MyoD-positive epiblast cells regulate skeletal muscle differentiation in the embryo

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MyoD mRNA is expressed in a subpopulation of cells within the embryonic epiblast. Most of these cells are incorporated into somites and synthesize Noggin. Ablation of MyoD-positive cells in the epiblast subsequently results in the herniation of organs through the ventral body wall, a decrease in the expression of Noggin, MyoD, Myf5, and myosin in the somites and limbs, and an increase in Pax-3–positive myogenic precursors. The addition of Noggin lateral to the somites compensates for the loss of MyoD-positive epiblast cells. Skeletal muscle stem cells that arise in the epiblast are utilized in the somites to promote muscle differentiation by serving as a source of Noggin.

Introduction

Skeletal muscle differentiation begins in the embryonic somites. Soon after their separation from the presomitic mesoderm, somites become partitioned into the dermomyotome and sclerotome (Christ and Ordahl, 1995; Stockdale et al., 2000; Pownall et al., 2002). Sclerotome cells form the cartilages of the vertebral bodies and ribs. The dermomyotome gives rise to the differentiated skeletal muscle of the myotome and the connective tissues of the dermatome. The dorsomedial region of the dermomyotome is the site of early expression of the skeletal muscle–specific transcription factors MyoD and Myf5 (Sassoon et al., 1989; Ott et al., 1991; Pownall and Emerson 1992) and is a source of cells for the myotome (Christ et al., 1978; Ordahl et al., 2000; Kalcheim and Ben-Yair, 2005).

Skeletal muscle differentiation in the somites is promoted by members of the Wnt family released from the neural tube and overlying ectoderm and by Sonic Hedgehog produced in the notochord (Stern et al., 1995; Munsterberg et al., 1995; Fan et al., 1997; Borycki et al., 1998, 1999; Tajbakhsh et al., 1998; Wagner et al., 2000). Myogenesis is also regulated by Noggin and Wnt5b that are synthesized within the segmental plate and somites (Pourquie et al., 1996; Hirsinger et al., 1997; Marcelle et al., 1997; Reshef et al., 1998; Tonegawa and Takahashi, 1998; Amthor et al., 1999). Although inductive molecules are required for the up-regulation of MyoD and Myf5 in the somite and the onset of skeletal muscle differentiation (Pownall et al., 2002), both transcription factors are weakly expressed in the presomitic mesoderm (George-Weinstein et al., 1996; Gerhart et al., 2000; Hirsinger et al., 2001; Kiefer and Hauschka, 2001). Cells expressing MyoD mRNA are also present in the epiblast of the chick embryo (George-Weinstein et al., 1996; Gerhart et al., 2000; Strony et al., 2005). The epiblast gives rise to all tissues of the embryo (Fontaine and Le Douarin, 1977; Bellairs, 1986; Stern and Canning, 1990) and is a source for embryonic stem cell lines (Smith, 2001). When MyoD-positive (MyoDpos) cells are isolated from the epiblast and placed in culture, nearly all differentiate into skeletal muscle (Gerhart et al., 2004a). This population recruits pluripotent epiblast cells to the skeletal muscle lineage in vitro by releasing an inhibitor of the BMP signaling pathway (Gerhart et al., 2004a). In this study, we examined the role that MyoD-expressing epiblast cells play in regulating myogenesis in vivo.

Results

Expression of Noggin by MyoDpos epiblast cells

Given that MyoD-expressing epiblast cells produce an inhibitor of the BMP signaling pathway in vitro, and that Noggin is...
important for muscle differentiation in vivo, we hypothesized that MyoDpos cells would be incorporated into the somites and produce Noggin. To test this hypothesis, we examined the sites of incorporation of MyoDpos epiblast cells in the developing chick embryo and determined whether they expressed Noggin. MyoDpos cells were tracked in the embryo by tagging them with the G8 mAb. G8 recognizes a surface antigen specifically expressed in cells that express MyoD mRNA in the epiblast and fetal organs (Gerhart et al., 2001, 2004a; Strony et al., 2005).

Most cells labeled with the G8 mAb in the epiblast (Fig. 1 A) were later found in the somites (Fig. 1, C–H). G8-positive (G8pos) cells were concentrated in the dorsomedial and ventrolateral regions of the dermomyotome and myotome (Fig. 1, C–F), and some expressed sarcomeric myosin, which is a marker for differentiation (Fig. 1, G and H).

The majority of cells that had been prelabeled with G8 in the stage 2 embryo expressed Noggin mRNA and protein in the somites, and most cells expressing Noggin mRNA were labeled with G8 (Fig. 1, C–F). Labeling for Noggin protein was more extensive than the distribution of G8pos cells (Fig. 1, E and F), most likely a reflection of diffusion. A few G8pos/Noggin-positive (Nogginpos) cells were also found in the mesenchyme of the head, neural tube, and eyes (Fig. 2, B, D, and E). The neural tube contained cells that expressed Noggin, but not G8 (Fig. 2 B). This pattern of Noggin expression is similar to that reported by Reshef et al. (1998). Our double-labeling experiments suggest that cells that express MyoD mRNA in the epiblast become a primary source of Noggin in the somite.

Effects of ablating MyoDpos cells in the epiblast on morphogenesis

To determine if MyoDpos epiblast cells are critical for skeletal myogenesis, they were ablated in the embryo by labeling them with the G8 mAb, followed by lysis with complement. Approximately 70 cells (~0.4% of the total epiblast cells) were ablated in the posterior epiblast of the stage 2 embryo (Fig. 3 A), which is where MyoD/G8-expressing cells are located (Fig. 1 A). Embryos treated with complement (Fig. 3 B) or G8 alone (not depicted) had only a few dead cells throughout the entire epiblast. The specificity of G8/complement treatment was demonstrated by incubating embryos with the E12 mAb and complement. E12 labels a subpopulation of epiblast cells that expresses the neurogenic transcription factor NeuroM, but not MyoD (Strony et al., 2005). Cells lysed with E12 and complement were located in the central/anterior and posterior regions of the epiblast (Fig. 3, C and D).

Morphogenesis appeared to progress normally for the first 2 d after ablation of MyoDpos epiblast cells with G8 and complement (unpublished data). Similar numbers of somites formed in treated and control embryos. However, differences were observed as development progressed. Whereas the ventral body wall was closed in 5-d control embryos, it remained open in G8/complement-treated embryos (Fig. 3, E, H, and I). Embryos died between the fifth and seventh day after elimination of MyoDpos cells in the epiblast, and all had herniations of the heart and abdominal organs through the ventral body wall. Treated embryos also exhibited malformations of both eyes, or more commonly, the right eye only (Fig. 3, E and H), and twisting of the neural tube at the cervical or sacral level (not depicted). In some embryos, abnormalities were observed in the facial prominences (Fig. 3 E). None of the control embryos had ventral body wall, eye, or facial defects (Fig. 3, F and G). The malformations resulting from ablation of MyoDpos cells in the epiblast are consistent with their locations in older embryos, as determined by cell-tracking experiments (Fig. 1).

Figure 1.  Expression of Noggin and myosin in MyoDpos cells originating in the epiblast. MyoDpos cells labeled with the G8 mAb were present in the posterior region of the stage 2 epiblast (red cells in A). 4–5 d after labeling with G8, stage 25 embryos were examined for expression of Noggin and sarcomeric myosin. Regions indicated on the right side of the embryo in the hematoxylin and eosin–stained section are shown at higher magnification in fluorescence photomicrographs of merged images of G8 mAb [labeled with Alexa Fluor 488 (green in C and D) and rhodamine (red in E–H)] and either Cy3/red-labeled dendrimers to Noggin mRNA (C and D) or Alexa Fluor 488/green–labeled antibodies to Noggin (E and F) or myosin (G and H). Nuclei were stained with Hoechst dye (blue). Double-labeled cells (overlay of red and green) appear yellow (arrows). (C–F) G8pos/Nogginpos cells were observed in the dorsomedial (dm) and ventrolateral (vl) dermomyotome and myotome. (G and H) G8pos/myosinpos cells were present in the myotome. Bar: (A and C–H) 9 μm; (B) 135 μm.
Histological analyses of embryos treated with G8 and complement revealed that the somites partitioned into the dermomyotome, myotome, and sclerotome. However, abnormalities were observed in the morphology of the dermomyotome and myotome, ranging from an enlargement of the dorsomedial and ventrolateral regions (not depicted) to a thickening along the entire length (Fig. 3 J) compared with controls (Fig. 3, K and L). The boundary between the dermomyotome and myotome in treated embryos was less discrete than that of control embryos. These morphological differences were paralleled by an increase in cell number in the dermomyotome and myotome-like structure of G8/complement-treated embryos (Table I). The dermatomes of treated embryos were also expanded, whereas the sclerotomes of control embryos contained more cells than those of treated embryos (Fig. 3 J; Table I). The notochord (Fig. 3 J) and cartilage rudiments of the limbs (not depicted) appeared similar in treated and control embryos. The neural tube was properly positioned in ablated embryos (Fig. 3, I and K), except for twisting at cranial or sacral levels (not depicted). In contrast, the somites of embryos treated with the E12 mAb and complement were similar to other control embryos, except that the neural tube was kinked along its length (Fig. 3 J) compared with controls (Fig. 3, K and L). The boundary between the dermomyotome and myotome; however, there was an abundance of Pax3–positive cells in the dermomyotome of treated embryos (Fig. 5, I–L). In contrast, in the 2.5-d embryo, more Pax3–positive cells were present in the somites of control embryos than those treated with G8 and complement (G and H).

Ablation of MyoDpos epiblast cells also resulted in a decreased expression of Noggin in the somites compared with control embryos (Fig. 6, C–F). The reduction in Noggin was more pronounced on the right side of the embryo than on the left (not depicted). In contrast, Noggin expression in the neural tube appeared increased in treated embryos compared with controls (Fig. 6, G and H).

Overall, these results complement those of published studies. First, an inverse relationship was found between expression of MyoD/Myf5 and Pax-3 in the somite (Goulding et al., 1994; Williams and Ordahl, 1994; Marcelle et al., 1995). Second, inhibition of MyoD and Myf5 expression in Pax-3-positive cells occurs in response to BMP signaling (Reshef et al., 1998). Third, Pax-3–positive cells are present in Noggin-null mice (McMahon et al., 1998).

Addition of exogenous Noggin to ablated embryos
To test whether exogenous Noggin could compensate for the loss of MyoDpos epiblast cells, Noggin-soaked beads were implanted into embryos 2 d after ablating G8pos/MyoDpos cells in the epiblast. 4 d later, the aforementioned gross malformations of the ventral body wall, neural tube, and facial prominences were not observed (Fig. 7, A and E). The eyes had normal pigmentation after Noggin supplementation, although in some embryos the right eye remained smaller than the left (Fig. 7 A).

The amount of myosin staining in the ventrolateral and dorsolateral myotomes of G8/complement-treated embryos supplemented with Noggin appeared similar to, or increased, compared with that of buffer-treated embryos implanted with control beads (Fig. 7, B, C, F, and G). Enhanced myogenesis in the myotomes may reflect the accumulation of Pax3–positive cells before the addition of Noggin. Exogenous Noggin also promoted muscle differentiation in the limbs of ablated embryos,

Effects of ablating MyoDpos cells in the epiblast on myogenesis
The effect of ablating G8pos/MyoDpos cells in the epiblast on skeletal muscle differentiation was examined by staining for sarcomeric myosin. Whereas control embryos contained an abundance of muscle in the dorsomedial and ventrolateral myotomes and limbs, G8/complement-treated embryos had reduced myosin staining in the dorsomedial myotome, and severely diminished or no detectable myosin in the ventrolateral myotome (Fig. 4, D–I; Table II). Differentiated skeletal muscle was also decreased in the limbs (Fig. 4, J–L). In all G8/complement-treated embryos, differentiation was affected more severely on the right side of the embryo than the left (Fig. 4, N and O; Table II).

The effect of ablating G8pos/MyoDpos epiblast cells on muscle differentiation was accompanied by a decrease in MyoD and Myf5 mRNAs in the 5-d embryo (Fig. 5, C–F). At this time, few cells in control embryos expressed the marker for myogenic precursor cells Pax-3 (Stockdale et al., 2000) in the dermomyotome; however, there was an abundance of Pax3–positive cells in the dermomyotome of treated embryos (Fig. 5, I–L). In contrast, in the 2.5-d embryo, more Pax3–positive cells were present in the somites of control embryos than those treated with G8 and complement (G and H).

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skeletal myogenesis in the somites. Initially, these cells were found in the posterior epiblast of the two-layered embryo (Gerhart et al., 2000; Strony et al., 2005). A few hours later, MyoDpos cells were present within and lateral to the primitive streak (Gerhart et al., 2000; Strony et al., 2005), in a pattern similar to that revealed by fate map analyses of prospective paraxial mesoderm cells (Rosenquist, 1966; Bortier and Vakaet, 1992; Hatada and Stern, 1994; Nicolas et al., 1996; Jouve et al., 2002). As expected, most MyoDpos epiblast cells were incorporated into the somites.

A few MyoDpos cells were observed in areas of the epiblast fated for nonsomatic tissues, and later, in the sclerotome, neural tube, and fetal organs lacking skeletal muscle (Gerhart et al., 2000, 2001; Strony et al., 2005). Cells expressing MyoD and Myf5 outside of skeletal muscle remain undifferentiated (Gerhart et al., 2000, 2001; Tajbakhsh and Buckingham, 1995). This raises the possibility that ectopically placed myogenic precursors, possibly originating in the epiblast, could be a potential source of rhabdomyosarcomas. These malignantities are characterized by the expression of myogenic genes and often arise in structures lacking skeletal muscle (Dagher and Helman, 1999).

MyoD-expressing cells of the epiblast become a major source of Noggin within the somites. Elimination of this population in the epiblast resulted in a reduction of Noggin and skeletal

### Table I. Number of cells in somite compartments of G8/complement-treated and control embryos

| G8/complement | Dermatome | Dermomyotome | Myotome | Sclerotome |
|---------------|-----------|--------------|---------|-----------|
| right         | 309 ± 113 | 192 ± 46     | 828 ± 370 | 228 ± 67 |
| left          | 289 ± 110 | 178 ± 42     | 704 ± 221 | 271 ± 55 |
| Buffer        |           |              |         |           |
| right         | 169 ± 34  | 144 ± 22     | 246 ± 47 | 503 ± 61 |
| left          | 144 ± 26  | 138 ± 9      | 246 ± 60 | 516 ± 107 |

Embryos were incubated with the G8 mAb and complement or Hanks’ buffer at stage 2 and fixed 4.5 d after treatment. The number of nuclei in each compartment of the somite was determined by microscopy using the Image-Pro Plus image analysis software. Values are the mean ± the SD of the number of sections (n) indicated in parentheses. Fewer myosin-positive cells were present in G8/complement-treated than control embryos (P ≤ 0.0005). More myosin-positive cells were present in the myotome-like structure on the left side of G8/complement treated embryos than the right (P ≤ 0.0005).

### Table II. Number of differentiated skeletal muscle cells in the myotomes of G8/complement-treated and control embryos

| G8/complement | Number of myosin-positive cells |
|---------------|--------------------------------|
| right (n = 12)| 18 ± 13                        |
| left (n = 11) | 32 ± 5                         |
| Buffer        |                                |
| right (n = 16)| 95 ± 9                         |
| left (n = 17) | 94 ± 11                        |

Embryos were incubated with the G8 mAb and complement or Hanks’ buffer at stage 2 and fixed 4.5 d after treatment. Transverse sections were stained with the MF20 mAb to sarcomeric myosin. The number of nuclei in the myotome-like structure of two G8/complement-treated embryos and the myotome of two buffer-treated embryos in sections through the wing level was determined by microscopy using the Image-Pro Plus image analysis software. Values are the mean ± the SD of the number of sections (n) indicated in parentheses. Fewer myosin-positive cells were present in G8/complement-treated than control embryos (P ≤ 0.0005). More myosin-positive cells were present in the myotome-like structure on the left side of G8/complement treated embryos than the right (P ≤ 0.0005).

**Discussion**

The purpose of this study was to determine whether cells that express MyoD mRNA in the epiblast play a role in regulating
muscle in the myotomes and limbs in older embryos. These results are consistent with previous studies demonstrating that Noggin regulates myogenesis in the somites by inhibiting BMPs (Pourquie et al., 1996; Zimmerman et al., 1996; Hirsinger et al., 1997; Marcelle et al., 1997; Dietrich et al., 1998; Reshef et al., 1998; Tonegawa and Takahashi, 1998; Amthor et al., 1999; Linker et al., 2003). Although some epiblast cells initiated the expression of MyoD and the G8 antigen after stage 2 (see Materials and methods), they were unable to completely compensate for those that were ablated earlier in development.

Ventrolateral hypaxial muscles were more severely affected by ablating MyoD<sup>pos</sup> epiblast cells than the dorsomedial epaxial muscles. This is opposite to what was observed in Noggin-deficient mice (McMahon et al., 1998); however, in G8/ complement-treated chick embryos, there was an alternative source of Noggin in the neural tube in close proximity to the epaxial myotome. The paucity of muscle in the ventral myotome of G8/complement-treated embryos was the most likely cause of herniation of organs through the body wall. Malformations of the eye and facial prominences arising from ablation of MyoD<sup>pos</sup> epiblast cells may be secondary to a disturbance in the differentiation of facial and extraocular muscles, although it is possible that Noggin produced by MyoD<sup>pos</sup> epiblast cells affects nonmyogenic cells.

The malformations that arise as a result of ablation of MyoD<sup>pos</sup> cells in the epiblast resemble those that are present in humans with Axenfeld-Rieger syndrome, an autosomal dominant haploinsufficiency of the Pitx2 gene (Jorgenson et al., 1978; Semina et al., 1996; Lines et al., 2002). Mutations in Pitx2 affect the development of left-sided structures (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; St. Amand et al., 1998; Yoshioka et al., 1998), whereas elimination of MyoD<sup>pos</sup> cells in the chick epiblast affected myogenesis, eye development, and Noggin expression more severely on the right side of the embryo. Although Pitx2 is involved in left– right asymmetry, it is expressed symmetrically in myogenic cells of the eye, limb, and myotome of the mouse (Campione et al., 1999; Yoshioka et al., 1998, Kitamura et al., 1999). Pitx2-null mice exhibit dysgenesis of the extraocular muscles and thinning of the abdominal wall (Kitamura et al., 1999). Given the similarities...
between the malformations in the chick embryo arising from ablation of MyoD<sup>pos</sup> epiblast cells and those that result from perturbation of Pitx2, it is possible that inhibition of BMP signaling by Noggin is involved in Pitx2 expression in the somite during myogenesis. This notion is supported by the fact that BMP2 induces, and Noggin inhibits, the expression of Snail/snrl which represses Pitx2 expression (Isaac et al., 1997; Patel et al., 1999; Piedra and Ros, 2002; Schlange et al., 2002).

Figure 5. Effect of ablating MyoD<sup>pos</sup>/G8<sup>pos</sup> epiblast cells on MyoD, Myf5, and Pax-3 expression. Stage 2 embryos were treated with the G8 mAb and complement [G8/cp] or Hanks' buffer (bc) and grown for 2 d (stage 14; G and H) or 4.5 d (stage 25; C–F and I–L). Areas outlined in hematoxylin and eosin–stained sections (A and B) are shown at higher magnification in fluorescence photomicrographs that are the merged images of Hoechst-stained nuclei and Cy3-labeled dendrimers to MyoD or Myf5 mRNA, or rhodamine-labeled antibody to Pax-3. Less MyoD and Myf5 mRNA were detected in the somites of G8/complement-treated embryos (C and E) than in embryos treated with buffer only (D and F). More Pax-3–positive cells were present in stage 14 control embryos than treated embryos [G and H], whereas the reverse was found in dorsomedial (dm) and ventrolateral (vl) dermomyotomes/myotomes of stage 25 embryos (I–L). Bar: (A and B) 135 μm; (C–L) 9 μm.

Figure 6. Effect of ablating G8<sup>pos</sup>/MyoD<sup>pos</sup> epiblast cells on Noggin expression. Noggin expression was analyzed 4.5 d after ablating stage 2 MyoD<sup>pos</sup> epiblast cells. Regions indicated in hematoxylin and eosin–stained sections (A and B) are shown in fluorescence photomicrographs of merged images of Hoechst-stained nuclei and Cy3-labeled dendrimers to Noggin mRNA (C–H). Less Noggin mRNA was detected in the dorsomedial (dm) and ventrolateral (vl) dermomyotome and myotome in G8/complement-treated embryos [G8/cp] (C and E) than in buffer-treated embryos (bc; D and F). (G and H) The reverse was found for Noggin expression in the neural tube (nt). Bar: (A and B) 135 μm; (C–H) 9 μm.

One possible explanation for the asymmetric effects of ablating G8/MyoD<sup>pos</sup> cells in the stage 2 embryo is that cells initiating expression of MyoD in the epiblast after treatment are preferentially incorporated into the left side of the embryo. It also is possible that MyoD<sup>pos</sup> epiblast cells are involved in signaling before their incorporation into the somites. This notion is consistent with our finding more MyoD<sup>pos</sup> cells on the right side of Hensen’s node (Gerhart et al., 2000), which is a structure located at the rostral end of the primitive streak that is a rich source of signaling molecules regulating laterality in the embryo (Chapman et al., 2002; Raya and Izpisua Belmonte, 2004). The importance of the node in myogenesis was illustrated in the mouse inverted viscerus mutant embryo, in which defects in the flow of molecules across the node reversed the asymmetric expression of α-skeletal actin and myosin light chain 3F in the myotome (Golding et al., 2004). Cell-tracking experiments that follow the pathways of migration of stages 1–3 MyoD<sup>pos</sup> epiblast cells may shed light on the mechanism whereby muscle differentiation is asymmetrically perturbed after their ablation.
We have identified skeletal muscle stem cells in the epi-
blast based on their expression of MyoD and demonstrated a
critical role for these cells in regulating myogenesis in vitro and
in vivo (George-Weinstein et al., 1996; Gerhart et al., 2004a;
Strony et al., 2005; this study). Choi et al. (1989) postulated that
the early chick embryo contains founder cells for the myogenic
lineage based on their observation that skeletal muscle emerges
in chick blastoderm cultures. In this context, founder cells were
defined as those that give rise to all cells of a given lineage. In
contrast, MyoD<sup>pos</sup> epiblast cells of the stage 2 embryo do not
appear to be the sole source of myogenic precursors in the
somite. In our cell-tracking experiments, the G8 mAb that had
been applied in the stage 2 embryo was not detected in most
cells of the myotome. However, cells expressing Pax-3 did emerge in the dermomyotome after ablat-
ing MyoD<sup>pos</sup> cells in the epiblast, and their ability to differenti-
ate was revealed when ablated embryos were supplemented
with exogenous Noggin. Therefore, under these experimental
conditions, most Pax-3 precursors do not appear to be the direct
descendants of MyoD<sup>pos</sup> epiblast cells.

Muscle founder/pioneer cells also have been defined in
the avian embryo as those that are the first to differentiate and
enter the myotome (Kahane et al., 1998). In invertebrate
embryos, founder/pioneer cells enter the myotome and serve as
a scaffold for myoblast fusion (Bate, 1990; Baylies and Michelson,
2001; Dworak and Sink, 2002). Although G8<sup>pos</sup>/MyoD<sup>pos</sup>
epiblast cells may be involved in both of these processes, their
influence clearly extends beyond that of playing a structural role
in the myotome. Collectively, the data indicate that the primary
function of MyoD<sup>pos</sup> epiblast cells within the somites is to promote
the differentiation of myogenic precursors by releasing Noggin.

**Materials and methods**

**Tracking MyoD<sup>pos</sup> cells from the epiblast into the somites**

White Leghorn chick embryos were obtained from BE Eggs and staged ac-
cording to the method of Hamburger and Hamilton (1951). Stage 2 em-
broys were removed from the shell on the yolk and placed in a tissue
culture dish. 100 μl of G8 mAb diluted 1:40 in PBS was applied to
the embryo for 45 min. After rinsing in PBS, embryos were incubated for
30 min in 100 μl rhodamine-conjugated goat anti–mouse FAb’2 fragments
(Jackson ImmunoResearch Laboratories) diluted 1:400 and rinsed. Embryos
on the yolk were poured into an empty host shell, covered, and incubated at
37°C for 2–4 d. Embryos were fixed in 4% formaldehyde overnight,
embedded in paraffin, sectioned transversely at 10 μm, and applied to
gelatin-coated slides. Sections were labeled with an antibody to Noggin
(R&D Systems) or processed for expression of Noggin mRNA as described
in the following paragraphs. Sections were mounted in Gelmount (Biomeda)
and observed with an epifluorescence microscope (Eclipse E800; Nikon)
using 4×/0.2 NA and 60×/1.4 NA oil objectives. Photomicrographs were
produced with the video camera (Evolution QE; Media Cybernetics) and ImagePro Plus image analysis software (Phase 3 Imaging Systems). Tracking of G8™ epitiblast cells was conducted in three embryos.

To test for the presence of residual unbound G8 mAb after the initial labeling period, we first determined the number of G8™ cells directly after labeling stage 2 embryos. An average of 76 cells in 75 embryos was labeled with G8. When embryos were labeled with G8 and an Alexa Fluor 488–conjugated secondary antibody, incubated for 3 h at 37°C, and labeled with a rhodamine-conjugated secondary antibody, there was an average of 77 cells labeled with both Alexa Fluor 488 and rhodamine, and no cells were labeled with either fluorochrome alone. A third group of embryos was labeled with G8 and Alexa Fluor 488 secondary antibody, incubated for 3 h, and then exposed to more G8 mAb, followed by the rhodamine secondary antibody. In this case, there was an average of 80 cells with both Alexa Fluor 488 and rhodamine, and 17 cells were labeled with rhodamine alone. These results demonstrate that additional cells are expressing the G8 antigen after the initial labeling period; however, there is insufficient residual G8 mAb present to label those cells. Therefore, unbound G8 mAb does appear to be washed out of the embryo during our labeling procedure, thereby demonstrating the feasibility of tracking MyoD™ cells from the stage 2 epiblast into the mesoderm.

Eliminating MyoD™ cells in the epiblast

Stage 2 embryos were removed from the shell on the yolk, labeled with the G8 mAb, and rinsed as described in the previous section. 100 μL of baby rabbit complement (Cedar Lane, Inc.) diluted 1:40 in Hanks’ buffer was applied to the embryo for 30 min at room temperature. Control embryos received Hanks’ buffer with BSA, G8 mAb, or complement alone. An additional control involved incubating embryos in the E12 mAb that labels a subpopulation of cells expressing NeuroM mRNA in the epiblast (Strony et al., 2005), followed by the addition of complement. The presence of unbound cells was determined directly after treatment by incubating embryos in 0.2% trypan blue in PBS for 15 min at 37°C and counting the number of blue cells. After treatment, embryos not exposed to trypan blue were poured into an empty shell and incubated for 2–4 d. Embryos were analyzed at the gross level and in transverse, 10-μm serial sections. The number of embryos in each treatment group is listed in Table III.

In situ hybridization

Paraffin sections were applied to Teflon-printed, three-well glass slides (Electron Microscopy Sciences) coated with 0.2% gelatin. The in situ hybridization procedure is described in detail in Gerhart et al. (2004b). Messenger RNAs for MyoD, Myf5, and Noggin were detected with DNA dendrimers conjugated with Cy3 and the following antisense oligonucleotide sequences: chicken MyoD, 5’-TTCTCAAGAGCAAATCTACCAT-TGTTGA TTCCGTGTA-3’ (L34006; Dechesne et al., 1994); chicken Noggin, 5’TTCGTAAAGACTCTCTCTGGTGGGCTAA-3’ (NM_204123; Tonegawa and Takahashi, 1998); and chicken Myf5, 5’-ATAATGGGATG-GGACGCTGGAGGATTCC-3’ (SS3719; Neville et al., 1992). Fluorescent dendrimers were obtained from Genisphere, Inc. Nuclei were stained with Hoechst dye. Double labeling with dendrimers and antibodies was performed as previously described (Gerhart et al., 2001, 2004a,b; Strony et al., 2005).

Immunofluorescence localization

Paraffin sections were labeled with the MF20 mAb to sarcomeric myosin heavy chain (Bader et al., 1982) diluted 1:60, a goat anti–mouse polyclonal antisemur to Noggin (R & D Systems) diluted 1:200, or a mAb to Pax-3 (Baker et al., 1999) diluted 1:150. Primary antibodies were labeled with rhodamine-conjugated goat anti–mouse Fab’2 fragments (Jackson Immunoresearch Laboratories) or fluorescein-conjugated donkey anti–goat IgG (CHEMICON International, Inc.), as previously described (George-Weinstein et al., 1994). The MF20 and Pax-3 mAbs were obtained from the Developmental Studies Hybridoma Bank.

Cell counting

The number of nuclei present in the dermatom, dermomyotome, myotome, and sclerotome of 20–24 sections through the wing level of two embryos treated with the G8 mAb and complement and two embryos incubated with Hanks’ buffer was determined in sections stained with Hoechst dye or hematoxylin and eosin using the Image-Pro Plus image analysis software. The accuracy of cell counting via software analysis was validated by comparing cell numbers to those obtained by manually counting cells. The number of myosin-positive cells was determined in 11–17 sections from the wing level of two treated and two control embryos by manually counting MF20-labeled cells via microscopy. Statistically significant differences in populations were determined using the t test.

Table III. Numbers of embryos analyzed by gross inspection and tissue sectioning

| Gross morphology | Number of Embryos | Histology |
|------------------|------------------|-----------|
| Gross morphology | 2-3 d | 4-5 d | 7 d | 2-2.5 d | 4-5 d | 7 d |
| Hanks’ buffer    | 7    | 7    | 2    | 2    | 3    | 1    |
| G8 mAb          | 1    | 6    |      | 1    | 1    | 1 |
| E12 mAb         |      |      |      | 1    | 1 |
| Complement      | 3    | 4    | 1    | 2    | 3 |
| E12 mAb + complement | 5    |      |      |      | 1 |
| G8 mAb + complement | 9    | 9    | 5    | 2    | 3    | 1 |
| Hanks’ Buffer Tx + PBS beads | 3 | 2 | 1 | 2 |
| G8/complement Tx + PBS beads | 5 | 2 | 1 | 2 |
| G8/complement Tx + Noggin beads | 4 | 6 | 2 | 3 |

Embryos were incubated with Hanks’ buffer, antibodies or complement alone, or antibodies and complement at stage 2 and fixed for 2-2.5 (stages 14–17), 4-5 (stages 25–26), or 7 d (stage 30) after treatment. Other embryos were treated at stage 2, grown to stages 11–13, and implanted with beads soaked in PBS or Noggin. All embryos were examined morphologically and some embryos were embedded and sectioned (histology). 90% of embryos treated with the G8 mAb and complement survived for 5 d.
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