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

Jacquelyn Gerhart,1 Christine Neely,1 Justin Elder,1 Jessica Pfautz,1 Jordanna Perlman,1 Luis Narciso,1 Kersti K. Linask,2 Karen Knudsen,3 and Mindy George-Weinstein1

1Center for Chronic Disorders of Aging, Philadelphia College of Osteopathic Medicine, Philadelphia, PA 19131
2University of South Florida, St. Petersburg, Fl 33701
3Lankenau Institute for Medical Research, Wynnewood, PA 19096

The epiblast of the chick embryo contains cells that express MyoD mRNA but not MyoD protein. We investigated whether MyoD-positive (MyoDpos) epiblast cells are stably committed to the skeletal muscle lineage or whether their fate can be altered in different environments. A small number of MyoDpos epiblast cells were tracked into the heart and nervous system. In these locations, they expressed MyoD mRNA and some synthesized MyoD protein. No MyoDpos epiblast cells differentiated into cardiac muscle or neurons. Similar results were obtained when MyoDpos 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 MyoDpos 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 Asamato, 1988; Christ et al., 1992; Ordahl and Le Douarin, 1992; Brand-Saberi et al., 1993; Pourquié 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; Morato 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 enzymic 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 (MyoDpos) 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 MyoDpos 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; Tonge and Takahashi, 1998; Amthor et al., 1999; Sela-Donenfeld and Kalcheim, 2002; Linker et al., 2003). Ablation of MyoDpos 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 MyoDpos 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; Tonge 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 MyoDpos epiblast cells was examined in two ways. First, cells that express MyoD in the epiblast were tracked into the heart and nervous system. Second, MyoDpos cells were isolated from the epiblast and microinjected into the precardiac mesoderm and neural plate. In both types of experiments, MyoDpos 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 (G8pos) 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.

MyoDpos 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 G8pos cells...
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 MyoDpos 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 G8pos 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 (G8neg) cells. Within the somites, all of the G8pos cells contained MyoD protein (Fig. 2 A), and the majority (73%) had synthesized sarcomeric myosin.

All of the G8pos 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 G8pos cells in the stage 16 heart (Fig. 2 E). Within the central nervous system (CNS) of the stage 16 embryo, all G8pos 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 G8pos cell contained sarcomeric myosin (Fig. 2 H). None of the G8pos 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 G8pos cells (~36 cells) were found in hearts directly labeled with the G8 mAb than the number of G8pos 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 G8pos 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 G8pos epiblast cells are not
induced to differentiate into cardiac muscle or neurons in the developing heart and nervous system.

**Microinjection of MyoDpos and MyoDneg 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 MyoDpos 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 G8pos and G8neg 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 G8pos or G8neg Hoechst-labeled epiblast cells were

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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<sup>pos</sup> and G8<sup>neg</sup> epiblast cells microinjected into the developing nervous system. G8<sup>pos</sup> (G8<sup>+</sup>) and G8<sup>neg</sup> (G8<sup>−</sup>) 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<sup>pos</sup> cells (arrowheads in E and F). MyoD mRNA (MyoD <sub>M</sub>) was present in G8<sup>pos</sup> cells in the brain (arrows in G and H). Some G8<sup>pos</sup> cells stained with the MyoD mAb (MyoD <sub>P</sub>; arrows in I and J). A host cell lacking the G8 marker was positive for MyoD mRNA (arrowheads in G and H). A G8<sup>pos</sup> cell expressing sarcomeric myosin is shown in K and L. G8<sup>neg</sup> 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<sup>pos</sup> and MyoD<sup>neg</sup> 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<sup>pos</sup> cells increased in number to a greater extent than G8<sup>neg</sup> cells (2.5- and 1.9-fold, respectively; P ≤ 0.05). Some clusters of two to four G8<sup>pos</sup> 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<sup>neg</sup> cells was present within the middle of the myocardium (Fig. 3, L and M), whereas
Host cells were stained with mAbs to MyoD and 12101 in hearts implanted with G8pos and G8neg cells (Table I). Some of the microinjected G8pos cells differentiated into cardiomyocytes (Fig. 3, N and O; and Table I). This raises the possibility that microinjected G8pos cells infl uence the pathway of 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.

G8pos 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 G8pos 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 G8pos 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 refl ect a delay in the accumulation of this antigen after terminal differentiation because the majority of G8pos cells contained sarcomeric myosin (Table I).

G8pos 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 MyoDpos 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 G8neg 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 G8pos cells, nearly all of the G8neg cells that were microinjected into the precardiac mesoderm differentiated into cardiac myocytes (Fig. 3, N and O; and Table I). A greater percentage of G8neg 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 G8pos cells than G8neg cells (Table I). Slightly more host cells were stained with mAbs to MyoD and 12101 in hearts implanted with G8pos cells than G8neg cells (Table I). This raises the possibility that microinjected G8pos cells infl uence the pathway of 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 G8pos and G8neg 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 G8pos cells increased in number to a greater extent than G8neg 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 fi xed 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 G8pos 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 G8pos cells found within the CNS (Fig. 4, G and H) and other embryonic tissues contained MyoD mRNA. Within the CNS, some G8pos 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 G8pos 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 G8pos cells, ~70% of G8neg 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 G8neg 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 G8neg cells found in the myogenic region of the somite did express MyoD mRNA (unpublished data).

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### Table I. Differentiation of G8pos and G8neg cells microinjected into the precardiac mesoderm

|                  | Myosin | 12101 | MyoD | CTpn |
|------------------|--------|-------|------|------|
| G8pos 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) |
| G8neg 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) |

G8pos and G8neg 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 MyoD and 12101 in hearts implanted with G8pos cells than G8neg cells (Table I). 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.
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 G8pos 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 G8neg epiblast cells were stained with the antibody to neurofilament-associated antigen, whereas approximately one third of the microinjected G8neg 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 MyoDpos 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 MyoDpos epiblast cells in culture

Although G8pos 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 G8pos 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 G8pos 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, MyoDpos 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 MyoDpos 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 MyoDpos 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 MyoDpos 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...
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 MyoDpos 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, MyoDpos epiblast cells may undergo self-renewal and produce offspring that differentiate in the somite.

Some MyoDpos epiblast cells were integrated in the heart, nervous system, and other structures. This observation is consistent with the locations of MyoDpos cells in the epiblast that correspond to areas fated to give rise to somites as well as nonsomatic 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, MyoDpos 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 posterior epiblast, as indicated by the expression of the preneural markers EN1 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 MyoDpos cells located throughout the epiblast differentiated into cardiac muscle when microinjected into the precardiac mesoderm.

We previously determined that small numbers of MyoDpos 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 MyoDpos 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).

MyoDpos 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 MyoDpos epiblast cells is critical for skeletal muscle differentiation (Gerhart et al., 2006). Noggin derived from MyoDpos 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 MyoDpos 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 MyoDpos 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<sup>pos</sup> 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 pre-gastrulating 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<sup>pos</sup> epiblast cells are also integrated into non-somitic 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<sup>pos</sup> 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 myogenic potentials (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 (Novoceastra) 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<sup>pos</sup> epiblast cells but not G8<sup>pos</sup> 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 antisemur to MyoD (a gift from S. Taspcott, 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 CDNA probes (Genisphere, Inc.) conjugated with antisense CDNA sequences for chicken Myf5 mRNA (S33719; 5′-ATATAGTGATCGACAGCTGGATTCCG-3′; Neville et al., 1992). Dendrimers lacking CDNA 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 (Biomedica) and observed with an epifluorescence microscope (Eclipse E800; Nikon) equipped with the following filters: excitation at 330–360 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 4X NA 0.2 and 60X NA 1.4 oil objectives (Nikon). Photomicrographs were produced with a video camera (Evolution QE; Media Cybernetics) and ImagePro Plus image analysis software (PhaseL Abs Imaging Systems). Figures were annotated and adjusted for contrast and brightness in Photoshop 6.0 (Adobe).

**Tracking MyoD<sup>pos</sup> epiblast cells in the embryo**

The tracking of MyoD<sup>pos</sup> 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 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 Bronner-Fraser, 1984], neural/fibroblast-associated antigens (12101 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 sections of pectoralis muscle and G8<sup>pos</sup> 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 pectoral muscle or G8<sup>pos</sup> 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′-TTCG-CAAGGACCAAATCTACCACTTGGTAGAATCCGGATGATA-3′ [L34006; Dechesne et al., 1994]). Nuclei were counterstained with Hoechst dye.

The sites of incorporation of G8<sup>pos</sup> epiblast cells were determined in two stage 16 embryos.

**Microinjection of G8<sup>pos</sup> and G8<sup>neg</sup> epiblast cells**

G8<sup>pos</sup> and G8<sup>neg</sup> cells were separated by magnetic cell sorting as described previously (Strony et al., 2003; Strony et al., 2005). In brief, epiblasts were isolated from the stage 1 embryo, dissociated in 0.25% trypsin-EDTA and collected, and G8<sup>pos</sup> and G8<sup>neg</sup> cells was performed in a MiniMACS column (Miltenyi Biotec). After centrifugation, cells were reuspended in PBS (1,000 cells/μl), 2 μl of fast green solution (0.1 g/l 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 (Nanoporject II; Drummond Scientific). 10 cells were microinjected into each of six sites in the precardiac mesoderm (Fig. 3 A). Bilateral injections were made just above and lateral to the rostral end of the primitive streak and across the midline of the developing neural folds above 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% CO2 for 48 h.

Embryos receiving microinjections into the precardiac mesoderm (two embryos with G8<sup>pos</sup> cells and two embryos with G8<sup>neg</sup> 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<sup>pos</sup> cells and two embryos with G8<sup>neg</sup> 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 formaldehyde, 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 G8pos or G8neg cells into the precardiac mesoderm (three experiments; two to three hearts pooled per experiment). Head and trunc Canal 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 test was used to compare populations.

Cell culture

2 d after Hoechst-labeled G8pos and G8neg 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 1.2 ml DMEM/F12 (Invitrogen). Cells were fixed in 2% formaldehyde after 4 d in culture and stained with the 12101 mAb.

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References

Amthor, H., B. Christ, and K. Patel. 1999. A molecular mechanism enabling continuous embryonic muscle growth-a balance between proliferation and differentiation. Development. 126:1041–1053.

Aoyama, H., and K. Asamoto. 1988. Determination of somite cells: independence of cell differentiation and morphogenesis. Development. 104:15–28.

Bachiller, D., J. Klingensmith, C. Kemp, J.A. Belo, R.M. Anderson, S.R. May, J.A. McMahon, A.P. McMahon, R.M. Harland, J. Rossant, and E.M. De Robertis. 2000. The organizer factors Chordin and Noggin are required for mouse forebrain development. Nature. 403:658–661.

Bader, D., T. Masaki, and D.A. Fischman. 1982. Immunochemical analysis of the chicken MyoD (CMD1) promoter reveal an indirect regulatory pathway. Mol. Cell. Biol. 4:34–44.

Dockter, J., and C.P. Ordahl. 2000. Dorsoventral axis determination in the somite: a re-examination. Development. 127:2201–2206.

Dudley, A.T., and E.J. Robertson. 1997. Overlapping expression domains of bone morphogenetic protein family members potentially account for limited tissue defects in BMP-7 deficient embryos. Dev. Dyn. 208:349–362.

Furler, A.J., S.B. Morton, D. Manaol, D. Karagogeos, J. Dodd, and T.M. Jessel. 1999. The axonal glycoprotein TAG-1 is an immunoglobulin superfamily member with neurite outgrowth promoting activity. Cell. 61:157–170.

George-Weinstein, M., J. Gerhart, F. Foti, and J.W. Lash. 1994. Maturation of myogenic and chondrogenic cells in the presomitic mesoderm of the chick embryo. Exp. Cell Res. 211:263–274.

George-Weinstein, M., J. Gerhart, R. Reed, J. Flynn, B. Callihan, M. Mattucci, C. Miehle, G. Foti, J.W. Lash, and H. Weintraub. 1996. Skeletal myogenesis: the preferred pathway of chick embryo epiblast cells in vitro. Dev. Biol. 173:279–291.

Gerhart, J., M. Baytion, S. DeLuca, R. Getts, C. Lopez, R. Niewenhuis, T. Nilsen, S. Oles, H. Weintraub, and M. George-Weinstein. 2000. DNA dendrimers localize MyoD mRNA in presomitic tissues of the chick embryo. J. Cell Biol. 149:825–833.

Gerhart, J., B. Bast, C. Neely, S. Iem, P. Amegbe, R. Niewenhuis, S. Miklasz, P.F. Cheng, and M. George-Weinstein. 2001. MyoD-positive myoblasts are present in mature fetal organs lacking skeletal muscle. J. Cell Biol. 155:381–391.

Gerhart, J., C. Neely, B. Stewart, J. Perlman, D. Beckmann, M. Wallon, K. Knudsen, and M. George-Weinstein. 2004a. Epiblast cells that express MyoD recruit pluripotent cells to the skeletal muscle lineage. J. Cell Biol. 164:739–746.

Gerhart, J., M. Baytion, J. Perlman, C. Neely, B. Hearon, T. Nilsen, R. Getts, J. Kadashin, and M. George-Weinstein. 2004b. Visualizing the needle in the haystack: in situ hybridization with fluorescent DNA dendrimers. Biol. Proced. Online. 6:149–156.

Gilbert, S.F. 2006. Developmental Biology, Eighth edition. Sinauer Associates, Inc., Sunderland, MA. 817 pp.

Hacker, A., and S. Guthrie. 1998. A distinct developmental programme for the cranial paraxial mesoderm in the chick embryo. Development. 125:3461–3472.

Hamburger, V., and H.L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88:49–92.

Hartley, K.O., Z. Hardcastle, Y. Cinnamon, I. Bachelet, and C. Kalcheim. 2001. The third wave transduction pathways control somite myogenesis. Curr. Top. Dev. Biol. 48:165–224.

Hogard, V., and H.L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88:49–92.

Hartley, K.O., Z. Hardcastle, Y. Cinnamon, I. Bachelet, and C. Kalcheim. 2001. The third wave

Hacker, A., and S. Guthrie. 1998. A distinct developmental programme for the cranial paraxial mesoderm in the chick embryo. Development. 125:3461–3472.

Hogard, V., and H.L. Hamilton. 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88:49–92.

Hartley, K.O., Z. Hardcastle, Y. Cinnamon, I. Bachelet, and C. Kalcheim. 2001. The third wave

Hacker, A., and S. Guthrie. 1998. A distinct developmental programme for the cranial paraxial mesoderm in the chick embryo. Development. 125:3461–3472.
Reshef, R., M. Mendelssohn, T.M., and Jessell, T.M. 1998. Neuronal pattern- 
ing by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. Genes Dev. 12:3394–3407.

Liem, K., G. Tremml, H. Roelink, and T.M. Jessel. 1995. Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. Cell. 82:969–979.

Linask, K.K., and J.W. Lash. 1988. A role for fibronectin in the migration of avian precartilaginous cells. II. Initiation of the heart-forming region during different stages and its effects. Dev. Biol. 129:324–329.

Linask, K.K., K.A. Knudsen, and Y.H. Gui. 1996. N-cadherin-catenin interaction: necessary component of cardiac cell compartmentalization during early vertebrate heart development. Dev. Biol. 185:148–164.

Linker, C., and C.D. Stern. 2004. Neural induction requires BMP inhibition only as a late step, and involves signals other than FGF and Wnt antagonists. Development. 131:5671–5681.

Linker, C., C. Lesbro, M. Stark, and C. Marcelli. 2003. Intrinsic signals regulate the initial steps of myogenesis in vertebrates. Development. 130:4797–4807.

Lough, J., M. Barron, M. Brogley, Y. Sugi, D.L. Bolender, and X. Zhu. 1996. Combined BMP-2 and FGF-4, but neither factor alone, induces cardiogenesis in non-precartilaginous embryonic mesoderm. Dev. Biol. 178:198–202.

Manasek, F.J. 1968. Embryonic development of the heart: a light and electron microscopic study of myocardial development in the early chick embryo. J. Morphol. 125:329–366.

Marcelle, C., J. Wolf, and M. Bronner-Fraser. 1995. The in vivo expression of the FGF receptor FRK mRNA in avian myoblasts suggests a role in muscle cell differentiation. Dev. Biol. 172:100–114.

Marcelle, C., M.R. Stark, and M. Bronner-Fraser. 1997. Coordinate actions of BMPs, Shh and Noggin mediate patterning of the dorsal somite. Development. 124:3955–3963.

Marcelli, C., C. Ahlgren, and M. Bronner-Fraser. 1999. In vivo regulation of somite differentiation and proliferation by Sonic hedgehog. Dev. Biol. 214:277–287.

Maroto, M., R. Reshef, A.E. Munsterberg, S. Koester, M. Goulding, and A.B. Lassar. 1997. Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. Cell. 89:139–148.

McMahon, J.A., S. Takada, L.B. Zimmerman, C.-M. Fan, R.M. Harland, and A.P. McMahon. 1998. Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. Genes Dev. 12:1438–1452.

Neville, C.M., M. Schmidt, and J. Schmidt. 1992. Response of myogenic determination factors to cessation and resumption of electrical activity in skeletal muscle: a possible role for myogenin in dedifferentiation sensitivity. Cell. Mol. Neurobiol. 12:511–527.

Nguyen, V.H., J. Trout, S.A. Connors, P. Andermann, E. Weinberg, and M.C. Mullins. 2000. Dorsal and intermediate neural cell types of the spinal cord are established by a BMP signaling pathway. Development. 127:1209–1220.

Orald, C.P., and N.M. Le Douarin. 1992. Two myogenic lineages within the spinal cord are established by a BMP signaling pathway. Development. 114:339–353.

Ott, M.O., E. Bober, G. Lyons, H. Arnold, and M. Buckingham. 1991. Early expression of the myogenic regulatory gene, myf-5, in precursor cells of skeletal muscle in the mouse embryo. Development. 111:1097–1107.

PisRKanen, A.J.C. Kiefer, and S.D. Hauschka. 2000. IGFs, insulin, Shh,bFGF, and TGF-beta 1 interact synergistically to promote myogenic somitogenesis in vitro. Dev. Biol. 224:189–203.

Pourquier, O., M. Coltey, M. Teillet, C. Orald, and N.M. Le Douarin. 1993. Control of dorsoventral patterning of somatic derivatives by notochord and floor plate. Proc. Natl. Acad. Sci. USA. 90:5242–5246.

Pourquier, O., C.M. Fan, M. Coltey, E. Hirsinger, Y. Watanabe, C. Breant, P. Francis-West, P. Brickell, M. Tessier-Lavigne, and N.M. Le Douarin. 1996. Lateral and axial signals involved in avian somite patterning: a role for BMP4. Cell. 84:461–471.

Pownall, M.E., and C.P. Emerson. 1992. Sequential activation of three myogenic regulatory genes during somite morphogenesis in quail embryos. Dev. Biol. 151:67–119.

Pownall, M.E., M.K. Gustafsson, and C.P. Emerson. 2002. Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. Annu. Rev. Cell Dev. Biol. 18:747–783.

Reshef, R., M. Maroto, and A.B. Lassar. 1998. Regulation of dorsal somatic cell fates: BMPs and Noggin control the timing and pattern of myogenic regulator expression. Genres Dev. 12:290–303.

Rudnicki, M.A., P.N.J. Schmegelsberg, R.H. Stead, T. Braun, H.-H. Arnold, and R. Jaenisch. 1993. MyoD or myf-5 is required for the formation of skeletal muscle. Cell. 75:1351–1359.