Cells that express MyoD mRNA in the epiblast are stably committed to the skeletal muscle lineage

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The epiblast of the chick embryo contains cells that express MyoD mRNA but not MyoD protein. We investigated whether MyoD-positive (MyoD^pos) epiblast cells are stably committed to the skeletal muscle lineage or whether their fate can be altered in different environments. A small number of MyoD^pos epiblast cells were tracked into the heart and nervous system. In these locations, they expressed MyoD mRNA and some synthesized MyoD protein. No MyoD^pos epiblast cells differentiated into cardiac muscle or neurons. Similar results were obtained when MyoD^pos cells were isolated from the epiblast and microinjected into the precardiac mesoderm or neural plate. In contrast, epiblast cells lacking MyoD differentiated according to their environment. These results demonstrate that the epiblast contains both multipotent cells and a subpopulation of cells that are stably committed to the skeletal muscle lineage before the onset of gastrulation. Stable programming in the epiblast may ensure that MyoD^pos cells express similar signaling molecules in a variety of environments.

Introduction

Commitment of a cell to develop along a prescribed pathway is thought to occur in two stages (Gilbert, 2006). The first stage, which is called specification, is characterized by the ability of a cell to differentiate autonomously in a neutral environment; however, the fate of the cell can still be redirected. During the determination phase, a cell will differentiate according to its prescribed fate even when placed in an environment that induces the development of other tissue types. In this case, commitment is considered to be stable and irreversible under normal circumstances.

Previous studies suggested that commitment to the skeletal muscle lineage occurs in the somites in response to factors released by surrounding structures and those produced within the somites themselves (Buckingham and Tajbakhsh, 1999; Linker et al., 2003; for reviews see Borycki and Emerson, 2000; Stockdale et al., 2000; Pownall et al., 2002). Stable commitment was operationally defined as the maintenance of myogenic potential when the muscle-forming region of the somite was exposed to factors that promote chondrogenesis (Watterson et al., 1954; Aoyama and Asamoto, 1988; Christ et al., 1992; Ordahl and Le Douarin, 1992; Brand-Saberi et al., 1993; Pourquie et al., 1993; Bober et al., 1994; Goulding et al., 1994; Dockter and Ordahl, 2000). At the

molecular level, commitment is driven by up-regulation of the skeletal muscle–specific transcription factors Myf5, MyoD, and Mrf4 in progenitor cells expressing the paired box transcription factors Pax-3 and/or Pax-7 (Sassoon et al., 1989; Ott et al., 1991; Pownall and Emerson, 1992; Rudnicki et al., 1993; Goulding et al., 1994; Williams and Ordahl, 1994; Marcelle et al., 1995; Maroto et al., 1997; Tajbakhsh et al., 1997; Kahane et al., 2001; Kassar-Duchossoy et al., 2004, 2005). Genetic manipulations of the mouse embryo revealed that Myf5 and Mrf4 are responsible for the initial activation of MyoD, whereas Pax-3 assumes this function later in development (Tajbakhsh et al., 1997; Kassar-Duchossoy et al., 2004).

Analyses of the chick embryo have yielded conflicting results regarding the sequence of expression of skeletal muscle transcription factors. In situ hybridization with enzymatic probes revealed that Myf5 but not MyoD was expressed in the presomitic mesoderm (Maroto et al., 1997; Hacker and Guthrie, 1998; Hirsinger et al., 2001; Kiefer and Hauschka, 2001). Low levels of Myf5 were detected in the primitive streak and adjacent epiblast of the stage 3 embryo (Kiefer and Hauschka, 2001). In contrast, in situ hybridization with fluorescent dendrimer probes and RT-PCR demonstrated the presence of MyoD mRNA in the presomitic mesoderm, stage 3 embryo, and stage 1 epiblast (George-Weinstein et al., 1996; Gerhart et al., 2000; Strony et al., 2005). Given that epiblast cells expressing low levels of MyoD

mRNA represent a small subpopulation within the presomitic mesoderm and somites (Gerhart et al., 2000), it is possible that they were not clearly visible with enzymatic probes. These MyoD-positive (MyoD^{pos}) cells may correspond to the small subpopulation of presomitic mesoderm cells that are capable of differentiating in cultured explants of presomitic mesoderm tissue (Stern and Hauschka, 1995; Stern et al., 1997).

The importance of MyoD expression in the epiblast of the chick embryo was demonstrated in vitro and in vivo. When epiblast cells that express MyoD mRNA were isolated from the embryo and cultured in serum-free medium, nearly all differentiated into skeletal muscle (Gerhart et al., 2004a). In vivo, most MyoD^{pos} epiblast cells were incorporated into the somites and synthesized Noggin (Gerhart et al., 2006). Noggin promotes skeletal muscle differentiation in the somites by blocking the bone morphogenetic protein (BMP) signaling pathway (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; Sela-Donenfeld and Kalcheim, 2002; Linker et al., 2003). Ablation of MyoD^{pos} cells in the epiblast resulted in a decrease in Noggin in the somites and a dramatic reduction in skeletal muscle in the trunk and limbs (Gerhart et al., 2006). The inhibition of muscle differentiation after ablation was averted with the addition of exogenous Noggin. Thus, cells that express MyoD mRNA in the epiblast regulate skeletal myogenesis in the somites by releasing Noggin.

The ability to differentiate in vitro and to promote muscle differentiation in vivo does not necessarily indicate that cells expressing MyoD mRNA in the epiblast are stably committed to the skeletal muscle lineage. Several studies have demonstrated that skeletal muscle transcription factors are transiently expressed during the early stages of development. Myf5 and MyoD mRNAs were initially expressed in nonmyogenic tissues of the chick and *Xenopus laevis* embryo, respectively, and gradually became restricted to muscle-forming regions of the somite (Rupp and Weintraub, 1991; Steinbach et al., 1998; Kiefer and Hauschka, 2001). Separation of the somites from surrounding tissues resulted in a down-regulation of MyoD and Myf5 and a failure of cells to differentiate (Maroto et al., 1997; Borycki et al., 1998; Dietrich et al., 1998; Teillet et al., 1998; Reshef et al., 1998; Marcelle et al., 1999; Pirskanen et al., 2000). Furthermore, some cells that express skeletal muscle transcription factors in adult muscle can be induced to differentiate into bone and adipocytes (for review see Chen and Goldhamer, 2003). These studies suggest that low levels of expression of skeletal muscle transcription factors may be the hallmark of specification but not of stable commitment to the skeletal muscle lineage. However, small numbers of cells in the presomitic mesoderm and myogenic precursors in the limb that have not up-regulated skeletal muscle–specific transcription factors do undergo skeletal myogenesis even when challenged with cartilage-promoting factors from the notochord (Williams and Ordahl, 1997, 2000).

The following studies were designed to determine whether MyoD^{pos} epiblast cells are stably committed to the skeletal muscle lineage or whether their fate can be altered in environments that induce the differentiation of nonskeletal muscle tissues. The environment of the developing heart is particularly challenging to skeletal myogenesis because BMPs are required for specification of the heart-forming fields and cardiomyocyte differentiation (Lough et al., 1996; Schultheiss et al., 1997), but they inhibit skeletal muscle differentiation (Pourquie 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; Sela-Donenfeld and Kalcheim, 2002; Linker et al., 2003). BMPs also play a role in the induction of dorsal cell fates in the neural tube (Basler et al., 1993; Dickinson et al., 1994; Liem et al., 1995; Hogan, 1996; Dudley and Robertson, 1997; Lee et al., 1998; Nguyen et al., 2000). Although BMP inhibitors are required for establishing ventral neuronal fates, neural tube closure, and anterior brain formation (McMahon et al., 1998; Bachiller et al., 2000; Hartley et al., 2001), skeletal muscle differentiation is not induced in the nervous system.

The state of commitment of MyoD^{pos} epiblast cells was examined in two ways. First, cells that express MyoD in the epiblast were tracked into the heart and nervous system. Second, MyoD^{pos} cells were isolated from the epiblast and micro-injected into the precardiac mesoderm and neural plate. In both types of experiments, MyoD^{pos} epiblast cells continued to express MyoD mRNA, some synthesized MyoD protein, and none differentiated into cardiac muscle or neurons. In contrast, cells that did not express MyoD in the epiblast differentiated according to their location.

Results

Expression of MyoD protein and Myf5 mRNA in the epiblast

MyoD mRNA is expressed in a small subpopulation of cells in the pregastrulating epiblast (George-Weinstein et al., 1996; Gerhart et al., 2000, 2006; Strony et al., 2005). To determine whether these cells produce MyoD protein, stage 1–4 embryos were double labeled with mAbs to MyoD and the G8 antigen. The G8 mAb binds to a cell surface antigen and is a specific marker for cells that express MyoD mRNA in the epiblast (Fig. 1 A; Gerhart et al., 2001, 2004a; Strony et al., 2005). MyoD protein was not detected in stage 1 or 2 embryos, and only a single G8-positive (G8[pos]) cell in the stage 4 epiblast was labeled with the MyoD antibody (Fig. 1, B–D). These results were confirmed with a rabbit polyclonal antiserum to MyoD. Therefore, MyoD mRNA either is not translated or the protein does not accumulate to detectable levels in the early epiblast.

MyoD^{pos} epiblast cells were further characterized by determining whether they express Myf5 mRNA. In agreement with the results of Kiefer and Hauschka (2001), Myf5 mRNA was not detected by in situ hybridization in the stage 1 or 2 epiblast (Fig. 1 E). The lack of detection of Myf5 mRNA in the pregastrulating epiblast suggests that MyoD is expressed before Myf5 in the chick embryo, although analyses of the embryo before laying would be required to demonstrate this definitively. Myf5 mRNA was detected in only a subpopulation of G8^{pos} cells
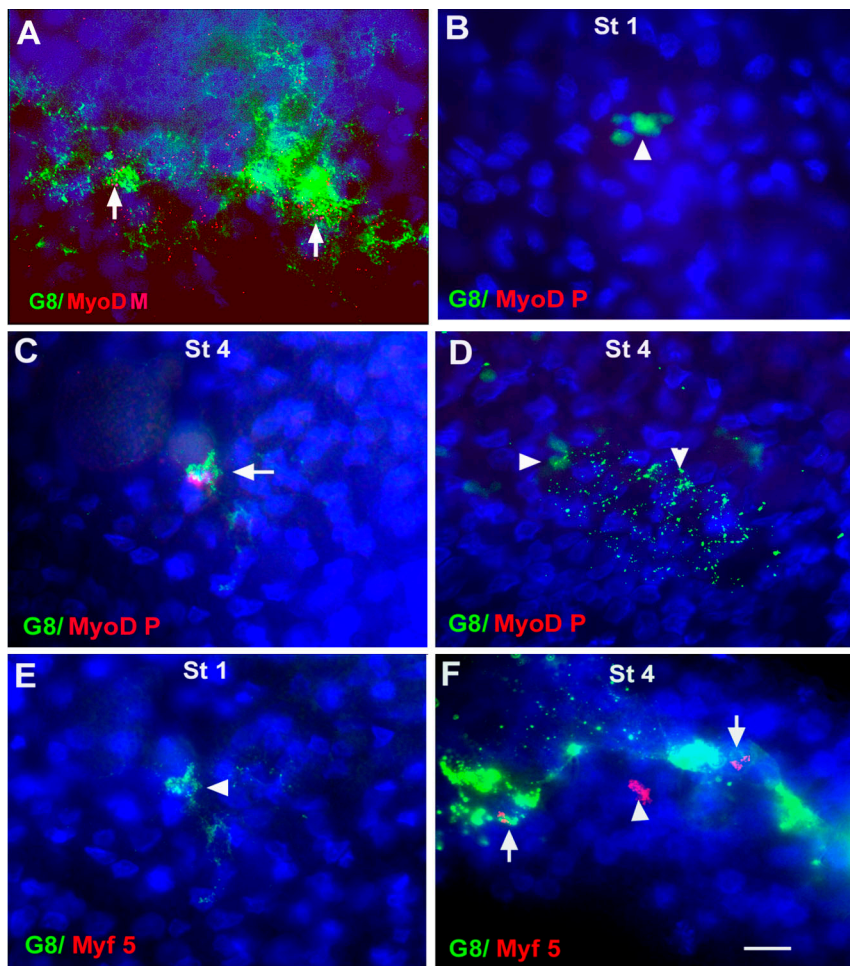
Figure 1. **Expression of G8, MyoD, and Myf5 in the pregastrulating and gastrulating epiblast.** Whole stage 1 and 4 embryos were double labeled for the G8 antigen (green) and MyoD mRNA (MyoD M), MyoD protein (MyoD P), or Myf5 mRNA (red). Nuclei were stained with Hoechst dye (blue). G8 and MyoD mRNA were expressed in the same cells in the stage 1 epiblast (arrows in A). The MyoD mAb did not label cells in the stage 1 epiblast or most cells of the stage 4 epiblast (arrowheads in B and D). One G8^{pos} cell in the stage 4 epiblast was stained with the MyoD mAb (arrow in C). Myf5 was not detected in the stage 1 epiblast (arrowhead in E). The stage 4 epiblast contained subpopulations of G8^{pos}/Myf5^{neg}, G8^{pos}/Myf5^{pos} (arrows), and G8^{neg}/Myf5^{pos} cells (arrowhead; F). Bar, 9 μm.

in the stage 4 epiblast (Fig. 1 F). Interestingly, a few stage 4 epiblast cells that did not appear to express the G8 antigen expressed Myf5 (Fig. 1 F). Therefore, epiblast cells of gastrulating embryos may be heterogeneous with respect to their expression of muscle regulatory factors.

Behavior of MyoD^{pos} epiblast cells tracked into the heart and nervous system

Cells expressing MyoD mRNA in the stage 2 epiblast were tracked into the heart and nervous system by fluorescently labeling them with the G8 mAb and incubating the embryos for 3 d in ovo (stage 16). Although the majority of G8^{pos} cells were incorporated into the somites (56%), ∼9 labeled cells were found in the heart, 10 were found in the neural tube, 9 were in the brain, and 27 were found in other nonsomitic tissues of the embryo, including the mesenchyme of the head. Most were present as single cells surrounded by G8-negative (G8^{neg}) cells. Within the somites, all of the G8^{pos} cells contained MyoD protein (Fig. 2 A), and the majority (73%) had synthesized sarcomeric myosin.

All of the G8^{pos} cells tracked into the heart continued to express MyoD mRNA, and a subpopulation (59%) was stained with the MyoD mAb (Fig. 2, B–D). Although most cells lacking the G8 label had synthesized cardiac troponin T, this marker for cardiac muscle differentiation was not detected in any G8^{pos}

cells in the stage 16 heart (Fig. 2 E). Within the central nervous system (CNS) of the stage 16 embryo, all G8^{pos} cells that originated in the epiblast continued to express MyoD mRNA, and ∼50% were stained with the MyoD mAb (Fig. 2, F and G). Only a single G8^{pos} cell contained sarcomeric myosin (Fig. 2 H). None of the G8^{pos} cells were labeled with an antibody to neurofilament-associated antigen (Fig. 2 I).

The CNS (Fig. 2 J) and heart contained a few cells that expressed MyoD but lacked the G8 tag that had been applied in the stage 2 embryo. This is consistent with the observation that more G8^{pos} cells (∼36 cells) were found in hearts directly labeled with the G8 mAb than the number of G8^{pos} epiblast cells tracked into the heart (nine cells). Some cells with MyoD mRNA but lacking the G8 tag applied in the epiblast were present in clusters containing G8-labeled cells, whereas others were surrounded by G8^{neg} cells (Fig. 2 J). Although it is possible that the G8 signal was lost in some cells as a result of proliferation, the expression of MyoD may have been initiated in a separate population after application of the antibody (Gerhart et al., 2006).

Importantly, no cell that expressed MyoD mRNA and the G8 antigen within the epiblast and were tracked into the heart or nervous system or any cells that may have initiated G8 synthesis sometime after stage 2 of development contained detectable levels of cardiac troponin T or neurofilament-associated antigen. These results indicate that G8^{pos} epiblast cells are not

Figure 2. Expression of skeletal muscle, cardiac muscle, and neuronal markers in G8^pos epiblast cells tracked into the heart and brain. Cells that expressed MyoD mRNA in the stage 2 epiblast were labeled with the G8 mAb (green) and tracked into the somite, heart, and brain of the 3-d embryo. Sections were stained for MyoD mRNA (MyoD M), MyoD protein (MyoD P), cardiac troponin T (CTpn), or neurofilament-associated antigen (NF; red). Nuclei were labeled with Hoechst dye (blue). Merged images with overlapping red and green fluorescence appear white. G8^pos cells that stained with the MyoD mAb were concentrated in the somites (arrows in A). G8^pos cells of the heart and brain contained MyoD mRNA (arrows in B and F), and some were labeled with the MyoD mAb (arrows in D and G). G8^pos cells that were not labeled with the MyoD mAb are shown in C and G (arrowheads). Other G8^pos cells in these organs did stain with the MyoD mAb (arrows in D and G). The brain contained G8^pos/sarcomeric myosin^pos (arrow) and G8^pos/sarcomeric myosin^neg cells (arrowhead; H). Cardiac troponin T or neurofilament-associated antigen was not detected in G8^pos cells in the heart and brain, respectively (arrowheads in E and I). MyoD mRNA was expressed in some G8^neg cells in the brain (arrowhead in J). Bar, 9 μm.
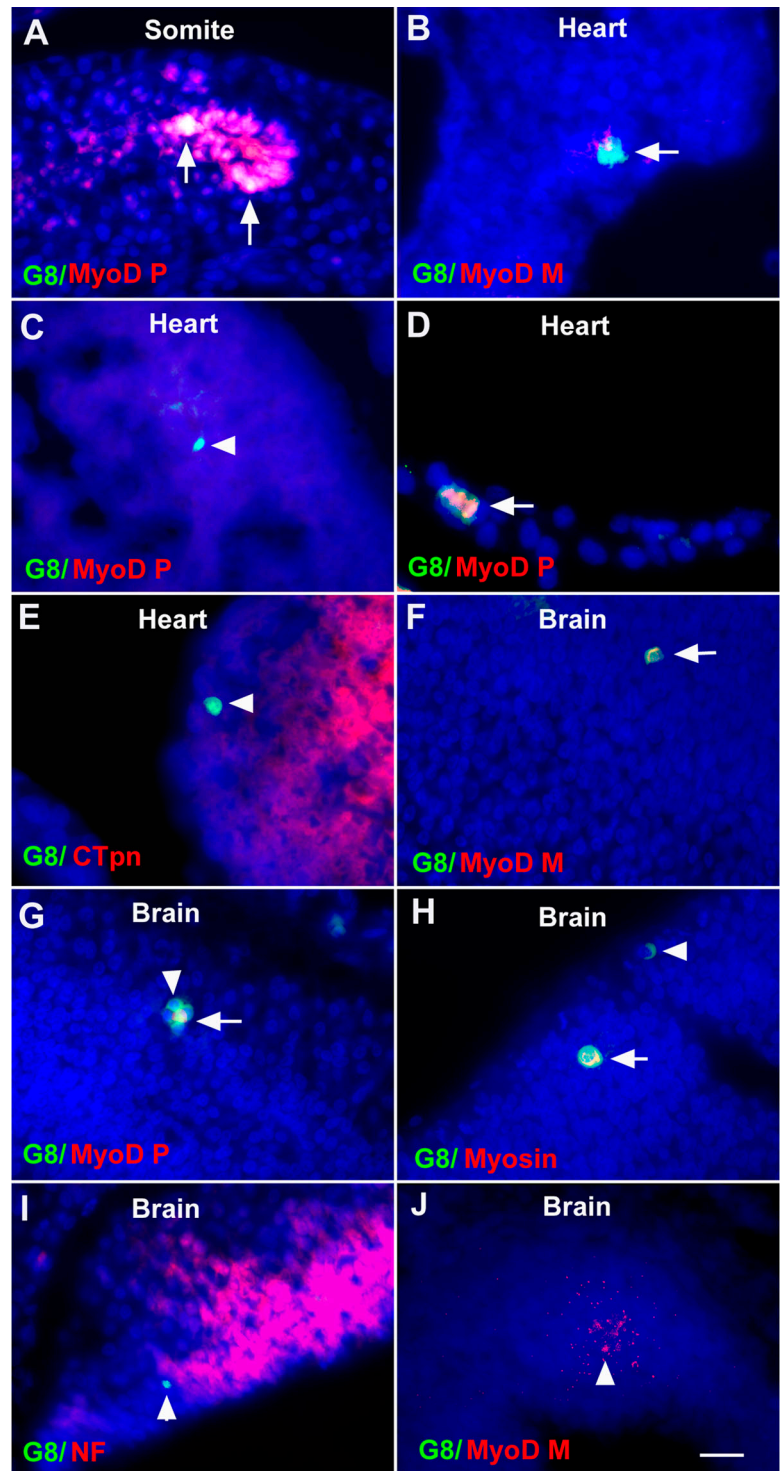

induced to differentiate into cardiac muscle or neurons in the developing heart and nervous system.

Microinjection of MyoD^pos and MyoD^neg epiblast cells into the precardiac mesoderm and neural plate

In the aforementioned tracking experiments, it is possible that the restriction of developmental potential may have occurred in cells on route to their final destination. Therefore, a second approach

was taken to challenge the behavior of MyoD^pos epiblast cells that involved microinjecting them directly into the precardiac mesoderm and neural plate. Stage 1 epiblasts were removed from the embryo, dissociated, labeled with the G8 mAb, and the G8^pos and G8^neg populations were isolated by magnetic cell sorting. The purity of both sorted populations was >97% (Gerhart et al., 2004a). Sorted cells were labeled with Hoechst dye, a procedure that did not affect their viability or ability to differentiate in vitro (unpublished data). 60 G8^pos or G8^neg Hoechst-labeled epiblast cells were
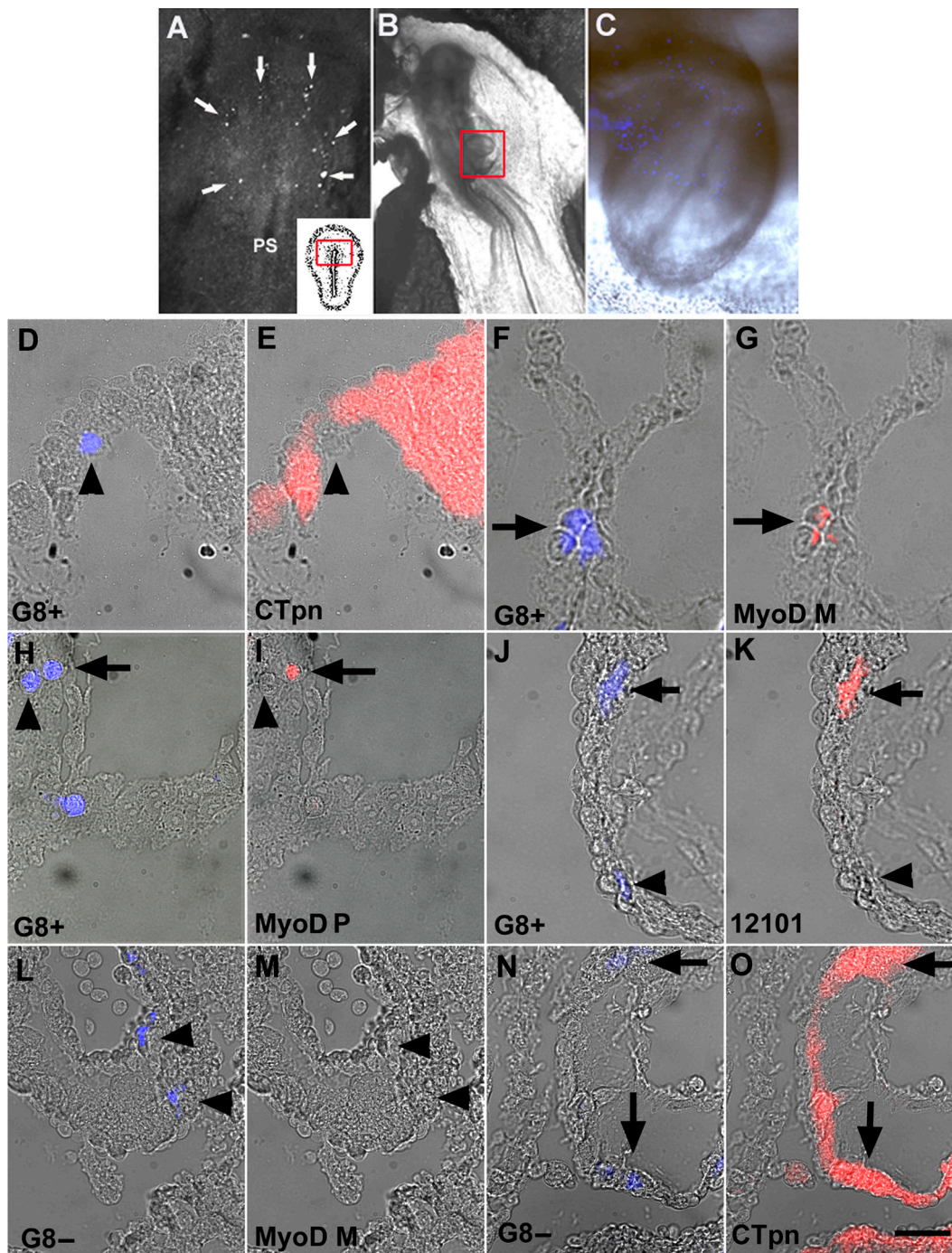
Figure 3. **Expression of skeletal and cardiac muscle markers in G8^{pos} and G8^{neg} epiblast cells microinjected into the developing heart.** G8^{pos} (G8+) and G8^{neg} (G8−) epiblast cells were labeled with Hoechst dye (blue) and microinjected into the precardiac mesoderm of the stage 4 embryo. (A) Microinjection sites (arrows) above and lateral to the primitive streak (PS) are shown in the photomicrograph of the area outlined in the inset. (B) Embryos developed normally after microinjection. (C) An enlargement of the heart containing G8^{pos} cells. (D–O) Sections were stained for markers of skeletal and cardiac muscle (red in E, G, I, K, M, and O). Cardiac troponin T was not detected in G8^{pos} cells (arrowheads in D and E). G8^{pos} cells expressed MyoD mRNA (arrows in F and G). Subpopulations of G8^{pos}/MyoD mAb^{pos} (arrows) and G8^{pos}/MyoD mAb^{neg} (arrowheads) were present in the heart (H and I). The 12101 marker of skeletal muscle differentiation was detected in some G8^{pos} cells (arrows in J and K) but not in others (arrowheads in J and K). G8^{neg} cells microinjected into the developing heart did not express MyoD mRNA (arrowheads in L and M). Instead, they differentiated into cardiac muscle (arrows in N and O). Bars (A and B), 56 μm; (C) 27 μm; (D–O) 9 μm.

microinjected into six sites (10 cells per site) of the precardiac mesoderm of stage 4–5 embryos (Fig. 3 A) or the neural plate of stage 6–7 embryos (Fig. 4 A). The microinjection procedure did not appear to affect morphogenesis of the heart or nervous system during the course of the experiment (Figs. 3, B and C; and 4, B–D). The expression of cell type–specific markers was analyzed in tissue sections and after the dissociation of tissues and centrifugation of the cell suspensions onto slides.
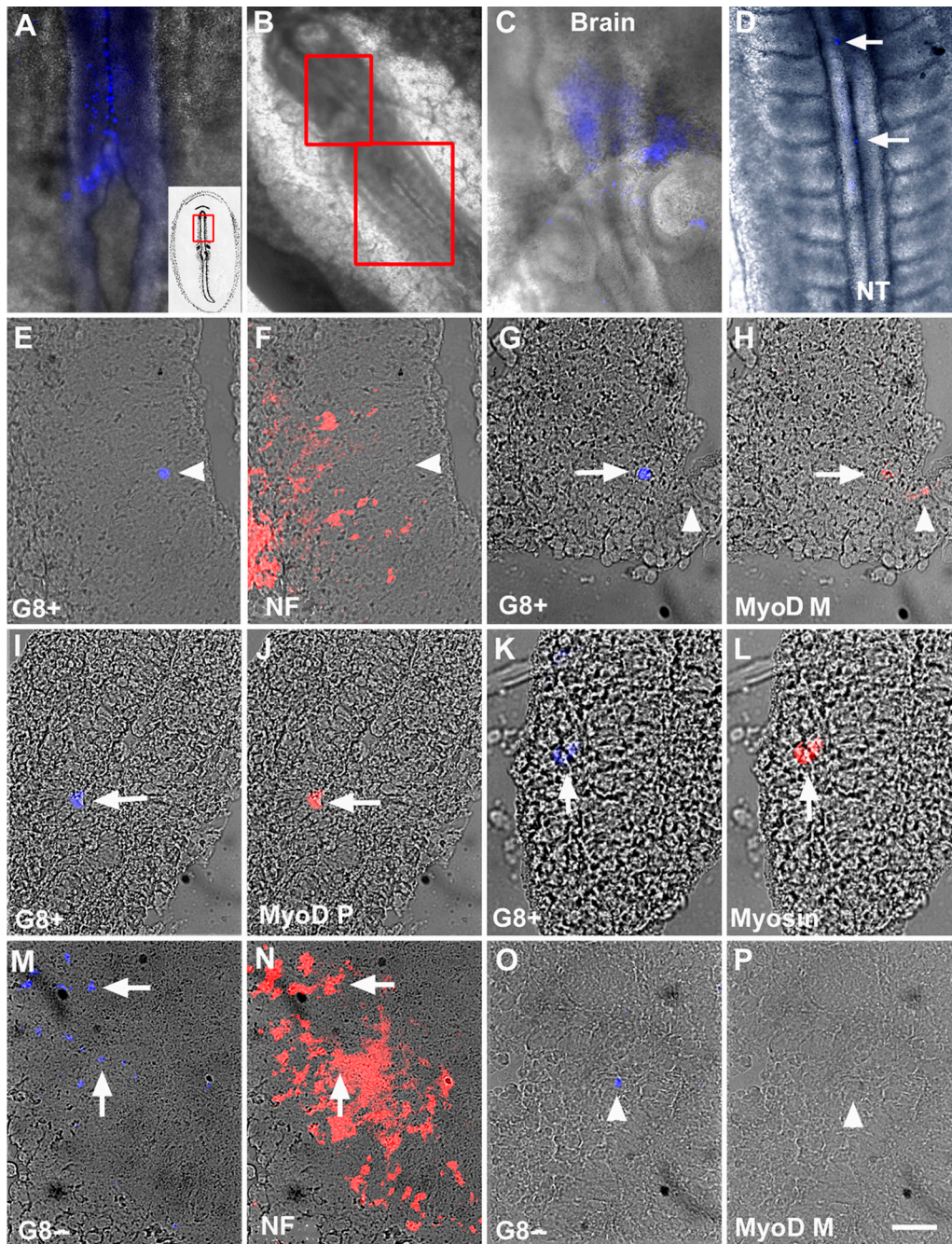
Figure 4. **Expression of skeletal muscle and neuronal markers in G8^{pos} and G8^{neg} epiblast cells microinjected into the developing nervous system.** G8^{pos} (G8+) and G8^{neg} (G8−) epiblast cells were labeled with Hoechst dye (blue) and microinjected into the neural plate of the stage 7 embryo. Microinjected cells are shown in the photomicrograph of the area outlined in the inset of A. Embryos were grown for 2.5 d. Areas outlined in B are shown at higher magnification in C and D. The brain (C) and neural tube (NT; D) contained microinjected cells. Sections through the brain were stained with markers for skeletal muscle and neurons (red in F, H, J, L, N, and P). Neurofilament-associated antigen (NF) was not detected in G8^{pos} cells (arrowheads in E and F). MyoD mRNA (MyoD M) was present in G8^{pos} cells in the brain (arrows in G and H). Some G8^{pos} cells stained with the MyoD mAb (MyoD P; arrows in I and J). A host cell lacking the G8 marker was positive for MyoD mRNA (arrowheads in G and H). A G8^{pos} cell expressing sarcomeric myosin is shown in K and L. G8^{neg} cells expressed neurofilament-associated antigen (arrows in M and N) but not MyoD mRNA (arrowheads in O and P). Bars (A and B), 56 μm; (C and D) 27 μm; (E–P) 9 μm.

Behavior of MyoD^{pos} and MyoD^{neg} epiblast cells microinjected into the precardiac mesoderm

After microinjecting cells into the precardiac mesoderm of stage 4–5 embryos, ∼94% of the Hoechst-labeled cells were later found in stage 12–14 hearts (Fig. 3 C). Microinjected G8^{pos} cells increased in number to a greater extent than G8^{neg} cells (2.5- and 1.9-fold, respectively; $P \le 0.05$). Some clusters of two to four G8^{pos} cells were found within the myocardium, although most were present as single cells surrounded by host cells (Fig. 3, D–K). The majority of microinjected G8^{neg} cells was present within the middle of the myocardium (Fig. 3, L and M), whereas

Table I. **Differentiation of G8^pos and G8^neg cells microinjected into the precardiac mesoderm**

	Percent positive			
	Myosin	**12101**	**MyoD**	**CTpn**
G8^pos cells	87 ± 11 (8)	40 ± 36 (8)	73 ± 13 (7)	0 (7)
Host cells	77 ± 16 (8)	1 ± 2 (8)	2 ± 1 (7)	82 ± 25 (8)
G8^neg cells	92 ± 9 (9)	0 (8)	0.4 ± 1 (7)	95 ± 8 (8)
Host cells	86 ± 10 (9)	0.4 ± 1 (9)	1 ± 1 (7)	88 ± 6 (8)

G8^pos and G8^neg cells were isolated from the stage 1 epiblast, labeled with Hoechst dye, and microinjected into the precardiac mesoderm of stage 4–5 embryos. Embryos were grown to stages 12–13 in culture. Hearts were removed and dissociated, and the cells were centrifuged onto slides. Cells were stained with mAbs to sarcomeric myosin, the skeletal muscle–specific 12101 antigen, MyoD, and cardiac muscle–specific troponin T (CTpn). More than 200 cells were scored per slide. The results are given as the mean ± SD. The number of slides scored is indicated in parentheses. G8^pos cells expressed markers of the skeletal muscle lineage and did not differentiate into cardiac muscle. G8^neg cells underwent cardiomyogenesis.

G8^pos epiblast cells often were found toward the periphery of the myocardium (Fig. 3, D–K), suggesting that some cell sorting may have occurred.

None of the Hoechst-labeled G8^pos cells contained detectable levels of cardiac troponin T (Fig. 3, D and E; and Table I). Instead, 99% contained MyoD mRNA (Fig. 3, F and G), and most were labeled with the MyoD mAb (Fig. 3, H and I; and Table I). Some of the microinjected G8^pos cells differentiated into skeletal muscle, as indicated by staining with the 12101 mAb (Fig. 3, J and K), although the percentage of these cells that expressed 12101 in vivo varied greatly between experiments (Table I). This may reflect a delay in the accumulation of this antigen after terminal differentiation because the majority of G8^pos cells contained sarcomeric myosin (Table I).

G8^pos epiblast cells microinjected into the precardiac mesoderm displayed a greater tendency to differentiate into skeletal muscle than those that were tracked from the epiblast into the heart. This suggests that the procedure for isolating and dissociating the epiblast in preparation for sorting and microinjection may have enhanced the ability of MyoD^pos cells to differentiate in foreign environments. Cell–cell interactions within the epiblast epithelium and a factor produced in the mesoderm are inhibitory for skeletal myogenesis (George-Weinstein et al., 1996).

The procedure for isolating epiblast cells is not sufficient to trigger skeletal myogenesis in epiblast cells that lack MyoD mRNA. Less than 1% of microinjected G8^neg cells or their progeny contained detectable levels of MyoD mRNA (Fig. 3, L and M) or MyoD protein, and none appeared to synthesize the 12101 antigen (Table I). Unlike the G8^pos cells, nearly all of the G8^neg cells that were microinjected into the precardiac mesoderm differentiated into cardiomyocytes (Fig. 3, N and O; and Table I). A greater percentage of G8^neg epiblast cells were labeled with the cardiac troponin antibody than host cells (P ≤ 0.03), illustrating their proclivity for differentiation.

A small decrease was found in the number of host cells that differentiated into cardiac muscle in embryos microinjected with G8^pos cells than G8^neg cells (Table I). Slightly more host cells were stained with mAbs to MyoD and 12101 in hearts implanted with G8^pos cells than G8^neg cells (Table I). This raises the possibility that microinjected G8^pos cells influence the pathway of the differentiation of host cells in the heart. The results obtained when epiblast cells were microinjected into the developing heart were consistent with the cell-tracking experiments.

That is, cells expressing MyoD mRNA in the epiblast continued to do so in the heart and were not redirected to the cardiac muscle lineage. In contrast, cells that lacked MyoD mRNA in the epiblast were capable of differentiating into cardiac muscle.

Behavior of G8^pos and G8^neg cells microinjected into the neural plate

The fate of epiblast cells that express MyoD mRNA was also tested in the developing nervous system. As was the case with implantations into the heart, the microinjected G8^pos cells increased in number to a greater extent than G8^neg cells (10-fold and ninefold, respectively). The higher rates of proliferation of cells microinjected into the neural plate than in the precardiac mesoderm is consistent with the observation that cardiomyocyte differentiation was nearly complete by the time the embryos were fixed for analysis (Manasek, 1968).

Greater than 80% of the microinjected cells were found in the head (Fig. 4 C). Within the head, 75% of Hoechst-labeled cells were present in the brain. Approximately 60% of the Hoechst-labeled cells in the trunk were found in the neural tube (Fig. 4 D). Most of the microinjected cells were surrounded by host cells, although some were present in clusters of two to four cells in the brain and neural tube (Fig. 4, E–P).

Neurofilament-associated antigen was not detected in any of the G8^pos cells microinjected into the neural plate even when they were surrounded by host cells containing this marker of neuronal differentiation (Fig. 4, E and F; and Table II). Instead, all G8^pos cells found within the CNS (Fig. 4, G and H) and other embryonic tissues contained MyoD mRNA. Within the CNS, some G8^pos cells were labeled with mAbs to MyoD protein (~70%; Fig. 4, I and J), sarcomeric myosin (~13%; Fig. 4, K and L), and the 12101 antigen (~7%). The majority (~55%) of Hoechst-labeled G8^pos cells present in the mesenchyme of the head or myogenic region of the somite had differentiated into skeletal muscle. A few host cells were found to express MyoD mRNA in the brain (Fig. 4, G and H).

In contrast to the behavior of G8^pos cells, ~70% of G8^neg cells that were microinjected into the neural plate and incorporated into nervous tissue expressed neurofilament-associated antigen (Fig. 4, M and N). Only 1% of the Hoechst-labeled G8^neg cells contained MyoD mRNA (Fig. 4, O and P), and none were stained with mAbs to MyoD protein or the 12101 antigen. All Hoechst-labeled G8^neg cells found in the myogenic region of the somite did express MyoD mRNA (unpublished data).

Table II. **Differentiation of G8^{pos} and G8^{neg} cells microinjected into the neural plate**

	Percent positive			
	Myosin	12101	MyoD	Neurofil.
Head				
G8^{pos} cells	35 ± 12 (8)	41 ± 27 (10)	70 ± 17 (8)	0 (8)
Host cells	9 ± 3 (8)	11 ± 1 (10)	15 ± 3 (8)	17 ± 2 (8)
G8^{neg} cells	17 ± 8 (8)	19 ± 10 (10)	15 ± 9 (10)	32 ± 6 (8)
Host cells	15 ± 6 (8)	9 ± 3 (10)	13 ± 3 (10)	19 ± 3 (8)
Trunk and limbs				
G8^{pos} cells	29 ± 45 (8)	57 ± 21 (10)	91 ± 12 (10)	0 (8)
Host cells	37 ± 4 (8)	13 ± 3 (10)	20 ± 4 (10)	16 ± 3 (8)
G8^{neg} cells	32 ± 33 (8)	40 ± 35 (11)	44 ± 30 (10)	27 ± 36 (8)
Host cells	41 ± 4 (8)	12 ± 6 (11)	29 ± 10 (10)	12 ± 6 (8)

G8^{pos} and G8^{neg} cells were isolated from stage 1 epiblasts, labeled with Hoechst dye, and microinjected into the neural plate of stage 6–7 embryos. Embryos were grown to stages 15–16 in culture. The heads were separated from the trunk, the tissues were dissociated, and the cells were centrifuged onto slides. Cells were stained with mAbs to sarcomeric myosin, the 12101 antigen, MyoD, and neurofilament-associated antigen (Neurofil). More than 200 cells were scored per slide. The results are the mean ± SD. The number of slides scored is indicated in parentheses. G8^{pos} cells expressed markers of the skeletal muscle lineage and did not differentiate into neurons. Subpopulations of G8^{neg} cells differentiated into skeletal muscle or neurons.

A precise determination of the percentages of cells microinjected into the neural plate that later differentiated into neurons or skeletal muscle throughout the embryo was calculated by separating the head from the trunk, dissociating the tissues to produce a single-cell suspension, centrifuging the cells onto slides, and staining with antibodies (Table II). Significantly more Hoechst-labeled G8^{pos} than G8^{neg} cells were stained with the MyoD mAb (head, P ≤ 0.000005; trunk, P ≤ 0.0003) and differentiated into skeletal muscle (head, P ≤ 0.02). Importantly, no microinjected G8^{pos} epiblast cells were stained with the antibody to neurofilament-associated antigen, whereas approximately one third of the microinjected G8^{neg} cells in the head and 10% in the remainder of the embryo expressed this marker of neurogenesis.

The results of experiments involving the microinjection of epiblast cells into the neural plate were consistent with those in which cells were tracked into the nervous system. They also mirrored the data obtained when epiblast cells were microinjected or tracked into the heart. Regardless of whether MyoD^{pos} cells were incorporated into the nervous system or heart, they either remained as skeletal muscle precursors or formed skeletal muscle. They did not differentiate into neurons or cardiac muscle.

Behavior of microinjected MyoD^{pos} epiblast cells in culture

Although G8^{pos} epiblast cells microinjected into the precardiac mesoderm and neural plate continued to express MyoD mRNA in the heart and nervous system, only a subpopulation synthesized sarcomeric myosin and the 12101 antigen. To test whether the population of microinjected G8^{pos} epiblast cells that remained undifferentiated was capable of undergoing skeletal myogenesis, cell cultures were prepared from hearts, heads, and trunks. Because epiblast cells lacking MyoD mRNA in vivo do not form skeletal muscle in this culture system, the conditions are permissive and not instructive for skeletal myogenesis (Gerhart et al., 2004a; Strony et al., 2005). 4 d after plating, 90–100% of Hoechst-labeled G8^{pos} epiblast cells that had been microinjected into the embryo synthesized the 12101 antigen (90 ± 5% from the heart, 95 ± 4% from the head, and 100% from the trunk; n = 3 cultures per

region; Fig. 5, A–C). Therefore, MyoD^{pos} epiblast cells microinjected into the precardiac mesoderm and neural plate are able to differentiate into skeletal muscle in a permissive environment.

Discussion

MyoD mRNA is expressed in a subpopulation of cells in the epiblast before the onset of gastrulation (George-Weinstein et al., 1996; Gerhart et al., 2000, 2006; Strony et al., 2005). When these cells are isolated from the stage 1 chick embryo and cultured in serum-free medium, >95% differentiate into skeletal muscle (Gerhart et al., 2004a). In this study, we tested whether MyoD^{pos} epiblast cells are stably committed to the skeletal muscle lineage by examining their behavior in environments that promote cardiomyogenesis and neurogenesis.

Cells expressing MyoD mRNA in the epiblast were either labeled in vivo and tracked into the heart and nervous system or isolated from stage 1 embryos and microinjected into the precardiac mesoderm and neural plate of gastrulating embryos. Both types of experiments revealed that MyoD^{pos} epiblast cells survived, proliferated, and continued to express MyoD in the heart and nervous system. A subpopulation of these cells differentiated into skeletal muscle in these ectopic locations. Nearly all MyoD^{pos} epiblast cells that were microinjected into the heart and nervous system differentiated into skeletal muscle when placed in culture. These findings indicate that epiblast cells expressing MyoD mRNA but lacking detectable MyoD protein or mRNA for Myf5 (this study), myogenin, or sarcomeric myosin (George-Weinstein et al., 1996; Gerhart et al., 2000) are stably committed to the skeletal muscle lineage before the onset of gastrulation. Therefore, incorporation into the mesoderm and somites is not required for the specification and determination of this enigmatic population of skeletal muscle stem cells.

Most cells that do not express MyoD in the epiblast appear to be uncommitted and multipotent because they differentiate into either cardiac muscle or neurons when microinjected into the precardiac mesoderm or neural plate. Their proclivity for differentiating according to environmental signals was revealed within the context of a developing tissue. Multipotent stem cells
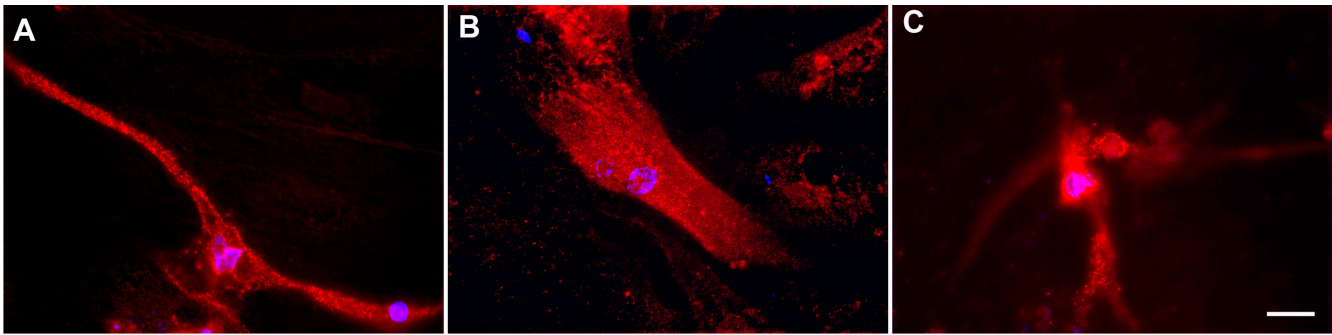
Figure 5. **Differentiation of microinjected G8^{pos} epiblast cells in vitro.** G8^{pos} cells were isolated from the stage 1 epiblast, labeled with Hoechst dye (blue), and microinjected into the stage 4 precardiac mesoderm and stage 7 neural plate. 2 d later, the hearts (A), heads (B), and trunks (C) were dissociated, and the cells were cultured for 5 d and stained with the 12101 mAb. Hoechst-labeled cells differentiated into skeletal muscle, whereas surrounding host cells were not stained with the 12101 mAb. Bar, 9 μm.

used for tissue regeneration may not display the same ability to differentiate into the desired cell type because the environment within diseased tissues of the adult may lack the molecules required for inducing specification, determination, and differentiation. Premixing multipotent cells with stably committed cells capable of producing factors that recruit unspecified cells to the appropriate lineage may improve the efficiency of tissue regeneration.

Our cell-tracking experiments revealed that the majority of MyoD^{pos} epiblast cells were incorporated into the somites (Gerhart et al., 2006; and this study). Although many of these cells differentiated into skeletal muscle, some were retained in the dermomyotome region of the somite that contains replicating myogenic precursors. Therefore, MyoD^{pos} epiblast cells may undergo self-renewal and produce offspring that differentiate in the somite.

Some MyoD^{pos} epiblast cells were integrated in the heart, nervous system, and other structures. This observation is consistent with the locations of MyoD^{pos} cells in the epiblast that correspond to areas fated to give rise to somites as well as nonsomitic tissues (Gerhart et al., 2000). Given their location in the epiblast, it is likely that cells with MyoD mRNA followed similar routes to those taken by other epiblast cells destined for integration into the heart and nervous system. In this case, MyoD^{pos} cells would be exposed to factors that regulate early stages in the specification of cells to the cardiogenic or neurogenic lineages.

Specification of the nervous system begins before gastrulation in the central epiblast, as indicated by the expression of the preneural markers ENRI and SOX3; however, the definitive neural marker SOX2 does not appear until neural plate formation (Streit et al., 2000; Wilson et al., 2000; Linker and Stern, 2004; Stern, 2005). Specification of cardiomyocytes also begins before the onset of gastrulation in the posterior epiblast (Yatskievych et al., 1997; Ladd et al., 1998), although the process continues in an anterior to posterior direction in the primitive heart (Linask and Lash, 1988; Linask et al., 1997). Exposure to early inducers of heart specification in the posterior epiblast may not be required for recruitment to the cardiomyocyte lineage because >95% of MyoD^{neg} cells located throughout the epiblast differentiated into cardiac muscle when microinjected into the precardiac mesoderm.

We previously determined that small numbers of MyoD^{pos} cells are present in the heart, brain, and other organs of the fetal chicken (Gerhart et al., 2001). Ectopically located skeletal muscle precursors of the fetus may be the direct descendants of MyoD^{pos} epiblast cells that were incorporated into all three germ layers during early stages of development. Murine embryos also contain ectopically located skeletal muscle precursors, as indicated by the expression of Myf5 in the nervous system and the emergence of skeletal muscle in cultures prepared from mouse neural tubes (Tajbakhsh et al., 1994; Tajbakhsh and Buckingham, 1995). It remains to be determined whether precursor cells expressing MyoD survive in ectopic locations of the adult. If so, they may be capable of proliferating in response to inflammatory cytokines and growth factors. Skeletal muscle precursors may be vulnerable to mutations leading to the development of rhabdomyosarcoma tumors, which often arise outside of skeletal muscle (Dagher and Helman, 1999).

MyoD^{pos} epiblast cells incorporated into seemingly ectopic locations may be involved in the formation of nonskeletal muscle tissues by serving as a local source of a BMP inhibitor. Cells that express MyoD in the epiblast produce Noggin both inside and outside of the somites (Gerhart et al., 2006). Within the somites, the release of Noggin from MyoD^{pos} epiblast cells is critical for skeletal muscle differentiation (Gerhart et al., 2006). Noggin derived from MyoD^{pos} epiblast cells also may be important for the development of other structures because ablation of these cells in the epiblast results in facial and eye defects (Gerhart et al., 2006).

The population of MyoD^{pos} epiblast cells appears to be distinct from those cells that become committed to undergo myogenesis within the somites. Uncommitted myogenic precursors in the somites express Pax-3 and Pax-7 but not Myf5 or MyoD (Goulding et al., 1994; Williams and Ordahl, 1994; Maroto et al., 1997; Tajbakhsh et al., 1997; Kassar-Duchossoy et al., 2005). Pax-3–positive precursor cells are present in the somites after MyoD^{pos} cells are ablated in the epiblast, and they differentiate in response to exogenous Noggin (Gerhart et al., 2006). Therefore, myogenic precursors that arise in the somites do not appear to be the direct descendants of cells that express MyoD in the stage 2 epiblast.

Another difference between MyoD^{pos} epiblast cells and the majority of myogenic precursors in the somite is that the latter express Myf5 before MyoD (Sassoon et al., 1989; Ott et al., 1991; Maroto et al., 1997; Hacker and Guthrie, 1998; Hirsinger et al., 2001; Kiefer and Hauschka, 2001). In contrast, MyoD but not Myf5 mRNA is present in stage 1 and 2 chick embryos (Kiefer and Hauschka, 2001; and this study). By stage 3, only a subpopulation of epiblast cells with MyoD coexpresses Myf5 and vice versa.

In conclusion, the expression of MyoD mRNA in the pregastrulating epiblast defines a unique population of stem cells that are committed to the skeletal muscle lineage and are capable of self-renewing and differentiating. These cells promote the differentiation of a separate population of skeletal muscle precursors that arise within the mesoderm (Gerhart et al., 2006). MyoD^{pos} epiblast cells are also integrated into nonsomitic tissues of the embryo. In these seemingly ectopic locations, they retain their identity as skeletal muscle stem cells (this study) and produce Noggin (Gerhart et al., 2006). Stable programming within the epiblast may ensure that MyoD^{pos} cells express similar regulatory molecules in a variety of environments.

Materials and methods

Double labeling of whole embryos

White Leghorn chick embryos (B E Eggs) were staged according to the method of Hamburger and Hamilton (1951). Three stage 1, four stage 2, four stage 3, and three stage 4 whole embryos were double labeled for the G8 antigen and MyoD protein. G8 is a cell surface antigen that is specifically expressed in epiblast cells that contain MyoD mRNA (Gerhart et al., 2001, 2004a,b; Strony et al., 2005). Embryos were incubated with 35 μg G8 mAb diluted in 100 μl of 10% goat serum in PBS and goat anti–mouse IgM antibodies conjugated with AlexaFluor488 (Invitrogen) diluted 1:1,000 in 10% goat serum in PBS. After permeabilizing with 0.5% Triton X-100, embryos were incubated with NCL-MyoD1 mAb to MyoD (Novacastra) diluted 1:1,000 and with goat anti–mouse IgG Fab'2 fragments conjugated with rhodamine (Jackson ImmunoResearch Laboratories) diluted 1:400 in 10% goat serum in PBS. The NCL-MyoD1 mAb labels pectoralis skeletal muscle and G8^{pos} epiblast cells but not G8^{neg} epiblast cells, cardiac muscle, or fibroblasts in culture. This mAb also stains the dermomyotome, myotome, and small numbers of cells that express MyoD mRNA outside of the somites in vivo. Stage 2 and 4 whole embryos also were labeled with the rabbit 6975B polyclonal antiserum to MyoD (a gift from S. Tapscott, Fred Hutchinson Cancer Research Center, Seattle, WA) and a goat anti–rabbit IgG conjugated with rhodamine (Chemicon). Nuclei were counterstained with 1 μg/ml of Hoechst dye in deionized water (Sigma-Aldrich) for 10 min. Labeling of embryos was performed at room temperature.

Three stage 1, two stage 2, two stage 3, and four stage 4 whole embryos were double labeled for the G8 antigen and Myf5 mRNA as described previously (Gerhart et al., 2001, 2004a,b; Strony et al., 2005). The G8 mAb was tagged with secondary antibodies conjugated with AlexaFluor488 as described in the previous paragraph. After permeabilizing in 0.1% Triton X-100 and 0.1% pepsin (Sigma-Aldrich), embryos were incubated in Cy3-labeled 3DNA dendrimers (Genisphere, Inc.) conjugated with an antisense cDNA sequence for chicken Myf5 mRNA (S53719; 5'-ATATAGTGGATGGCAGAGCTGAGGATTTCG-3'; Neville et al., 1992). Dendrimers lacking cDNAs for a specific mRNA were used as a negative control for background fluorescence. Nuclei were counterstained with Hoechst dye as described in the previous paragraph.

Embryos were mounted in Gelmount (Biomeda) and observed with an epifluorescence microscope (Eclipse E800; Nikon) equipped with the following filters: excitation at 530–560 nm and barrier at 573–648 nm for Cy3 and rhodamine; excitation at 465–495 nm and barrier at 515–555 nm for AlexaFluor488; and excitation at 330–380 nm and barrier at 435–485 nm for Hoechst dye using 4× NA 0.2 and 60× NA 1.4 oil objectives (Nikon). Photomicrographs were produced with a video camera (Evolution QE; Media Cybernetics) and Image-Pro Plus image analysis software (Phase 3 Imaging Systems). Figures were annotated and adjusted for contrast and brightness in Photoshop 6.0 (Adobe).

Tracking MyoD^{pos} epiblast cells in the embryo

The tracking of MyoD^{pos} epiblast cells was performed as described previously (Gerhart et al., 2006). In brief, stage 2 embryos were removed from the shell on the yolk and incubated for 45 min at room temperature in 35 μg G8 mAb/100 μl HBSS (Invitrogen) and rinsed three times in PBS. 100 μl of secondary antibodies conjugated with rhodamine diluted 1:400 in Hanks buffer or AlexaFluor488 diluted 1:1,000 in Hanks buffer was added for 45 min, and the embryos were rinsed three times in PBS. Labeled embryos still on the yolk were placed in an empty shell, incubated at 37°C, fixed in formaldehyde, embedded in paraffin, and sectioned transversely at 10 μm.

Paraffin sections were labeled with mAbs to sarcomeric myosin heavy chain (MF20 mAb diluted 1:60; Bader et al., 1982), the skeletal muscle–specific 12101 antigen (12101 mAb diluted 1:10; Kintner and Brockes, 1984), neurofilament-associated antigen (3A10 mAb diluted 1:400; Furley et al., 1990), MyoD1 (NCL-MyoD1 diluted 1:150), or cardiac troponin T (AB-1 mAb diluted 1:400; Neomarkers). The 12101 mAb stained skeletal muscle in cultures of pectoralis muscle and G8^{pos} epiblast cells and in sections through the myotomes and limbs. 12101 did not stain cardiac muscle in vivo or in vitro. The cardiac troponin T mAb labeled cardiac muscle in culture and in sections through the heart but did not stain skeletal muscle in sections through the somites and limbs or cultures of pectoralis muscle or G8^{pos} epiblast cells (Gerhart et al., 2004a).

Primary antibodies were labeled with secondary antibodies conjugated with rhodamine or AlexaFluor488. All antibodies were diluted in 10% goat serum in PBS. The MF20, 12101, and 3A10 mAbs were obtained from the Developmental Studies Hybridoma Bank. Most sections were double labeled with antibodies and Cy3-labeled dendrimers conjugated with an antisense oligonucleotide sequence to chicken MyoD (5'-TTCT-CAAGAGCAAATACTCACCATTTGGTGATTCCGTGTAGTA-3' [L34006; Dechesne et al., 1994]). Nuclei were counterstained with Hoechst dye. The sites of incorporation of G8^{pos} epiblast cells were determined in two stage 16 embryos.

Microinjection of G8[pos] and G8^{neg} epiblast cells

G8[pos] and G8^{neg} cells were separated by magnetic cell sorting as described previously (Gerhart et al., 2004a; Strony et al., 2005) In brief, epiblasts were isolated from the stage 1 embryo, dissociated in 0.25% trypsin-EDTA (Invitrogen) for 10 min, incubated with the G8 mAb for 45 min, and labeled with rat anti–mouse IgM microbeads (Miltenyi Biotec). Nuclei were counterstained with 1 μg Hoechst dye/ml Hanks buffer. Sorting of G8[pos] and G8^{neg} cells was performed in a MiniMACS column (Miltenyi Biotec). After centrifugation, cells were resuspended in PBS (1,000 cells/μl). 2 μl of fast green solution (0.1 μl of fast green/μl of 70% glycerol; both were obtained from Sigma-Aldrich) was added to the cell suspension.

Embryos were rinsed in Hanks buffer and placed on nucleopore filters. Microinjections were performed with a microinjector (Nanoject ll; Drummond Scientific). 10 cells were microinjected into each of six sites in the precardiac mesoderm (Fig. 3 A). Bilateral injections were made just above the rostral end of the head process, lateral to the head process, and lateral to the rostral end of the primitive streak. Implantations into the neural plate of stage 6–7 embryos consisted of six microinjections along the midline of the developing neural folds above the rostral end of the primitive streak (Fig. 4 A). Embryos on the nucleopore filters were transferred to nine-well 2.5-cm plates containing thin egg albumen and a piece of ashless filter paper (Whatman) with a hole cut in its center. The nine-well plate was placed in a Petri dish on filter paper moistened with PBS and incubated at 37°C with 5% CO_2 for 48 h.

Embryos receiving microinjections into the precardiac mesoderm (two embryos with G8^{pos} cells and two embryos with G8^{neg} cells) were fixed at stages 12–14, embedded in paraffin, and serially sectioned. Embryos that were microinjected into the neural plate (two embryos with G8^{pos} cells and two embryos with G8^{neg} cells) were fixed at stages 15–16. Sections were labeled with dendrimers to MyoD mRNA and/or mAbs to MyoD, MF20, 12101, cardiac troponin T, or 3A10 as described above (see Double labeling of whole embryos).

Other embryos receiving microinjections into the precardiac mesoderm had their hearts removed and were dissociated in 0.25% trypsin-EDTA (Sigma-Aldrich) for 10 min to produce a single-cell suspension. Embryos that were microinjected in the neural plate were cut to separate the head from the trunk. The heart was then removed from the trunk so that analyses of myosin expression would reflect skeletal and not cardiac muscle. Heads and trunks were also dissociated in trypsin-EDTA. Cells were fixed in formalin, centrifuged onto gelatin-coated glass slides for 4 min in a Cytofuge 2 centrifuge (Statspin), and stained with antibodies as described above (see Double labeling of whole embryos).

Hearts were obtained from seven stage 10–14 embryos that received microinjections of G8[pos] or G8^{neg} cells into the precardiac mesoderm (three experiments; two to three hearts pooled per experiment). Head and trunk cells were obtained from seven stage 15–17 embryos that received microinjections into the neural plate (two experiments; three to four embryos pooled per experiment). A *t* test was used to compare populations.

Cell culture

2 d after Hoechst-labeled G8[pos] and G8[neg] stage 1 epiblast cells were microinjected into the precardiac mesoderm or neural plate, the hearts, heads, and trunks from two embryos were dissociated in trypsin-EDTA. Cells were plated at a density of 20,000/15 µl of medium on gelatin- and fibronectin-coated dishes as described previously (George-Weinstein et al., 1994, 1996). Dishes were flooded with 1.5 ml DME/F12 (Invitrogen). Cells were fixed in 2% formaldehyde after 4 d in culture and stained with the 12101 mAb.

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