Landmesser, 1991). In agreement with previous fate maps of limb level somites (Chevallier et al., 1977; Christ et al., 1977; Lance-Jones, 1988; Ordahl and Le Douarin, 1992; Pardanaud et al., 1996; Schramm and Solursh, 1990; Wilting et al., 1995), AP-positive cells were found either within the muscles or the endothelium of blood vessels of the hind limb.

A total of 9375 cells were picked. An initial product was amplified from 5298 of these (56% of cells picked), 3775 of which (40% of cells picked) yielded a readable 24 bp oligonucleotide sequence. In most cases, a single tag was recovered from each cell pick. However, in some instances, amplified cells yielded multiple tags; a clonal identity to these cells was not assigned. A total of 260 different tags, or clones, were identified. As expected, given the complexity of the library (estimated to minimally contain 10^5 members with an approximately equal representation; Golden et al., 1995), the same viral tag was not recovered from more than one independent infection.

Dynamics and Overall Pattern of Muscle Cells Populating the Hind Limb

Injections of CHAPOL into somite 31 at somite stages I, V, and VII resulted in 3–111 muscle clones per injection (Table 1). Since each clone represents cells descended from a single infected precursor, this indicates that greater than 100 myogenic precursors can emigrate from somite 31. These myogenic cells ultimately contributed to 2–244 myotubes in st 32–33 limbs. Cells labeled at somite st VII (as myogenic cells are emigrating from the somite) gave rise to 2–50 cells 5 days later. Given that only 40% of the tags are recovered, we estimate that individual myogenic cells can give rise to approximately 125 cells during this period. If cells divide at a constant rate, this suggests that myogenic cells divide approximately every 17 hr.

Myogenic cells labeled in somite 31 ultimately populate multiple muscles in the dorsal and ventral thigh, shank, and foot (see Supplemental Table S1 at http://www.developmentalcell.com/cgi/content/full/3/4/533/DC1). In agreement with Lance-Jones's (1988) fate map of somite 31, labeled cells from somite 31 are found in all but the anterior-most muscles of the hind limb. This distribution of labeled cells reflects the relatively posterior position of somite 31 among the somites (26–33) that contribute to hind limb muscles and the stereotyped path of myogenic cell migration.

Myogenic Precursors Are Not Committed to Forming Particular Anatomical Muscles or Muscles in Particular Dorsal/Ventral or Proximal/Distal Regions of the Limb

Myogenic cells labeled at early somite stages, I or V, can give rise to both dorsal and ventral muscle (Table 1A). At both somite stages, most (23) groups of clonally related cells were found either in only dorsal or ventral muscles (representative clones are diagrammed in Figures 1B–1D). However, two clones derived from cells labeled at somite st I and one from a somite st V-labeled cell had multiple members in both dorsal and ventral muscles (e.g., Figures 1E–1G and 2A). On average, the dorsal-only and ventral-only clones are smaller than the dorsal/ventral clones and simply may be found only dorsally or ventrally because of their small clone size. This indicates that myogenic cells in the early somite are not restricted, and so not committed, to a dorsal or ventral fate.

By somite st VII, myogenic cells are largely restricted to forming only dorsal or ventral muscles (Table 1A). Of 111 clones derived from cells labeled at somite st VII and 89 clones labeled in the proximal limb, all members of most of these clones (108 somite st VII clones and 82 proximal limb clones) were found in only dorsal or ventral muscles (Figure 1A). In a small number of cases (three somite st VII and seven proximal limb clones), clones were found where most members were in either dorsal or ventral muscles, but one or two cells were in muscles on the other side of the limb. The D/V restriction, we generally found could, in principle, reflect the commitment of somite st VII myogenic cells to a dorsal or ventral fate. However, it is more likely to be a consequence of the myogenic cells' stereotyped path of migration and their rates of proliferation. DiI labeling studies have determined that once myogenic cells migrate dorsally or ventrally to the chondrogenic cores, they never again move across the D/V boundary (Hayashi and Ozawa, 1991). Based on their rates of proliferation (one division every 17 hr; see above) and the relatively short time between exiting the somite and migrating dorsally or ventrally around the chondrogenic cores (generally less than 24 hr), myogenic cells labeled at somite st VII or in the proximal limb would not generally be expected to give rise to two daughter cells in time for them to migrate to opposite sides of the limb. Thus, the general D/V restriction of myogenic cells labeled in older somites or in the proximal limb probably does not reflect an intrinsic commitment to a dorsal or ventral fate, but rather is a consequence of the lack of multicell clones at the time the cells migrate dorsally and ventrally.

Descendants of myogenic cells labeled at somite st I, V, VII, or in the proximal limb can populate multiple P/D regions of the limb (Table 1A). Of the 234 total muscle clones, members of 181 were found in only one P/D region (Figures 1A and 1B). However, members of 45 clones were found in two P/D regions (thigh and shank or shank and foot; see Figures 1C–1E), and members of eight clones were found in all three P/D regions (Figures 1F, 1G, and 2A). On average, larger clones spanned more P/D regions and therefore clones found in only one P/D region are probably simply a result of the clones' small size. Thus, neither when in the somites

Table 1. Analysis of Anatomical, Slow/Fast, and Muscle/Endothelial Fate of Clones

A

Somite st injected	Total number of clones	Range of clone size	Average clone size	Number of clones found in dorsal and/or ventral regions of the limb			Number of clones found in one or more proximal/distal regions of the limb						Number of clones found in one or more individual anatomical muscles		
				Dorsal only	Ventral only	Dorsal and ventral	Thigh only	Shank only	Foot only	Thigh and shank	Shank and foot	Thigh, shank, and foot	1 muscle	2–6 muscles	7–12 muscles
I	17	2–244	69	5	3	2[1]	4	0	1	6	2	4	0	9	8
V	17	3–62	28	8	7	1[2]	4	6	0	4	3	0	2	14	1
VII	111	2–50	10	68	40	0[3]	55	25	11	8	12	0	57	54	0
Proximal limb	89	2–52	8	47	35	0[4]	48	22	5	5	5	4	50	39	0

B

Somite st injected	Number of clones containing different proportions of fast/slow myotubes										
	100/0% fast/slow	90/10% fast/slow	80/20% fast/slow	70/30% fast/slow	60/40% fast/slow	50/50% fast/slow	40/60% fast/slow	30/70% fast/slow	20/80% fast/slow	10/90% fast/slow	0/100% fast/slow
I	9	4	0	0	2	0	0	1	1	0	0
V	9	0	1	0	1	1	0	0	0	3	2
VII	72	6	4	6	1	4	1	3	0	0	14
Proximal limb	36	9	5	3	0	5	3	2	3	1	22

C

Clone type	Total number of clones	Range of clone size	Average clone size	Number of clones found in dorsal and/or ventral regions of the limb			Number of clones found in one or more proximal/distal regions of the limb					
				Dorsal only	Ventral only	Dorsal and ventral	Thigh only	Shank only	Foot only	Thigh and shank	Shank and foot	Thigh, shank, and foot
Muscle only	74	2–50	8	41	30	0[1]	37	15	6	6	10	0
Endothelial only	26	2–11	4	17	6	0[2]	16	2	0	2	6	0
Muscle and endothelial	36	2–42	15	19	14	1[3]	13	11	3	5	4	0

(A) Analysis of muscle clones found in different regions of the limb. Shown are the total number, size range, average size of muscle clones resulting from cells labeled in somite 31 at somite stage I, V, VII, or in the proximal limb; number of clones found in dorsal (D) and/or ventral (V) regions; number of clones found in one or more proximal/distal (P/D) regions; and number of clones found within one or more different anatomical muscles of the limb.

[1] In addition to the two clones with multiple D and V members, also not included: six primarily V clones with one or two D members and one primarily D clone with two V members.

[2] In addition to the one clone with multiple D and V members, also not included: one primarily V clone with one D member.

[3] No clones with multiple D and V members, but also not included: one primarily D clone with one V member, one primarily V clone with one D member, and one small clone with one D and one V member.

[4] No clones with multiple D and V members, but also not included: three primarily V clones with one or two D members, two primarily D clones with one V member, and two small clones with one or two D and one or two V members.

(B) Analysis of slow and fast fiber type of muscle clones in the limb. Shown are the number of muscle clones containing different proportions of fast/slow myotubes resulting from cells labeled in somite 31 at somite stage I, V, VII, or in the proximal limb.

(C) Analysis of myogenic and endothelial-producing clones in the limb. Shown are the total number, size range, average size, and D/V and P/D distribution of muscle only, endothelial only, and muscle and endothelial clones resulting from cells labeled in somite 31 at somite stage VII.

[1] No clones with multiple dorsal (D) and ventral (V) members, but also not included: one primarily D clone with one V member, one primarily V clone with one D member, and one small clone with one D and one V member.

[2] No clones with multiple D and V members, but also not included: one primarily V clone with one D member and two primarily D clones with one V member.

[3] One muscle/endothelial clone with multiple D and V endothelial members, but also not included: two primarily D muscle/endothelial clones with one V endothelial member.

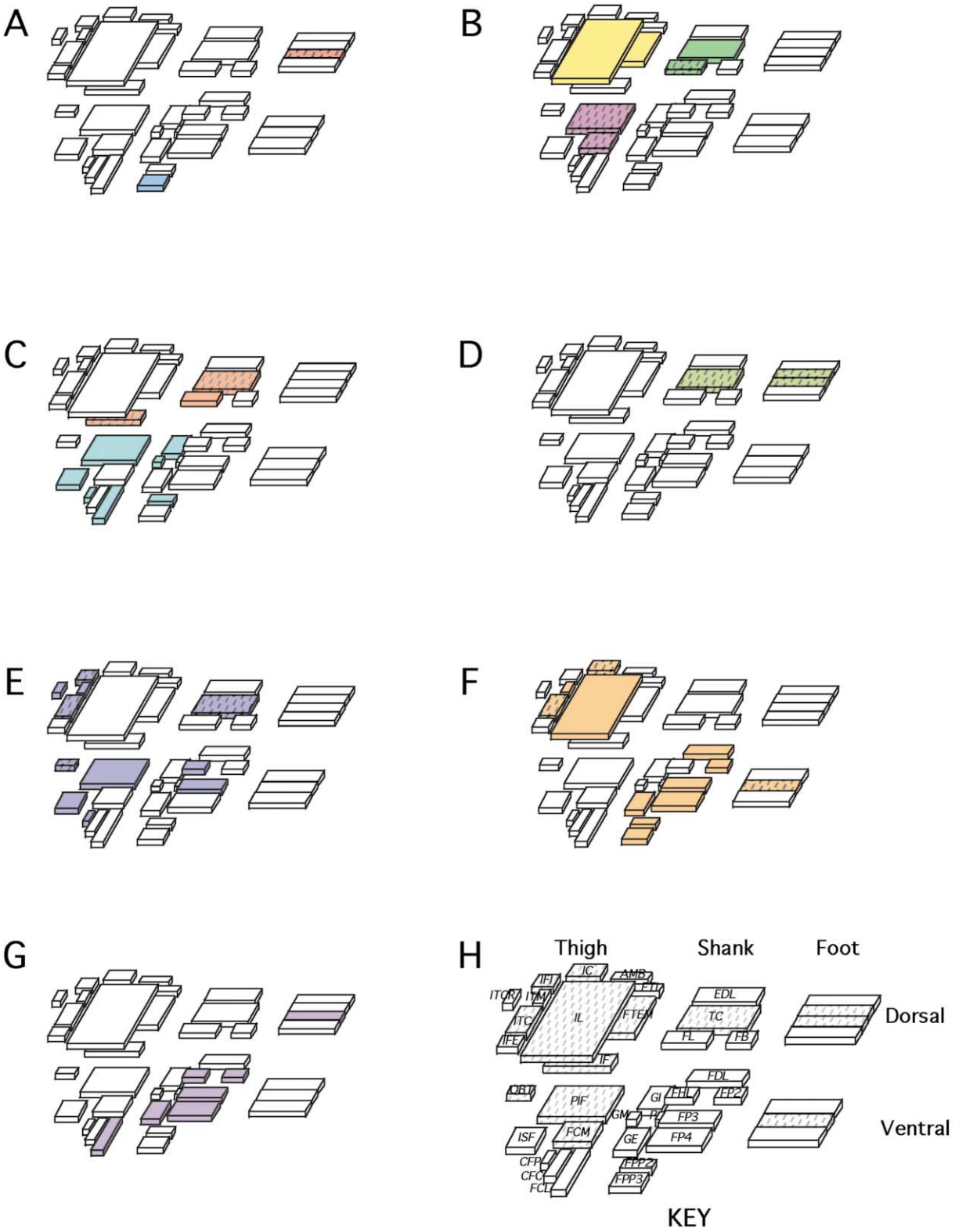

Figure 1. Schematic Presentation of Representative Muscle Clones Seen in Lineage Analysis

Clonally related cells labeled in somite 31 at somite st I, V, VII, or in the proximal limb are found in the dorsal and ventral regions of the limb; in the thigh, shank, and foot; and within multiple muscles of slow or fast fiber type. Shown is the distribution of clonally related cells of representative clones. Each box represents one anatomical muscle. Boxes shaded in the same color show muscles that contain clonally related cells. Boxes colored and stippled show muscles with clonally related cells that contain some slow myotubes. (H) shows a key to the muscles. Abbreviations are explained in the Supplemental Data. Stippled boxes show muscles that normally contain at least some slow myotubes.

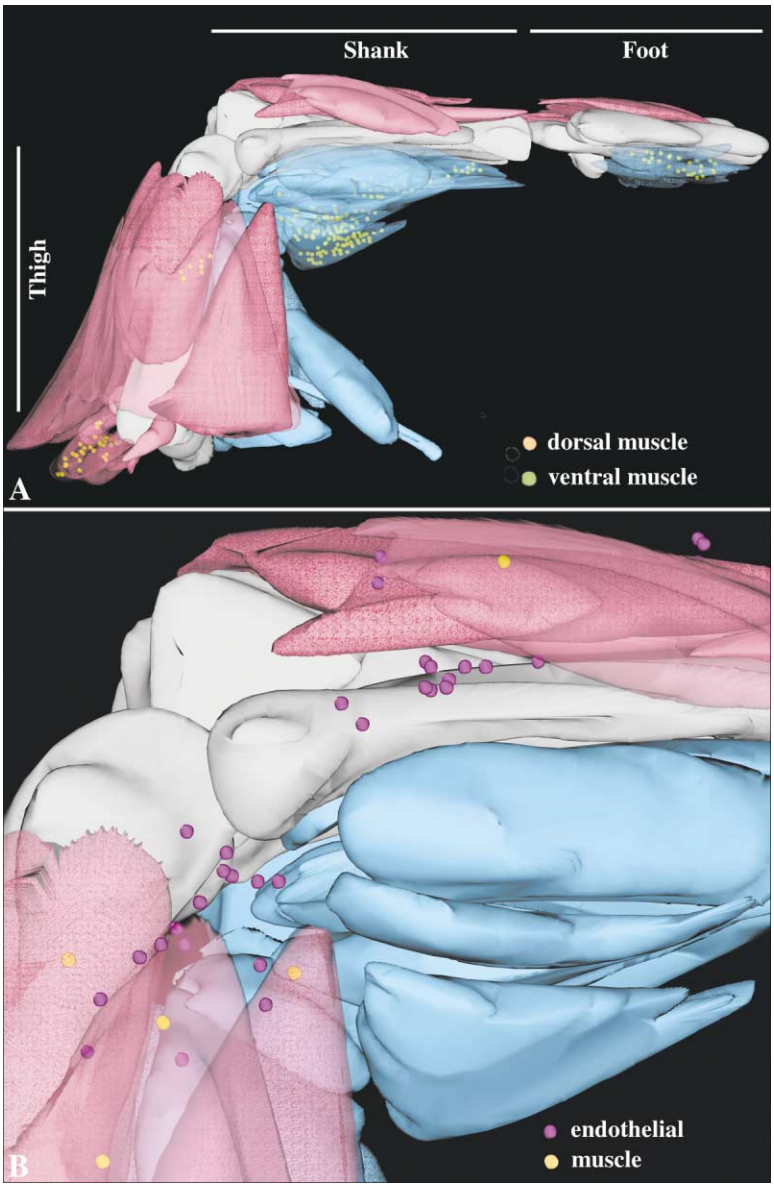

Figure 2. Distribution of Representative Clones Labeled in Somite 31 at Somite St I and St VII

(A) Three-dimensional reconstruction of a st 32 hind limb in which clonally related cells labeled in somite 31 at somite st I are broadly distributed in the dorsal thigh and ventral shank and foot within multiple slow and fast muscles. Balls represent single, clonally related cells. Balls with a yellow-orange tint represent myotubes in dorsal muscles, while balls with a yellow-green tint represent myotubes in ventral muscles. Most balls are found in unstippled muscles and so represent fast myotubes. Some of the balls found within stippled muscles (such as ventral foot muscle) represent slow myotubes.

(B) Reconstruction of a st 32 hind limb knee region in which clonally related cells labeled in somite 31 at somite st VII differentiate within close proximity into muscle (represented as yellow balls) and endothelial cells (represented as purple balls). Purple and yellow balls are all clonally related. Yellow balls are either fast myotubes (found in muscle that is unstippled) or slow myotubes (found in muscle that is stippled). Purple balls shown extrinsic to dorsal shank muscles are endothelial cells lying in the vessels in between the muscles and the ectoderm.

For both reconstructions, dorsal muscles are shown in pink, and ventral muscles in blue. Muscles that are stippled contain at least some slow myotubes. Bones are shown in gray.

nor in the proximal limb are myogenic cells restricted, and so committed, to forming muscles in one particular P/D region of the limb.

Myogenic cells labeled at somite st I, V, VII, or in the proximal limb were also found in multiple individual anatomical muscles (Table 1A). Of the 234 total muscle clones, 109 were limited to one individual muscle (Figure 1A), while 125 clones were found in at least two different muscles (Figures 1B–1G and 2A). There was a general trend for the smaller clones resulting from labeling cells

(A) Two clones labeled at somite st VII, which contain cells in just one muscle. The red clone contains only slow myotubes in the dorsal foot muscle, and the blue clone contains fast myotubes only within the ventral shank FPP3.

(B) Three clones with cells in only one P/D segment in either the dorsal or ventral limb. The yellow clone labeled at somite st I contains only fast myotubes in the dorsal thigh. The green clone labeled at somite st V contains both slow and fast myotubes in only the dorsal shank. The purple clone labeled in the proximal limb contains only slow myotubes in the ventral thigh.

(C and D) Three clones in two P/D segments in either the dorsal or ventral limb. The orange clone (C) labeled at somite st V contains both slow and fast myotubes in the dorsal thigh and shank. The blue clone (C) labeled at somite st I contains fast myotubes in the ventral thigh and shank. The green clone (D) labeled at somite st V contains only slow myotubes in the dorsal shank and foot.

(E–G) Three clones labeled at somite st I, which contain myotubes in at least two P/D segments in both the dorsal and ventral limb. The blue clone (E) contains slow and fast myotubes in the dorsal and ventral thigh and shank. The orange clone (F) contains slow and fast myotubes in the dorsal thigh and ventral shank and foot. The purple clone (G) contains fast myotubes in the ventral thigh and shank and dorsal foot.

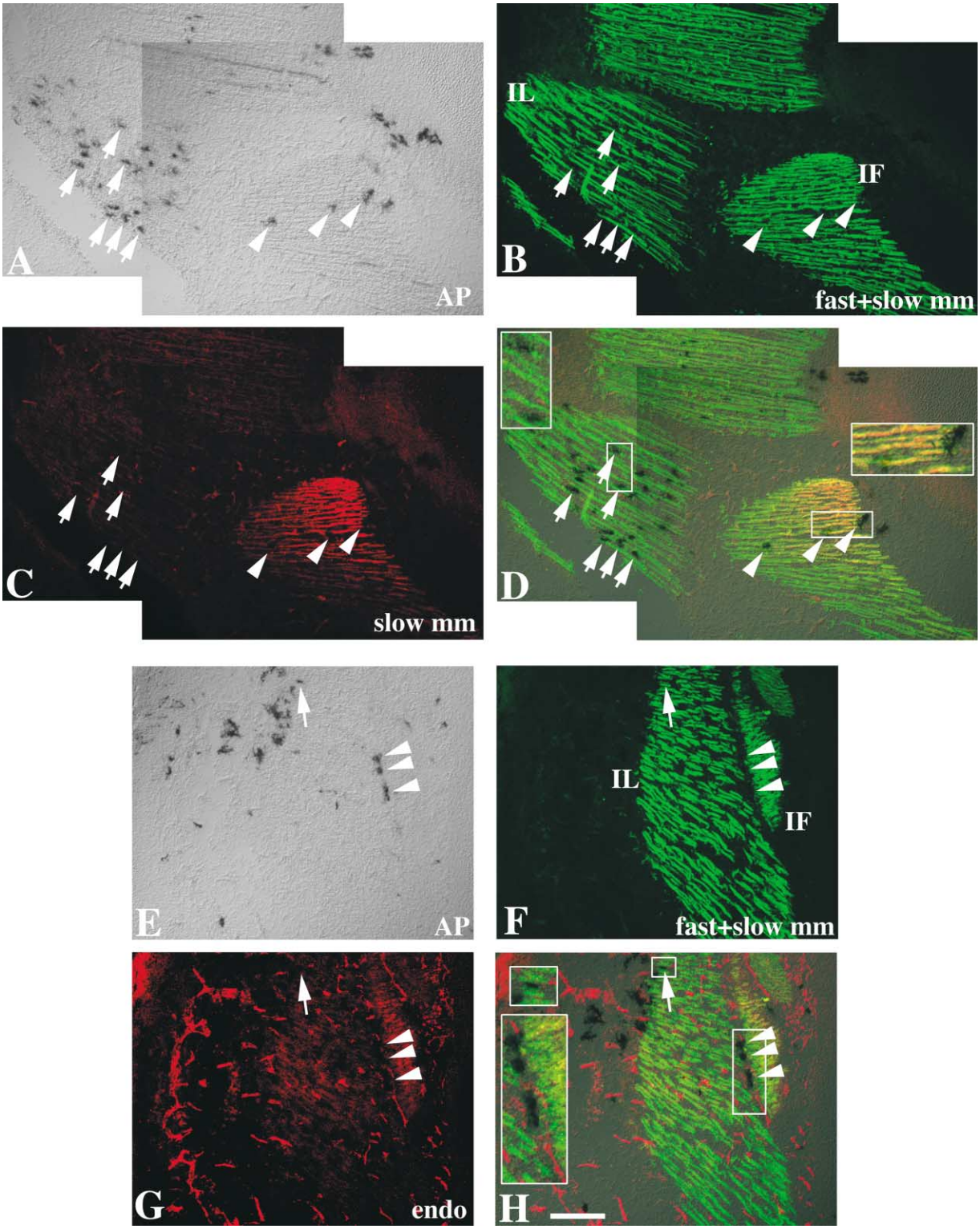

Figure 3. Clonally Related Cells Differentiate into Both Slow and Fast Myotubes and into Both Muscle and Endothelial Cells

(A–D) Clonally related cells differentiate into both slow and fast myotubes. A single section is shown, triply labeled, through the dorsal thigh of a st 32 limb with cells labeled adjacent to somite 31 in the proximal limb at st 20.

(A) DIC image showing cells labeled by CHAPOL and expressing AP.

(B) Green fluorescent image showing fast and slow myotubes labeled with MF20.

(C) Red fluorescent image showing only slow myotubes labeled with NA8.

(D) Merged image of DIC, green, and red fluorescence. Arrows and arrowheads show nine AP-positive cells picked and shown to contain the same 24 bp tag. Arrows show fast myotubes in the fast IL muscle and arrowheads show slow myotubes in the slow IF muscle, all of the same clone.

at somite st VII or in the proximal limb to be found in fewer muscles. However, at all stages, larger clones were found in many anatomical muscles, and smaller clones were generally found in only one muscle. Therefore, even after entering the proximal limb myogenic cells are not restricted, and so not committed, to forming individual anatomical muscles.

Myogenic Cells in the Somite or Proximal Limb Are Not Committed to Forming Slow or Fast Myotubes

Upon entering the limb, myogenic cells differentiate into fast myotubes (which contain fast MHCs) or slow myotubes (which contain fast MHCs as well as slow MHC3; Page et al., 1992). In the chick limb, fast and slow myotubes differentiate in a distinct and stereotyped pattern, with the majority of muscles containing only fast myotubes, some containing only slow myotubes, and a few muscles containing both fast and slow myotubes, but with the two types clearly regionalized within these muscles (see Supplemental Data).

Myogenic cells labeled at all somite stages and in the proximal limb can differentiate into both fast and slow myotubes (Table 1B). Some myogenic clones labeled at all stages contained only fast myotubes, and some myogenic clones labeled at somite st V and VII and in the proximal limb contained only slow myotubes. However, at all stages of labeling, 23%– 47% of myogenic clones contained both fast and slow myotubes (Table 1B; Figures 3A–3D). Clones containing only slow myotubes are small (1–23 cells) and localized in only one or two muscles. The fast-only clones are generally larger (1–189 cells) than the slow-only clones, but localized in regions (e.g., ventral shank) where there are few slow muscles. The size and distribution of the slow-only and fast-only clones suggest that these clones may contain only one fiber type simply as a result of their small size or localization in the limb. Therefore, the large number of slow/fast clones, in combination with the small size and localization of slow-only or fast-only clones, suggests that individual myogenic cells in the somite and the proximal limb are not restricted, and so not committed, to a slow or fast fate.

Despite this lack of commitment of individual myogenic cells to a slow or fast fate, a lower percentage of slow myotubes are derived from myogenic cells labeled at somite st VII than from cells labeled at st I. While 17% of all 1100 myogenic cells labeled at somite st I were slow myotubes, only 7% of all 1072 myogenic cells labeled at somite st VII were slow. A χ^2 test of independence confirms that the proportion of slow myotubes is significantly different ($p < 0.01$) between cells labeled at somite st I versus st VII.

Dynamics and Pattern of Endothelial Cells Populating the Hind Limb

To analyze the lineage of limb endothelial cells derived from somites, we also examined in one embryo the endothelial descendents from cells in somite 31 labeled with CHAPOL at somite st VII. A total of 62 clones gave rise to endothelial cells, 26 of which gave rise to only endothelial cells and 36 which gave rise to muscle and endothelial cells (see below; Table 1C). The endothelial-only clones ranged in size from 2 to 11 cells. Given that only 40% of the tags are recovered, we estimate that each stage VII somite cell can give rise to 27 endothelial cells. Assuming these cells divide at a constant rate, this suggests that endothelial cells divide approximately every 27 hr.

The D/V distribution of most endothelial cells within these endothelial or muscle/endothelial clones appears generally similar to the D/V distribution of muscle-only clones labeled at st VII. Of 62 endothelial or muscle/endothelial clones, all endothelial members of most (56) of these clones were found only on the dorsal or ventral side of the limb (Table 1C). In a small number of clones (five), most endothelial members were found on either the dorsal or ventral side, but a single member was found on the opposite side. Interestingly, we did find one muscle/endothelial clone that clearly contains multiple dorsal and ventral endothelial members in the foot. Although there are no studies tracking the paths of migration of endothelial cells, these data suggest that endothelial cells (unlike muscle cells) can cross between the dorsal and ventral sides of the foot.

The P/D distribution of endothelial cells within endothelial or muscle/endothelial clones also appears similar to that of the muscle-only clones labeled at st VII. Of 62 endothelial or muscle/endothelial clones, endothelial members of 45 clones were found in only one P/D region (Table 1C). However, members of 17 clones (which on average were larger than clones in one P/D region) were found in two P/D regions (thigh and shank or shank and foot). Thus, like myogenic cells, endothelial cells are not restricted and so are not committed to forming endothelium in one particular P/D region of the limb.

Cells Labeled at Somite St VII Are Not Committed to Forming Muscle or Endothelial Cells

Surprisingly, we found a large number of clones labeled at somite st VII that contained both muscle and endothelial cells (Table 1C; Figures 3E–3H). A total of 136 clones

(E–H) Clonally related cells differentiate into both muscle and endothelial cells. A single section is shown, triply labeled, through the dorsal thigh of a st 32 limb with cells in somite 31 labeled at somite st VII.

(E) DIC image showing cells labeled by CHAPOL and expressing AP.

(F) Green fluorescent image showing fast and slow myotubes labeled with MF20.

(G) Red fluorescent image showing endothelial cells labeled with DiI acetylated LDL.

(H) Merged image of DIC, green, and red fluorescence. Arrow and arrowheads show four AP-positive cells picked and shown to contain the same 24 bp tag. Arrow shows fast myotube in IL muscle and arrowheads show endothelial cells in the blood vessel between the IL and IF muscles, all members of the same clone. Note that in (G), the AP staining locally quenches the DiI acetylated LDL signal, but the DiI acetylated LDL signal is clear in endothelial cells just anterior and posterior to the AP-positive endothelial cells. The scale bar in (H) equals 200 μm and applies to (A–H). Insets in (D) and (H) are 2× magnifications.

were recovered in the embryo analyzed for endothelial cells (Table 1C); 74 of these included only muscles cells and 26 only endothelial cells. Thirty-six clones (26% of the total clones recovered) contained both muscle and endothelial cells. The ratio of slow/fast myotubes within these muscle/endothelial clones is similar to the ratio of slow/fast myotubes within muscle-only clones labeled at somite st VII. Twenty-three of the muscle/endothelial clones contained only fast myotubes, eight contained only slow myotubes, and five contained both fast and slow myotubes. The latter five clones indicate that single cells labeled at the lateral edge of st VII somites are capable of giving rise to slow and fast myotubes and endothelial cells.

The average clone size of muscle-only, endothelial-only, and muscle/endothelial clones differs significantly (Table 1C). On average, endothelial-only clones are the smallest (average size of four), muscle-only clones the next largest (average size of eight), and muscle/endothelial clones the largest (average size of 15). An ANOVA comparison of clone sizes confirms that the sizes are significantly different ($p < 0.001$). These differences in clone size indicate that there is heterogeneity in rates of proliferation among somite st VII cells populating the limb. Larger clones may simply have a higher probability of differentiating into both muscle and endothelial cells. Therefore, it is likely that all somite st VII cells are not restricted, and so not committed, to a myogenic (whether fast or slow) or endothelial cell fate.

A comparison of the distribution of clonally related muscle and endothelial cells reveals that clonally related cells are generally found in the same D/V and P/D regions, and often in close proximity, sometimes within a few cell diameters of each other (Figures 2B and 3E–3H). This close proximity of related cells that acquire different cell fates suggests that local extrinsic cues within the limb environment are determining the muscle versus endothelial fate of the somitic cells.

Discussion

Myogenic Cells Are Patterned with Respect to Their Anatomical Fate by Extrinsic Cues within the Limb Environment

To delimit when and where individual myogenic cells acquire patterning information, we followed the anatomical fate in the hind limb of cells labeled at different somite stages and in the proximal limb. Within the somites or the proximal limb, myogenic cells are not restricted to forming muscle in one P/D region of the limb or restricted to forming individual muscles. At somite stages I and V, myogenic cells are also unrestricted to a dorsal or ventral fate. By somite stages VII and in the proximal limb, myogenic cells are largely restricted to a dorsal or ventral fate. Although this could reflect the commitment of somite st VII myogenic cells to a dorsal or ventral fate, it more likely is simply a consequence of the cells' stereotyped path of migration and rate of proliferation. Thus, individual myogenic cells within the somites and in the proximal limb are uncommitted with respect to their anatomical fate. Individual myogenic cells must acquire patterning information and commit to a particular anatomical fate as they migrate from the proximal limb but before they differentiate into myotubes, when muscle patterning is complete (Figure 4; Kardon, 1998). The source of this patterning information is likely to be signals from the surrounding limb mesoderm (Jacob and Christ, 1980). However, neither the specific limb mesodermal tissue source nor the molecular nature of these signals is known.

Specification of Slow and Fast Myotubes Involves Extrinsic and Intrinsic Mechanisms

We found that commitment to a slow/fast fate occurs after myogenic precursors enter the limb. We found that a large number of myogenic cells labeled at all somite stages and in the proximal limb gave rise to both slow and fast myotubes and thus are uncommitted to a slow/fast fate. We also found myogenic clones labeled at most somite stages and in the proximal limb which contained either only fast or only slow myotubes. It could be argued that these clones reveal intrinsic heterogeneity within early myogenic precursors such that these precursors are committed to a slow or fast fate. However, the size and distribution of the slow-only and fast-only clones suggest that these clones may be restricted to only one fiber type, simply as a result of their small size and/or localization in the limb. Thus, it is most likely that none of the myogenic precursors in the somites and proximal limb are committed to a slow or fast fate (Figure 4). This conclusion contrasts with the conclusion by Nikovits and colleagues (2001) that myogenic precursors are committed to a fast or slow fiber type within the somite. Their conclusion was based on quail/chick chimera studies in which quail somites transplanted into a chick host gave rise to limb musculature with a quail-specific profile of slow and fast myotubes. In light of our current results, we alternately suggest that species differences in the timing of migration of myogenic precursors from the somites (see below) or differences in their responsiveness to extrinsic influences in the limb environment may account for their results.

Previous in vitro clonal analysis (Miller and Stockdale, 1986) and in vivo transplantation studies (DiMario et al., 1993) have determined that embryonic myoblasts are committed to a slow or fast fate by st 24–28 within the limb. Therefore, our lineage analysis, in combination with these studies, determines that myogenic cells in the chick become committed to a slow or fast fate within the limb bud between approximately st 20–24.

The commitment of myogenic precursors to a slow or fast fate within the early limb bud strongly suggests that local, extrinsic cues within the limb bud environment determine the slow/fast fate of these cells. Surgical manipulations of avian somites and lateral plate previously indicated that the lateral plate ultimately defines the pattern of slow and fast myotubes within the limb and body wall (Butler et al., 1988; Lance-Jones and Van Swearingen, 1995; Nikovits et al., 2001), and changes in the lateral plate can predictably respecify the pattern (Robson et al., 1994; Duprez et al., 1999). Therefore, signals from the limb bud environment can both determine the initial locations where myogenic cells will differentiate into either slow or fast myotubes and also respecify myogenic cells which are potentially committed

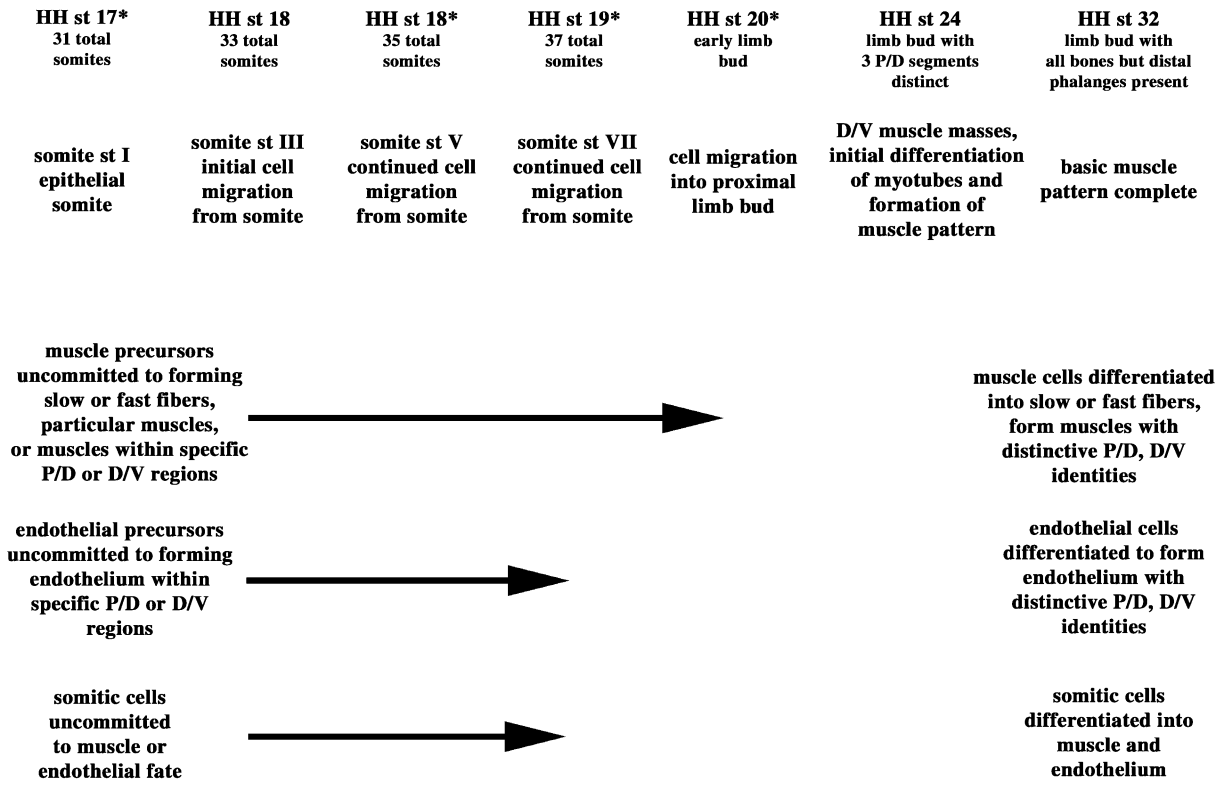

Figure 4. Summary of Cell Fate and Patterning of Hind Limb Muscle and Endothelial Cells Derived from Somite 31

Asterisks indicate stages at which cells were labeled with the CHAPOL virus.

to a particular slow/fast fate. In agreement with these data on the importance of the lateral plate for determining the overall pattern of slow and fast muscles, we find that local, extrinsic factors determine the slow/fast fate of individual myogenic cells.

Nevertheless, we did discover that overall, significantly fewer slow myotubes are derived from myogenic cells labeled at somite stage VII than from cells labeled at somite stage I. This reduction in the number of slow myotubes could result from changes in the extrinsic limb environment into which the myogenic cells migrate and differentiate or reflect an intrinsic bias in the ability of myogenic cells from early and late somites to form slow myotubes. We favor this latter alternative because it agrees with the results of Lance-Jones and Van Swearingen (1995). Using coelomic grafts of chick limb buds or isochronic transplantations of limb buds between quail and chick, they also found that myogenic cells emigrating early from the somites differentiate predominantly into slow myotubes, while later migrants differentiate predominantly into fast myotubes. Moreover, using heterochronic transplantations of limb buds, they explicitly tested and demonstrated that differences in slow/fast fate generally result from intrinsic properties of early versus late migrants and not from changes in the extrinsic influences in the limb environment. Therefore, it is likely that the reduction in the number of slow myotubes derived from somite stage VII versus I somites we see reflects intrinsic differences in the myogenic cells within the somites. Thus, our data support a model in which some component of the slow/fast pattern is also determined by mechanisms intrinsic to myogenic cells within the somites.

Endothelial Precursors Are Patterned with Respect to Their Dorsal/Ventral and Proximal/Distal Fate by Extrinsic Cues within the Limb Environment

We analyzed when and where endothelial precursors acquire patterning information by following the D/V and P/D fate in the hind limb of cells labeled in stage VII somites. As with myogenic cells labeled at this somite stage, endothelial precursors were not restricted and therefore were not committed to forming endothelium in one particular P/D region. The D/V distribution of endothelial cells in general was similar to the D/V distribution of muscle cells labeled at somite st VII; most clones were restricted to just the dorsal or ventral side of the limb. However, unlike the myogenic clones, one clone contained multiple dorsal and ventral endothelial members. This indicates that endothelial precursors are not committed to a dorsal or ventral fate at somite st VII (Figure 4). The lack of commitment of endothelial precursors within somite stage VII somites to a D/V or P/D fate shows that, like myogenic cells, these cells are patterned later within the limb bud. Whether the same signals patterning myogenic cells are involved in patterning endothelial precursors remains an open question.

Muscle and Endothelial Cells Are Derived from a Common Somitic Precursor and Extrinsic Signals in the Limb Environment Determine Muscle versus Endothelial Cell Fate

Fate maps based on transplantation of whole somites between quail and chick embryos have established that somites, in particular the lateral dermomyotome, give rise to both limb muscle and endothelial cells (Chevallier et al., 1977; Christ et al., 1977; Ordahl and Le Douarin, 1992; Eichmann et al., 1993; Wilting et al., 1995; Pardanaud et al., 1996). Based on the expression of characteristic transcription factors such as *Pax3* (see below), it has been assumed that individual cells are specified to a muscle or endothelial cell fate within the somite.

Our in vivo lineage analysis has unexpectedly revealed that myogenic and endothelial cells are derived from a common precursor population within the somites. We found that 26% of lateral dermomyotomal cells labeled at somite st VII give rise to both muscle and endothelial cells and suggest that all cells emigrating from the somites are bipotential, and not committed to either a myogenic or endothelial cell fate (Figure 4). We did find that a large number of clones contained only muscle or endothelial cells, which in principle could reflect true heterogeneity among cells emigrating from the somite. However, muscle/endothelial clones were significantly larger than muscle-only and endothelial-only clones. Thus, it is likely that differentiation of progenitors into both muscle and endothelial cells is simply a probabilistic event, depending on the microenvironment where the individual migrating cell ends up, with the chance of a single progenitor giving rise to both cell types increased with a larger number of overall progeny.

Studies of the potential commitment of myogenic precursors to a muscle fate when they reside within the somite have focused on the role of the transcription factor *Pax3* in myogenesis. The early expression of *Pax3* in muscle precursors (Goulding et al., 1994; Williams and Ordahl, 1994), its requirement for limb muscle formation (Franz et al., 1993), and its ability to activate *MyoD* and myogenesis (Heanue et al., 1999; Maroto et al., 1997; Ridgeway and Skerjanc, 2001) have suggested that its expression in the dermomyotome marks an early stage of commitment to the myogenic lineage (e.g., Maroto et al., 1997). In our experiments, we have followed the cell fate of individual lateral dermomyotomal cells from a population where the vast majority express *Pax3* as they leave the somite and migrate into the chick hind limb. Our data strongly suggest that *Pax3*-positive cells give rise to both muscle and endothelial cells in the limb. Thus, *Pax3* does not commit lateral dermomyotomal cells to a muscle cell fate.

Our data further suggest that it is local extrinsic cues within the limb environment that ultimately determine the cell fate of the bipotential, *Pax3*-positive lateral dermomyotomal cells. Clonally related muscle and endothelial cells, labeled in the somite, generally differentiated in close proximity to one another within the limb. This indicates that local signals, which direct differentiation into either muscle or endothelial cells, can differ over short distances (on the order of a few cell diameters). Based on the timing of *MyoD* expression (Lin-Jones and Hauschka, 1996), these local cues must operate prior to st 23–24, when progenitors start becoming committed to myogenesis in the limb. The nature of the positive or negative signals in the limb committing progenitors to adopt a myogenic versus an endothelial cell fate is currently unknown. Also unknown is whether these signals determining muscle/endothelial cell fate are the same as those specifying the pattern of muscles and endothelium in the limb.

Implications for Muscle Stem Cell Biology

Skeletal muscle contains two interrelated muscle subpopulations that exhibit stem cell characteristics (reviewed in Bailey et al., 2001; Deasy et al., 2001; Seale et al., 2001; Seale and Rudnicki, 2000; Zammit and Beauchamp, 2001). One population, muscle satellite cells, are mononucleated cells, lying beneath the basal lamina of skeletal muscle, which are normally mitotically quiescent but in response to stress are activated, undergo division, and form new myofibers (reviewed in Bischoff, 1994). Recently, another population, known as muscle-derived stem cells (MDSCs), has also been isolated by fluorescent-activated cell sorting on the basis of Hoechst dye exclusion and shown to participate in muscle regeneration (Gussoni et al., 1999; Jackson et al., 1999). The exact relationship between satellite cells and MDSCs is unclear, but it has been hypothesized that satellite cells are derived from the MDSCs (Seale et al., 2000). Surprisingly, there appears to be much plasticity between myogenic stem cells and hematopoietic derived stem cells and endothelial cells. MDSCs can both contribute to regenerating myofibers and efficiently reconstitute the hematopoietic system (Gussoni et al., 1999; Jackson et al., 1999). Conversely, hematopoietic stem cells derived from the bone marrow can participate in muscle regeneration (Bittner et al., 1999; Ferrari et al., 1998). In addition, two reports suggest that the satellite cell and endothelial lineages are interrelated (De Angelis et al., 1999; Minasi et al., 2002). Cells cultured from the embryonic dorsal aorta express both endothelial and muscle markers, and satellite cells express this same set of markers. Moreover, these cultured aorta cells can give rise to satellite cells which, when implanted into regenerating muscle, contribute to postnatal muscle growth and regeneration. Also, embryonic dorsal aorta cells directly implanted into a developing limb bud contribute both to muscle fibers and the endothelial cells in the vessels surrounding the fibers. This plasticity between myogenic stem cells and hematopoietic and endothelial cells has prompted interest in the developmental origin of MDSCs and satellite cells. Early studies suggested that satellite cells, like other myogenic lineages, are derived from the somites (Armand et al., 1983). However, De Angelis and colleagues (1999; Cossu and Mavilio, 2000) have suggested that some satellite cells may be derived from the dorsal aorta or more generally from the embryonic vasculature.

Our in vivo lineage analysis of somitic cells gives some further insights into the relationship between muscle and endothelial cells. First, in light of our lineage analysis and experiments of Pardanaud and colleagues (1996), the finding that cells cultured from the dorsal aorta give rise to muscle and endothelium is not surprising. Quail/chick chimera experiments have shown that the somites give rise not only to the endothelial cells of the limb and

body wall, but also the dorsal aorta (Pardanaud et al., 1996). Since we have found that somitic-derived cells in the limb are bipotential, it is not unreasonable that the somitic cells within the dorsal aorta may also maintain their bipotentiality to form muscle and endothelium. Cossu and colleagues (Cossu and Mavilio, 2000; De Angelis et al., 1999) have further suggested that satellite cells may not only be derived from the dorsal aorta, but more generally from the embryonic vasculature. We would predict that the bipotentiality to form muscle and endothelium is restricted to endothelial cells derived embryonically from the somites. For instance, cells in the ventral aorta, which are derived from the splanchnic mesoderm (Pardanaud et al., 1996), may not possess this bipotentiality. Finally, local cues appear to determine whether transplanted dorsal aorta cells differentiate into muscle or endothelium, as differentiated cells of both types are found in close proximity to one another (Minasi et al., 2002). This is reminiscent of our finding that local limb mesodermal signals determine muscle versus endothelial cell fate. Whether the signals are the same in both instances has yet to be determined.

An important, outstanding problem in muscle stem cell research is the origin of muscle satellite cells. Two different embryonic sources have been suggested. Muscle satellite cells may arise from the somites, which give rise to all other (embryonic and fetal) myogenic lineages (Armand et al., 1983). Alternatively, satellite cells may be derived from the embryonic vasculature (DeAngelis et al., 1999; Cossu and Mavilio, 2000). We have found that a common somitic precursor gives rise to both muscle and endothelial cells. It will be interesting to explicitly examine whether some of the muscle cells clonally related to endothelial cells are in fact muscle satellite cells. In our present analysis, we cannot distinguish whether the oligonucleotide tags of muscle cells picked and analyzed are derived from myonuclei within fused myotubes or nuclei of satellite cells lying adjacent under the myofiber basal lamina. In the future, this issue will be resolved by analyzing older embryos in which satellite cells can be more easily distinguished (with markers such as the antibody to Pax7; Seale et al., 2000).

Experimental Procedures

Lineage Analysis Using the Retroviral Library CHAPOL

The CHAPOL retroviral library has been described (Cepko et al., 1998; Golden et al., 1995). Concentrated CHAPOL viral stocks were injected into chick embryos (SPAFAS) at st 17–20 into the ventrolateral edge of somite 31 at somite stages I, V, and VII (Christ and Ordahl, 1995) and into the proximal limb just lateral to somite 31. Eggs were sealed and incubated until st 32–33. To visualize endothelial cells, 10 μl of dil acetylated LDL (200 μg/ml; Biomedical Technologies; Yablonka-Reuveni, 1989) was injected into the jugular vein of st 32–33 embryos, which were incubated at 37°C for an additional 2 hr before harvesting. Harvested hind limbs were fixed for 6–8 hr at 4°C with 4% paraformaldehyde, washed in PBS, and histochemically stained for AP in whole mount to detect retrovirally labeled cells. Limbs with labeled cells were embedded in OCT and sectioned at 20–30 μm.

Limb sections were restained for AP to enhance detection of individual retrovirally labeled cells and labeled with antibodies to detect slow and fast myotubes (see below). Sections containing AP-positive cells were photographed. Individual or small clusters of AP-positive cells were picked from the sections, their location annotated on the photographs, and placed into individual wells with proteinase K solution (Golden et al., 1995) in a 96-well plate. The cells were digested and the unique 24 bp insert was amplified as previously described (Golden et al., 1995). An aliquot of DNA was run on a 2% agarose gel to determine whether the inserts had amplified, and amplified DNA was purified with the QIAquick 96-well PCR purification kit. Sequencing reactions were performed with the Applied BioSystems Big Dye termination reaction kit, purified with the 96-well Edge BioSystems gel filtration kit, and analyzed on an Applied BioSystems 3700 DNA analyzer. The unique 24 bp insert in each viral genome that was amplified and sequenced was used to establish the clonal identity of each cell picked.

During cell picking, several wells were left containing just buffers and primers to check for possible contamination of stock solutions with plasmid DNA. Also, tissue pieces without AP-positive cells were picked and analyzed for potential non-AP-expressing viral genomes (Golden and Cepko, 1996). No such "silent" virally infected cells were found.

Immunohistochemistry

CHAPOL-injected embryos harvested at st 32–33 were labeled with the monoclonal antibodies MF20 (Developmental Studies Hybridoma Bank), which recognizes myosin heavy chain (MHC) and labels all primary myotubes (Bader et al., 1982), and NA8 (gift of E. Bandman), which recognizes just slow myosin heavy chains 2 and 3 and labels only slow primary myotubes (Bourke et al., 1995; Page et al., 1992). After AP staining, sections were labeled sequentially: NA8 (1:200), overnight at 4°C, followed by PBS washes; Cy3 goat anti-mouse IgG (1:400; Jackson ImmunoResearch), 2 hr at room temperature, PBS washes; MF20 (1:20), overnight at 4°C, PBS washes; Cy2 goat anti-mouse IgG (1:400; Jackson ImmunoResearch), 2 hr at room temperature, PBS washes.

Identification of Muscle and Endothelium

Individual muscle cells were identified by their expression of MF20 and NA8 antibodies. Where the AP histochemical reaction locally quenched the fluorescently labeled antibodies, individual cells were identified by their position within muscles. Each muscle was identified on sections by its characteristic position, shape, size, myotube orientation, and fiber type, and points of origin and insertion. In this study, the seven individual foot muscles were not distinguished. The distribution and pattern of slow myotubes was found to be highly stereotyped for all limbs examined. Descriptions of the slow/fast composition of hind limb muscles can be found in the Supplemental Data.

Endothelial cells were identified based on their uptake of dil acetylated LDL. Because the AP histochemical reaction routinely quenched the local fluorescence of the dil, we also identified AP-labeled endothelial cells by their morphological position within vessels (in which dil is not quenched adjacent to the AP-labeled cells).

Details of how the three-dimensional reconstructions of hind limb muscles and clones were constructed and rendered can be found in the Supplemental Data.

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