Notch and bone morphogenetic protein differentially act on dermomyotome cells to generate endothelium, smooth, and striated muscle

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We address the mechanisms underlying generation of skeletal muscle, smooth muscle, and endothelium from epithelial progenitors in the dermomyotome. Lineage analysis shows that of all epithelial domains, the lateral region is the most prolific producer of smooth muscle and endothelium. Importantly, individual labeled lateral somitic cells give rise to only endothelial or mural cells (not both), and endothelial and mural cell differentiation is driven by distinct signaling systems. Notch activity is necessary for smooth muscle production while inhibiting striated muscle differentiation, yet it does not affect initial development of endothelial cells. On the other hand, bone morphogenetic protein signaling is required for endothelial cell differentiation and/or migration but inhibits striated muscle differentiation and fails to impact smooth muscle cell production. Hence, although different mechanisms are responsible for smooth muscle and endothelium generation, the choice to become smooth versus striated muscle depends on a single signaling system. Altogether, these findings underscore the spatial and temporal complexity of lineage diversification in an apparently homogeneous epithelium.

Introduction

The somites are epithelial structures arising in a metameric pattern from the paraxial mesoderm. In the course of development, somites undergo successive phases of deep epithelialization concomitant with the acquisition of diverse cell fates. Initially, the ventral somite dissociates to generate the sclerotome, which forms the vertebral, ribs, and tendons. The remaining dorsal part, the dermomyotome (DM), contributes cells to the myotome, the precursor of skeletal muscles and, upon dissociation, also generates the dorsal dermis (Scaal and Christ, 2004).

Previously, we mapped the origin of muscle and dermis from the DM. The initial myotome is established by a population of early specified pioneer myoblasts resident in the medial epithelial somite (Kahane et al., 1998b, 2007). Subsequent myofibers form from all four lips of the DM (Kahane et al., 1998a, 2002; Cinnamon et al., 1999, 2006; Huang and Christ, 2000; Gros et al., 2004), and their proper patterning is determined by the initial scaffold of pioneer fibers (Kahane et al., 2007). In addition to the formation of unit-length myofibers, the DM produces progenitors that remain mitotically active within the myotome (Kahane et al., 2001; Ben-Yair and Kalcheim, 2005) and later develop into either fibers or muscle satellite cells (Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005). These are generated from the extreme lips of the DM (Kahane et al., 2001) and from the dissociating DM sheet that also produces dermis (Ben-Yair et al., 2003; Ben-Yair and Kalcheim, 2005). Notably, both mitotic myotomal precursors and dermis originate from single cells residing in the central DM sheet. The diversification of these two lineages is accompanied by a striking shift in the plane of epithelial cell division that becomes perpendicular to the mediolateral aspect of the DM. This shift is coupled to the asymmetrical segregation of N-cadherin to the apical daughter cells, which will become muscle, but not to the basal cells, which will give rise to dermis (Ben-Yair and Kalcheim, 2005; Cinnamon et al., 2006).

To better understand the mechanisms responsible for the segregation of the DM epithelium into its derivatives, we turned our attention in this study to the generation of two additional lineages, endothelial and mural cells (vascular smooth muscle and pericytes; Pardanaud et al., 1996; Scaal and Christ, 2004; Esner et al., 2006; Pouget et al., 2006; Wilting and Becker, 2006). The somite-derived endothelium is composed of at least two lineages, blood vessel (BV) cells characterized by the expression of resident Notch activity and bone morphogenetic protein.
of VEGF receptor (VEGFr) 2 (flk-1) and lymphatic endothelial cells defined by expression of VEGFr2 and 3 and Prox-1 (Wiling et al., 2001). VEGFr2 is expressed in the lateral epithelial somite, and this expression pattern, as well as the normal development of BVs, depends on bone morphogenetic protein (BMP) signaling from the intermediate and lateral plate mesoderm (Nimmagadda et al., 2004, 2005). In vitro studies demonstrated that VEGFr2-positive cells can give rise to both endothelial and smooth muscle (Yamashita et al., 2000; Ema et al., 2003). Moreover, VEGFr2-positive mouse cells injected into avian embryos generate both lineages in vivo (Yamashita et al., 2000). In contrast, work on transgenic mice carrying the lacZ reporter under the control of the flk-1 promoter demonstrated that in normal development, flk-1–positive cells contribute only to endothelium, hematopoietic cells, and a subset of skeletal muscle but not to mural cells (Motoike et al., 2003). Clonal analysis in avian embryos established that the lateral portion of hindlimb somites harbors progenitors common for both limb endothelial and striated muscle cells (Kardon et al., 2002). Furthermore, recent work demonstrated that mural, endothelial, and skeletal muscle cells share a common lineage (Esnier et al., 2006). It is, however, still unclear at which point in development these common lineages diverge and whether they segregate in response to similar or distinct factors.

The possibility that endothelial and mural cells originate from a single DM progenitor seems sensible, as both cell types are of somitic origin and contribute to the walls of the same vessels. Such a scenario would bear resemblance to the segregation of dermal and myotomal fates. Alternatively, these cells may arise from distinct progenitors in the DM. To answer this question, we fate mapped the flank DM epithelium using focal GFP expression. To determine if both endothelial and mural derivatives develop from a single DM progenitor, we performed lineage analysis of five discrete DM domains, the four extreme lips and the central sheet. Five groups of embryonic day (E)–2.5 (30–32 somite pairs) embryos were injected with GFP-DNA and embryos were further incubated for 40 h until E4. Successful injections gave rise to progeny composed of 1–34 GFP-expressing cells. Results of the lineage analysis are summarized in Fig. 1 A. Of the five domains analyzed, the lateral DM was the greatest source of endothelial and mural lineages (12 and 40%, respectively). The caudal and rostral DM lips contributed to a lesser extent, producing endothelial cells (6.6 and 2.6%, respectively) and mural cells (20 and 18.4%, respectively). The medial DM had a minor contribution to mural cells (5%) and, although the central DM sheet also generated mural derivatives (16.6%), neither the medial DM nor the central sheet produced endothelial cells under these experimental conditions (Fig. 1 A).

Typical locations of endothelial and smooth muscle phenotypes produced by the DM were the cardinal veins (CVs) and vitelline arteries and the mesonephric, dermal, and somatopleural vessels (Fig. 1, C–F; and not depicted). Specifically, lateral DM-derived BV cells were characterizedly located in the walls of mesonephric and great vessels, whereas the contribution of the rostral lips and DM sheet was limited almost exclusively to mural cells in dermal BVs. Interestingly, both endothelial and mural cells generated from lateral injections to single somites extended beyond the injected segment to colonize BVs as far as two and one segments away from the site of labeling, respectively; therefore,
in contrast to myotomal cells that display a segmentally restricted behavior, these cells visibly migrate along the rostrocaudal axis (unpublished data). It is also worth mentioning that both rostral and caudal DM lips, as well as the central DM sheet, were found to be the major contributors to myotome development (fibers and myoblasts altogether) when compared with the medial and lateral domains (Fig. 1A and G), which is consistent with previous findings (Kahane et al., 1998a, 2002; Ben-Yair and Kalcheim, 2005). Moreover, in addition to the known contribution of the central DM sheet to dermis (Ben-Yair et al., 2003), which was further substantiated here, the medial DM (Olivera-Martinez et al., 2002) and rostral lip also significantly contributed to dermis. The latter source was not recognized previously (Fig. 1A).

These results show for the first time a direct contribution of the DM epithelium to the smooth muscle lineage. In addition, they point to a differential contribution of DM domains to both BV lineages and to the colonization of specific vessels. For this reason, they raise basic questions regarding the mechanisms of...
cell diversification in the DM. To begin exploring these issues, we focused on the contribution of the most productive DM domain in terms of BV lineages, the lateral DM. First, we asked whether its contribution to BVs changes during development. To this end, the lateral portions of epithelial somites or DMs were focally labeled with GFP at E2 (25 somite pairs) and E3 (43–45 somite pairs). 40 h later, successful injections gave rise to 2–8 or 1–31 GFP-expressing cells per segment, respectively. Production of endothelial cells was maximal in the lateral epithelial somite (E2, 22.6%) and progressively diminished to 12% by E2.5 and to 6.6% by E3. The proportion of mural cells was highest at E2 (47.2%) and 2.5 (40%) when compared with that at E3 (26.6%). Notably, late lateral DM (E3) injections produced mostly myotomal derivatives (80%; Fig. 1 B). These findings were further supported by results of more extensive lateral labelings performed by electroporation at E2 or 2.5. The latter produced visibly higher proportions of myotomal cells (mostly fibers) than E2 electroporations, which gave rise to a mixed population of myotomal and BV cells (unpublished data). These results suggest an ordered time course of lineage segregation from the lateral portions of the somite and subsequent DM.

Early segregation of endothelial, smooth, and striated muscle lineages

To further examine the relationship between the endothelial, smooth, and striated lineages, discrete injections of GFP-encoding DNA (one to two cells transfected per injection) were delivered to the lateral domain of single epithelial somites per embryo. The resulting progeny, containing all labeled cells per embryo whether segmentally restricted or not, was analyzed 2 d later (labeled cells were observed in 26 out of 223 embryos with a mean of 12, 3, and 3.8 smooth muscle, endothelial, and myotomal cells per injection, respectively). In 20 out of 26 cases, labeled cells were in BVs. Notably, these BVs contained either GFP+ endothelial cells or smooth muscle cells but not both, suggesting they derived from distinct progenitors (P < 0.0001; Fig. 1 H). Mural and myotomal cells were, however, occasionally detected in the progeny of single injections (Fig. 1 H). In contrast, a 50% overlap between dermal and myotomal populations was observed in the progeny derived from the central DM sheet, which is consistent with previous clonal analysis (Ben-Yair and Kalcheim, 2005; Fig. 1 I). Collectively, these data strongly suggest that in the lateral domain, endothelial and mural lineages have begun to segregate from a common precursor by the time of somite formation.

Notch signaling plays an essential role in the choice between mural and myotomal fates but not in initial development of endothelial cells

Notch signaling in the DM. A major question arising from our lineage analysis studies is how cells in the lateral somite and DM give rise to three separate lineages (muscle, mural, and endothelial cells). We examined the possibility that Notch signaling governs cell fate decisions in the lateral DM. Several components of the Notch pathway were shown to be expressed in the DM. The receptor cNotch1 is expressed at low levels throughout the DM (Hirsinger et al., 2001). One of its ligands, cDelta-1, is expressed in the caudal lateral corner of the DM. Another ligand, cSerrate-2, is expressed by myotomal cells (Hirsinger et al., 2001; Holowacz et al., 2006).

To complete this picture, we performed in situ hybridizations for additional Notch signaling components. cNotch2 is expressed at high levels throughout the DM (Fig. 2 A). Members of the HES family of transcriptional repressors are well known as direct mediators of Notch signaling (Kageyama et al., 2007). We therefore performed in situ hybridizations with probes for five HES family members. One of the chicken HES-1 homologues, chairy2, is strongly expressed in the lateral half of the DM (Fig. 2 B), whereas another homologue, chairy1, is not expressed by DM cells but is expressed in the lateral sclerotome (not depicted). Three additional HES family members were detected mainly in the neural tube (HES-5, HES-5/7, and HES-6). HES-6 appeared in sporadic DML cells only at late stages of DM development (unpublished data). The expression of chairy2 indicates that Notch signaling is active in lateral DM cells. Along this line, overexpression of constitutively active Notch1 (N1–intracellular domain [ICD])/GFP or Notch2 (N2-ICD)/GFP, but not of control GFP, strongly up-regulated chairy2 mRNA (n = 16 and 5, respectively; Fig. 2, C and D; and not depicted). We therefore proceeded to explore the biological roles of Notch signaling in this domain.

Activation of Notch signaling biases lateral DM cells to a mural fate. Constitutively active Notch1 (N1-ICD) or Notch2 (N2-ICD) constructs were coelectroporated with a GFP-encoding plasmid into the lateral DM and embryos were incubated for an additional 40 h. Electroporation of either Notch construct increased the proportions of mural cells out of total GFP+ cells by threefold when compared with control GFP (n = 4 embryos out of 20 with a similar phenotype; Fig. 3, A–C and E–G). Many Notch-overexpressing cells were located in the walls of the great vessels, especially in the CV, causing a small but consistent increase in SMA/desmin staining in the vein on the treated compared with the untreated side or to control GFP-treated segments (Fig. 3, B, C, and E–G). Interestingly, many Notch-overexpressing cells that stained positive for SMA and desmin were apparent between the ventrolateral lip (VLL) of the DM and the CV. This feature was not detected under control conditions although, occasionally, a few control GFP-expressing cells lacking smooth muscle markers were visible at this location (Fig. 3, E–G, insets). We suggest that these are migrating cells en route to the CV and that Notch signaling strikingly increases their number while prematurely inducing expression of smooth muscle markers. Consistent with the latter notion, premature expression of SMA was already detected around BVs 16–20 h after N2-ICD transfection, which is before the onset of normal expression of this marker under control conditions (Fig. 4, A and B).

Reciprocally, two- and threefold reductions in the proportion of myotomal cells was monitored upon overexpression of N2-ICD and N1-ICD, respectively (Fig. 3, A–C). This effect was visible even in whole embryos observed under a fluorescent binocular. In addition, fewer cells were detected in the residual VLL epithelium. Notably, similar results were already evident 16 h after transfection (unpublished data). Hence, the most significant
of VEGFr2 mRNA, an early marker of angioblasts and subsequently of endothelial cells (Wilting et al., 1997), was neither up-regulated nor down-regulated by Notch misexpression (Fig. 4, C and D). Collectively, we suggest that Notch signaling primarily affects the balance between production of mural and myotomal cells but has no direct effect on the acquisition of an endothelial fate. Hence, the reductions observed upon overexpression of either of these factors might result from secondary effects on proliferation rates or survival of endothelial cells.

Different factors regulate the production of endothelial and mural cells from the lateral DM

Production of endothelial cells by the lateral somite and DM is correlated with the dynamic expression of VEGFr2. VEGFr2 was shown to be expressed in the lateral epithelial somite (Eichmann et al., 1993; Nimmagadda et al., 2004). We thus examined the dynamics of VEGFr2 expression in relation with results of our fate map analysis (Fig. 1 B). At E2, expression was detected in a broad lateral cluster of epithelial cells adjacent to the intermediate mesoderm (Fig. 5 A, arrow). At E2.5, expression in the DM was apparent in a few VLL cells residing in the extreme portion of the epithelium.
E2 embryos. 12 h after transfection, Noggin-expressing segments exhibited no change in \(hairy2\) levels, suggesting that BMP is not required for mural cell differentiation (\(n = 5\); Fig. 6, A and A'). In contrast, electroporation of noggin-DNA into the lateral mesoderm opposite the segmental plate of E1.5 embryos revealed, 12 h later, a marked reduction of \(VEGFr2\) expression already in the lateral epithelial somite where its expression is maximal (\(n = 8\); Fig. 5 and Fig. 6 B), confirming that BMP is required early during endothelial development.

To directly assess the effects of endogenous BMP on mural versus endothelial cell differentiation, noggin-DNA was electroporated, along with GFP, into the lateral region of flank epithelial somites and embryos were reincubated for 16 h to minimize possible long-term effects. Inhibition of BMP activity resulted in a twofold reduction of \(VEGFr2\) expression already in the lateral epithelial somite where its expression is maximal (\(n = 8\); Fig. 5 and Fig. 6 B), confirming that BMP is required early during endothelial development.

BMP signaling is required for differentiation and/or migration of endothelial, but not of mural, cells. BMP was shown to drive the differentiation of endothelial cells in vitro from embryonic stem cells (Park et al., 2004) and in vivo from the lateral somite (Nimmagadda et al., 2004, 2005). We asked whether it is also important for proper differentiation of the mural lineage. First, we examined whether inhibiting BMP signaling had any effect on expression of \(hairy2\) mRNA. Hairy2 is a transducer of Notch signaling whose mRNA level is stimulated by Notch overexpression (Fig. 2, C and D), an effect accompanied by enhanced mural cell development (Fig. 3). Noggin-DNA was electroporated into the lateral somite of E2 embryos. 12 h after transfection, Noggin-expressing segments exhibited no change in \(hairy2\) levels, suggesting that BMP is not required for mural cell differentiation (\(n = 5\); Fig. 6, A and A').
revealed, however, that in spite of not affecting the proportion of total QH1+/GFP+ endothelial cells, the latter remained preferentially within the DM epithelium and failed to migrate toward the nascent BVs (Fig. 6, D, E, and G). To get further insight into a possible function of BMP on cell migration and homing to BVs, we focused our analysis on the colonization of the CV (see Materials and methods), a vessel which receives a significant contribution of cells from the lateral somite and DM (Fig. 3). Accordingly, a marked decrease in CV diameter was observed in noggin-electroporated embryos when compared with GFP controls (n = 9; Fig. 6, F and G). We then asked whether the ratio of mural to endothelial cells in this vessel changes upon inhibition of BMP activity. Noggin stimulated by twofold the ratio of mural to endothelial cells in the wall of the CV (from 1.24 mural cells per endothelial cell in embryos that received control GFP to 2.46 mural cells per endothelial cell in noggin-electroporated embryos; n = 9 and 10, respectively; Fig. 6 H). Because noggin does not affect the proportion of mural cells or the expression of hairy2 but severely down-regulates VEGFr2 and the ability of endothelial progenitors to delaminate from the epithelium and/or to migrate, we conclude that the increased proportion of mural/endothelial cells is accounted for by a decrease in the contribution of the DM to endothelial cells. Collectively, these data suggest that lateral BMP is required for the proper differentiation and/or migration of endothelial, but not of mural, cells.

**Discussion**

The DM, although displaying an overall homogeneous pattern of growth (Ben-Yair et al., 2003), cannot be regarded as a uniform epithelium. Rather, it is composed of subdomains distinguished by gene expression patterns and generation of various cell fates.
with endothelial cells being an early segregated population. In addition, because the relative level of endothelial cell production drops first, in association with the disappearance of VEGF^+ cells from the lateral DM, we suggest that in addition to being spatially limited, the pool of endothelial precursors is finite and temporally restricted.

The second part of this study demonstrates that Notch signaling plays a physiological role in the generation of smooth versus striated muscle from the lateral DM and has little if any effect on initial specification of the endothelium. Reciprocally, BMP signaling, despite its effect on the differentiation and/or migration of somite-derived endothelial cells via regulation of VEGF^R2 expression, does not seem to affect smooth muscle development yet inhibits differentiation of myotomal myofibers (Pourquie et al., 1996). Thus, we suggest that initial development of mural and endothelial cells is governed by different mechanisms. In contrast, development of mural and striated muscle sublineages is a binary choice that depends upon a single signaling system (Fig. 7).

The DM origin of cells that contribute to the walls of BVs

The contribution of the lateral somite to endothelial and mural lineages has already been reported (Eichmann et al., 1993; Pardanaud et al., 1996; Kardon et al., 2002; Scaal and Christ, 2004; Esner et al., 2006; Wiegreffe et al., 2007); however, a comprehensive fate map of the DM contribution to these lineages has not been available until now. Although a DM origin of endothelial cells was established (Scaal and Christ, 2004), we report for the first time that the DM is also a direct source of smooth muscle. Moreover, we find that endothelial and mural cell production is not entirely restricted to the lateral DM. To a smaller extent, endothelial cells are also produced by rostral and caudal lips but not by the DM sheet or DML. Mural cells are generated from all DM domains but much more so from the lateral region. Notably, mural cells stemming from different DM domains contributed to distinct vessels in a stereotypic fashion.

When the composition of GFP-expressing cells is considered, a surprising aspect of endothelial and mural cell differentiation is revealed: most injections gave rise to only one type of derivative, even though injections were performed as early as the epithelial somite stage. This result raises the possibility that the progenitors at the origin of these two lineages are fate restricted already at the epithelial somite stage, being able to generate, e.g., smooth and striated muscle but not endothelium and smooth muscle. This does not exclude the option that bipotent progenitors may still exist at this stage; however, our data would argue that they are a minor subset. In contrast to the separation between mural and endothelial lineages, we found many cases containing both dermis and myotomal cells (Ben-Yair and Kalcheim, 2005) and also additional phenotypic combinations. Collectively, we sustain that the observed absence of cases with both endothelium and smooth muscle is of physiological significance. In spite of a putative fate restriction, these progenitors are not necessarily fully or irreversibly committed. In fact, our experimental data show that we can still affect lineage decisions between smooth and striated muscle and between striated muscle and endothelium.

Figure 5. Dynamics of VEGF^R2 expression in the lateral somite and DM.
(A) Strong expression is detected in progenitors residing in the lateral region of flank-level epithelial somites (arrow). (B) Few cells expressing VEGF^R2 are apparent in the lateral-most epithelium of E2.5 flank-level DMs (arrow). (C) By E3, the DM epithelium is VEGF^R2 negative. The lateral border of the somite–DM epithelium is marked by a red dashed line. DA, dorsal aorta; NT, neural tube; Scl, sclerotome. Bars: (A) 17 μm; (B and C) 35 μm.

(Pardanaud et al., 1996; Scaal and Christ, 2004; Kalcheim and Ben-Yair, 2005; Esner et al., 2006). This motivated us to investigate the mechanisms underlying the development of mural and endothelial cells that contribute to the walls of BVs in comparison to myotomal cells. The first part of this study entailed a comprehensive lineage analysis of DM subdomains, which established that in the somite and early DM, the lateral domain is the major contributor of both endothelial and mural cells. In addition to unraveling a direct derivation of smooth muscle cells from the DM, these experiments reveal a time-dependent generation of cell fates from the lateral domain and suggest that mural and endothelial cells arise from distinct somitic progenitors.
Nevertheless, endothelium and smooth muscle appear to be differentially affected by BMP and Notch, respectively, further validating the notion that their respective progenitors are already restricted in their developmental potential. Moreover, the transient expression of VEGF-R2 in the lateral-most somite and the rapid exhaustion of VEGF-R2+ endothelial progenitors from the lateral DM favor the view that, at least, the endothelial precursors are an early specified cell population.

Fate analysis of lateral somite cells, performed at hindlimb levels of the axis, proved that a significant proportion of single progenitors produce both endothelial and striated muscle cells (Kardon et al., 2002). Our lateral injections generated, instead, only a small proportion of both endothelial and muscle cells even if injections were not clonal, suggesting that in flank somites, the existence of a common progenitor for the two derivatives is not a common event. Possibly, fate segregation at hindlimb levels is a later process as lateral progenitors delaminate and migrate extensively into the limb before overt differentiation. Retroactive lineage analysis in transgenic mice using the lacZ reporter showed that endothelial, smooth muscle, and striated muscle cells share a common early progenitor present before somitogenesis (Esner et al., 2006). Our data, stemming from direct labeling of epithelial somites and DM tissue, suggest that the majority of progenitors are already fate restricted at the epithelial somite stage. Collectively, these results would argue that fate restriction begins sometime during the transition from the presomitic to the segmented paraxial mesoderm.

The generation of derivatives from the lateral DM follows a stereotypic temporal pattern with endothelial cells being produced mostly at early stages (somite and early DM). This correlates with the observed pattern of VEGF-R2 expression in the lateral somite and young DM. Nevertheless, mural cell production is significant also at later stages of DM development after VEGF-R2 expression in the DM had disappeared. We therefore reason that VEGF-R2 activity in somites mediates development of endothelial, but not of mural, cells, although we cannot rule out the possibility that some VEGF-R2-positive cells also contribute to the latter. Our results, stemming from lineage analysis as well as from responsiveness to different signals, are in agreement with results obtained in transgenic mice expressing LacZ under the control of the VEGF-R2 promoter in which lacZ-expressing cells contributed to endothelial but not to mural derivatives (Motoko et al., 2003). VEGF-R2-positive cells are capable, however, of also producing mural derivatives under several experimental conditions (Yamashita et al., 2000; Ema et al., 2003).

Notch signaling plays a role in cell fate determination in the lateral DM

We find that overexpression of either Notch1 or 2 strongly promotes the differentiation of mural cells from the DM, which is accompanied by a significant increase in eHairy2 mRNA and premature expression of SMA protein. Reciprocally, inhibition of endogenous Notch with Numb strongly biased cells to muscle fiber fate at the expense of other fates, particularly smooth muscle. This is consistent with a physiological role for Notch proteins in the binary choice between smooth versus striated muscle fates. Thus, Notch acts not only by inhibiting striated muscle differentiation (Hirsinger et al., 2001), it also positively stimulates differentiation of smooth muscle. Our data are consistent with recent results implicating Notch in differentiation of smooth muscle from embryonic stem cells in vitro (Doi et al., 2006) and from neural crest cells in vivo (High et al., 2007), thus emphasizing the...
to stimulate premature MyoD transcription and lateral myofiber differentiation. Likewise, inhibition of BMP activity was found leading to a reduction in cell number within generating a myofiber or remaining a mitotic muscle precursor within the sublineages. Of the three lineages, endothelial cells constitute a population of early segregated progenitors whose differentiation and/or migration depends on lateral mesoderm-derived BMP. In turn, we postulate the existence of a common intermediate progenitor for smooth and striated muscle sublineages from which separate fates are generated over a more extended period. The segregation of these fates is a binary choice that depends on Notch signaling. Elevated activity of Notch stimulates smooth muscle development, whereas lower levels or inhibition of Notch signaling drive generation of striated muscle. Under experimental conditions, abrogation of Notch function by overexpression of Numb results in myofiber differentiation in the myotome at the expense of mitotic muscle progenitors (satellite cell or myofiber progenitors). It is possible, however, that in the embryo, more subtle differences in levels of Notch activity modulate the balance between generating a myofiber or remaining a mitotic muscle precursor within the striated muscle sublineage. Likewise, inhibition of BMP activity was found to stimulate premature MyoD transcription and lateral myofiber differentiation (Kahane et al., 2007) leading to a reduction in cell number within myotomes (Fig. 6). Hence, the development of striated muscle precursors is negatively modulated by Notch as well as by BMP signaling cascades. Phenotypic segregation in the DM, therefore, exemplifies how two signaling cascades produce three distinct lineages by combining agonistic and antagonistic interactions.

Notch signaling has been shown to regulate production of alternative cell fates by lateral inhibition (Chitnis et al., 1995; Bray, 2006). In other situations, dividing cells segregate Numb protein asymmetrically, thereby inhibiting Notch signaling in the daughter cells that inherited Numb, a process that results in production of alternative fates (Rhyu et al., 1994; Guo et al., 1996; Cayouette and Raff, 2003). Because Numb isoforms are known to be expressed in the DM (Venters and Ordahl, 2005; Holowacz et al., 2006), theoretically any one of these two mechanisms could play a role in the choice between striated versus smooth muscle production from lateral DM cells. Our lineage analysis data exclude the possibility that single dividing cells in the lateral DM produce two daughters bearing alternative fates because injections have a strong tendency to give rise to only one of these fates. Yet the possibility remains open that lateral inhibition is operative in this system. Along this line, somite precursors expressing a Notch ligand would become striated muscle, whereas their neighboring cells, in which the Notch pathway is activated, would generate smooth muscle. A third possibility to explain our observations is that Notch has opposite effects on the actual differentiation of progenitors for smooth versus striated muscle. This would be consistent with data suggesting that Notch signaling acts downstream of Myf5 to inhibit muscle differentiation via inhibition of MyoD transcription (Hirsinger et al., 2001) and with additional results suggesting a positive effect of Notch on smooth muscle development (see previous paragraph). In such a case, the responsive progenitors could already be at least partially specified at the epithelial somite stage or, alternatively, bear unique positional information. This would explain why injected cells or cell clusters tend to produce only one fate in our lineage-tracing experiments.

Collectively, the present findings further highlight the spatial and temporal complexity of lineage segregation in the somitic epithelium. Elucidating the precise mechanisms underlying lineage segregation in the various domains of the epithelium will be the focus of future studies.

Materials and methods

Embryos
Fertile quail (Coturnix coturnix japonica) eggs from commercial sources (Moshav Mata) were used.

Expression vectors and electroporation

The five expression vectors used were the following: pCAGGS-AFP (Momose et al., 1999); Numb (pMW-CN1C89, N1-ICD; obtained from Y. Wakamatsu, Tsukuba University, Ibaraki, Japan; Wakamatsu et al., 1999); mouse activated Notch2 (N2-ICD; obtained from S. Chiba, Tokyo University, Tokyo, Japan; Shimizu et al., 2002); and xNoggin (Endo et al., 2002). The latter two were subcloned into pCAGGS. Electroporations were performed under a dissecting microscope (MB; WILD). 1–4 μg/μl DNA was microinjected into the center of flank-level epithelial somites or young DMs (Ben-Yair et al., 2003). For lateral-plate injections, the DNA was introduced between the two layers of mesoderm. Electrodes were positioned bilaterally in the intraembryonic coelom and parallel to the rostrocaudal axis. A four-parameter PulseAgile square wave electroporator (PA-4000; Cyto Pulse Sciences, Inc.) was used to deliver three groups of sequential pulses: 3 x 30 V, 20 ms each; 1 x 38 V, 5 ms each; and 3 x 30 V, 20 ms each.

Transfection of a GFP-encoding plasmid into discrete DM cell populations: specificity controls

Direct injections of GFP-DNA to somite and DM cells were performed as previously described (Ben-Yair and Kalcheim, 2003). All injections were directed to flank somites 20 through 25. For epithelial somite labelings, embryos aged 25 somites were used, and for DM injections, embryos were 30–32 somites old (E2.5) or ~43 somites (E3). Although originally developed for clonal transfection, we aimed instead to trace small cell subsets rather than performing clonal analysis. To this end, a concentration of 1 μg/μl DNA was used. Based on the initial number of GFP+ cells monitored at fixation, we estimate that 1–2 cells were labelled/injected.

To assess that injections hit the DM as opposed to the sclerotome, which also produces smooth muscle and endothelium, 40 embryos were injected with GFP-DNA in the lateral DM, the region closest to the sclerotome, and fixed 4 h later. In 34 out of 40 cases, labelled cells were found in the lateral DM and in 1 out of 40 in both the lateral DM and myotome. In addition, in 1 case, the lateral mesoderm was labelled and in 4 out of 40 segments the lateral myotome was attainted. No sclerotomal transfection was observed under these conditions, thus validating the specificity of the method (Fig. S1, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200707206/DC1). Likewise, more prominent transfections of GFP-DNA, performed by electroporations to the lateral DM, revealed 4 h later that only this epithelial domain contained labelled cells. Occasionally, a few DM cells were located more medially and, in only one case, a few sclerotomal progenitors were attained (n = 8; Fig. S1 C).

Figure 7. A simplified model for lineage segregation in the lateral somite/DM. Endothelial (E), smooth muscle (M), and striated muscle (StM) lineages arise from a common progenitor (Esner et al., 2006). Direct lineage analysis of lateral somite and DM show that fate-restricted progenitors are already detected at the epithelial somite stage. Of the three lineages, endothelial cells constitute a population of early segregated progenitors whose differentiation and/or migration depends on lateral mesoderm-derived BMP. In turn, we postulate the existence of a common intermediate progenitor for smooth and striated muscle sublineages from which separate fates are generated over a more extended period. The segregation of these fates is a binary choice that depends on Notch signaling. Elevated activity of Notch stimulates smooth muscle development, whereas lower levels or inhibition of Notch signaling drive generation of striated muscle. Under experimental conditions, abrogation of Notch function by overexpression of Numb results in myofiber differentiation in the myotome at the expense of mitotic muscle progenitors (satellite cell or myofiber progenitors). It is possible, however, that in the embryo, more subtle differences in levels of Notch activity modulate the balance between generating a myofiber or remaining a mitotic muscle precursor within the striated muscle sublineage. Likewise, inhibition of BMP activity was found to stimulate premature MyoD transcription and lateral myofiber differentiation (Kahane et al., 2007) leading to a reduction in cell number within myotomes (Fig. 6). Hence, the development of striated muscle precursors is negatively modulated by Notch as well as by BMP signaling cascades. Phenotypic segregation in the DM, therefore, exemplifies how two signaling cascades produce three distinct lineages by combining agonistic and antagonistic interactions.
Embryo processing and in situ hybridization

Embryos were fixed with either 4% formaldehyde or Fornoy and processed for paraffin wax embedding (Cinnamom et al., 1999). In situ hybridization was performed with probes for VEGF2 (Eichmann et al., 1993), cHairy2 (obtained from I. Palmeirim, Minho University, Braga, Portugal; Jouve et al., 2000), and cNotch1 (Myat et al., 1996), as well as with the following probes from the BBSRC ChickEST database (ARK Genomics): two HES5 homologues (clones CHET3958 and CHET83636); HES6 (clone CHET1453); and Notch2 (clones CHET823m1 and CHEST1007g3). Some sections were further subjected to anti-GFP immunohistochemistry.

Immunohistochemistry and image processing

Immunostaining was performed with monoclonal and polyclonal antibodies to desmin (1:100; MP Biomedicals); and 1:20 [Sigma-Aldrich]) that recognize both smooth and striated muscle lineages (Gerhardt and Betscholtz, 2003), polyclonal antibodies to GFP [1:200; Invitrogen], antibodies to quail endothelial marker QH1 [1:10]; Developmental Studies Hybridoma Bank), monoclonal antibodies to SMA (1:400; Sigma-Aldrich), and polyclonal antibodies to chicken Numb (1:1,000; Wakamatsu et al., 1999). Secondary antibodies coupled either to Cy3 or Rhodamine were used (1:200; Jackson ImmunoResearch Laboratories). DakoCytoMount was used as a mounting medium. Micrographs were taken from a microscope (BX51; Olympus) with U plan FLN 10x, 20x, or 40x, and 40x/0.75 dry objectives (Olympus) at RT, using DP controller v1.2.1108 acquisition software (Olympus) coupled to a cooled charge-coupled digital camera (DP70; Olympus). For figure preparation, images were exported into Photoshop CS2 (Adobe). If necessary, the brightness and contrast were adjusted to the entire image, and images were cropped without color correction adjustments or γ adjustments. Final figures were prepared using Photoshop CS2.

Data analysis

Lineage analysis of DM domains. The fates of distinct DM regions were quantified from micrographs, and cells were counted as described in the previous paragraph. To ensure uniformity, sections spanning several segments with labeled cells were photographed and counted. The proportion of a given derivative was calculated from the number of GFP + cells and visible HOECHST-positive nuclei (ranging from 2 to 5) distribution to determine the goodness of fit of the model.

Electroporations. Cells expressing GFP and cell type-specific markers were counted as described in the previous paragraph, to ensure uniformity, sections spanning several segments with labeled cells were photographed and counted. The proportion of a given derivative was calculated as the number of labeled cells out of the total number of GFP + cells in all sections and expressed as mean ± SEM. 174–1,101 cells were counted per embryo in quantitative presentations that were made 48 h after treatments, or 24–276 cells were counted per embryo in quantitative presentations made 16 h after treatments. In nogo-in-electroporated embryos, a range of 296–755 (total GFP + cells) or 21–188 (GFP + cells in CV) cells per embryo were counted, and results are expressed as the ratio of labeled derivatives out of the total number of GFP + cells in all sections or as the ratio of mural to endothelial cells ± SEM, respectively. Significance of the results was determined using the unpaired t test.

Online supplemental material

Fig. S1 shows the specificity of GFP-DNA injections (A and B) or electroporation (C) to the lateral DM. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200707206/DC1.

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