JCB Article

# MyoD-positive myoblasts are present in mature fetal organs lacking skeletal muscle

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The epiblast of the chick embryo gives rise to the ectoderm, mesoderm, and endoderm during gastrulation. Previous studies revealed that MyoD-positive cells were present throughout the epiblast, suggesting that skeletal muscle precursors would become incorporated into all three germ layers. The focus of the present study was to examine a variety of organs from the chicken fetus for the presence of myogenic cells. RT-PCR and in situ hybridizations demonstrated that MyoD-positive cells were present in the brain, lung, intestine, kidney, spleen, heart, and liver. When these organs were dissociated and placed in culture, a subpopulation of cells differentiated into skeletal muscle. The G8 antibody was used to label those cells that expressed MyoD in vivo and to follow their fate in vitro. Most, if not all, of the muscle that formed in culture arose from cells that expressed MyoD and G8 in vivo. Practically all of the G8-positive cells from the intestine differentiated after purification by FACS®. This population of ectopically located cells appears to be distinct from multipotential stem cells and myofibroblasts. They closely resemble quiescent, stably programmed skeletal myoblasts with the capacity to differentiate when placed in a permissive environment.

## Introduction

Fate map analyses demonstrate the origin and destination of cells during development; however, they do not necessarily reveal the potential of cells to differentiate along a particular pathway. For example, the area opaca of the chick embryo that normally forms the extraembryonic mesoderm can be induced to differentiate into neural tissue when Hensen's node is ectopically placed in this region (for review see Storey et al., 1992). In this case, it was assumed that cells of the area opaca were multipotential and could be induced to change fates by factors released from Hensen's node.

The dissociation of developmental potential from location also has been demonstrated in studies of the myogenic lineage. Most skeletal muscles are formed from cells derived from the embryonic somites (for review see Christ and Ordahl, 1995). Somites arise during gastrulation when cells of the epiblast layer ingress through the primitive streak to form the mesoderm (Bellairs, 1986). The region of the epiblast surrounding the rostral end of the primitive streak contains those cells that will become incorporated into the somites (Rosenquist, 1966). Although this region of the epiblast is fated to form muscle, a small number of cells expressing the skeletal muscle–specific transcription factor MyoD are present throughout the epiblast, mesoderm, and endoderm (Gerhart et al., 2000). Myf-5, a member of the MyoD family, was also detected in the nonmyogenic mesoderm (Kiefer and Hauschka, 2001). Furthermore, skeletal muscle develops in cultures prepared from different regions of the epiblast (George-Weinstein et al., 1996). These results led to the hypothesis that cells with the potential to differentiate into skeletal muscle would become incorporated into fetal organs derived from all three germ layers, but would remain as precursors in a nonpermissive environment.

The notion that skeletal muscle precursors would come to lie in ectopic locations after gastrulation is supported by the fact that MyoD-positive cells were found later in development in tissues of the chick embryo that do not give rise to skeletal muscle, including the neural tube (Gerhart et al., 2000). MyoD and Myf-5 mRNAs were detected in the chondrogenic region of the somite called the sclerotome (Gerhart et al., 2000; Kiefer and Hauschka, 2001). Myf-5 expression also was observed in the brain and spinal cord of

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Key words: MyoD; myoblasts; chicken; fetal; organs

mouse embryos in which the lacZ reporter gene was targeted into the myf-5 locus (Tajbakhsh et al., 1994). Brain cells from these animals transiently coexpress neuronal and muscle proteins suggesting that they are at least bipotential.

Cells with myogenic potential are also present in non-skeletal muscle tissues of the adult. Myofibroblasts derived from the kidney (BHK/mesangial cells) and liver stellate cells synthesize skeletal muscle contractile proteins when placed in culture, although they do not assemble sarcomeres or fuse to form multinucleated myotubes (Mayer and Leinwand, 1997). Smooth muscle cells can convert to skeletal muscle in vivo and in vitro (Volpe et al., 1993; Patapoutian et al., 1995; Borirakchanyavat et al., 1997; Link and Nishi, 1998; Graves and Yablonka-Reuveni, 2000). Dermal fibroblasts and stem cells from the bone marrow and brain fuse with existing myofibers in vivo (Gibson et al., 1995; Ferrari et al., 1998; Bittner et al., 1999; Gussoni et al., 1999; Galli et al., 2000). Whereas fibroblasts and smooth muscle cells undergo transdifferentiation, bone marrow and neuronal stem cells appear to be multipotential. These cells have not been reported to express myogenic transcription factors before their conversion to muscle. For this reason they are considered less mature than myoblasts, the immediate precursors to differentiated myocytes that fuse to form myofibers (Miller et al., 1999; Cossu and Mavilio, 2000; Seale and Rudnicki, 2000).

Based on the prediction that cells expressing MyoD in the embryonic epiblast would be incorporated into tissues derived from all three germ layers, we have searched for myogenic cells in a variety of mature organs from the chicken fetus by reverse transcription (RT)*-PCR, in situ hybridization, cell culture analyses, and FACS®. A small number of MyoD-positive cells were found in seven fully differentiated organs in vivo. Each of these organs gave rise to skeletal muscle in vitro. When cells with MyoD were purified from the intestine, >90% formed skeletal muscle. These results demonstrate that cells resembling stably programmed skeletal myoblasts are ectopically located in the fetus and are capable of differentiating in a permissive environment.

# Results

## Analysis of MyoD expression by RT-PCR

RT-PCR was performed on 13 different fetal organs (Fig. 1). MyoD was detected in extracts from all of these organs except the lens and liver. When the intensity of the MyoD bands was compared with that of GAPDH by scanning densitometry, adipose tissue, spinal cord, skin, brain, and lung appeared to contain relatively greater amounts of MyoD mRNA than the heart, spleen, tendon, intestine, and kidney, but less than that present in pectoralis skeletal muscle. Eight organs were analyzed for the expression of myogenin and sarcomeric myosin heavy chain (Fig. 1). Only pectoralis muscle and the heart contained detectable amounts of sarcomeric myosin mRNA. Myogenin was found only in pectoralis muscle.

*Abbreviations used in this paper: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription.

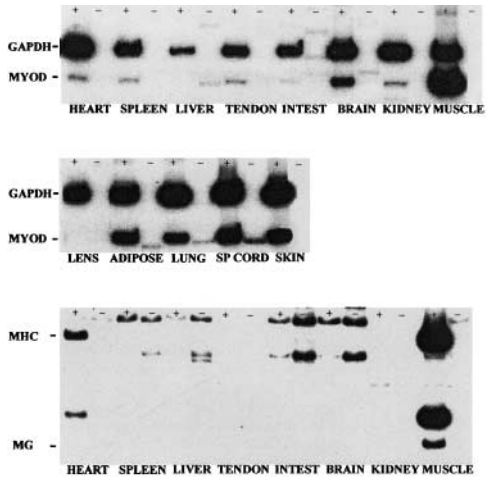


Figure 1. **RT-PCR analysis of MyoD expression in fetal organs.** mRNA from fetal organs was incubated with primers for MyoD, embryonic fast myosin (MHC), myogenin (MG), or GAPDH in the presence (+) or absence (−) of reverse transcriptase. MyoD (263 bp) was detected in all samples except the liver and lens. Myosin (616 bp) was expressed in pectoralis muscle and the heart. Myogenin (278 bp) was only detected in pectoralis muscle.

## Localization of MyoD mRNA in fetal organs

Fluorescently labeled DNA dendrimers were used to localize MyoD mRNA in sections from seven different organs. Previous experiments demonstrated that dendrimers were sensitive and precise reagents for detecting low levels of mRNA and/or small numbers of expressing cells by in situ hybridization (Gerhart et al., 2000). Consistent with the results obtained by RT-PCR, MyoD-positive cells were detected in all 7 organs (Fig. 2). All sections from the kidney, heart, intestine, and brain, and 13 out of 15 sections from the lung contained labeled cells. The kidney, heart, lung, and intestine sections had 1–3 cells labeled with >5 dendrimers, and 5–10 other cells with 1 or 2 dendrimers. In the intestine, the most abundantly labeled cells were located in the muscularis externa and the weakly labeled cells were present in the submucosa. In the kidney and heart, the intensely fluorescent cells were located in the tubules and myocardium, respectively. A thorough analysis of the distribution of MyoD expression in the brain will be conducted in a separate study; however, the optic tectum contained relatively intense labeling, as did small groups of cells in other cortical regions. Five out of nine sections from the spleen contained MyoD-positive cells that were, for the most part, weakly labeled, although half of these sections contained a single brightly fluorescent cell. The fewest number of MyoD-expressing cells was found in sections of the liver. Only 2 out of 15 sections contained one cell with >3 dendrimers. By contrast, pectoralis muscle tissue was abundantly labeled with MyoD dendrimers (Fig. 2c).

Dendrimers with a recognition sequence for embryonic fast myosin bound to cells of the heart, whereas the other organs contained only 0–2 myosin dendrimers over the entire section (Fig. 2 P). A similar level of background fluorescence was obtained with dendrimers lacking a specific recognition sequence. This, and the fact that myosin and myogenin mRNAs were not detected by RT-PCR, suggest that the MyoD-positive cells were undifferentiated.

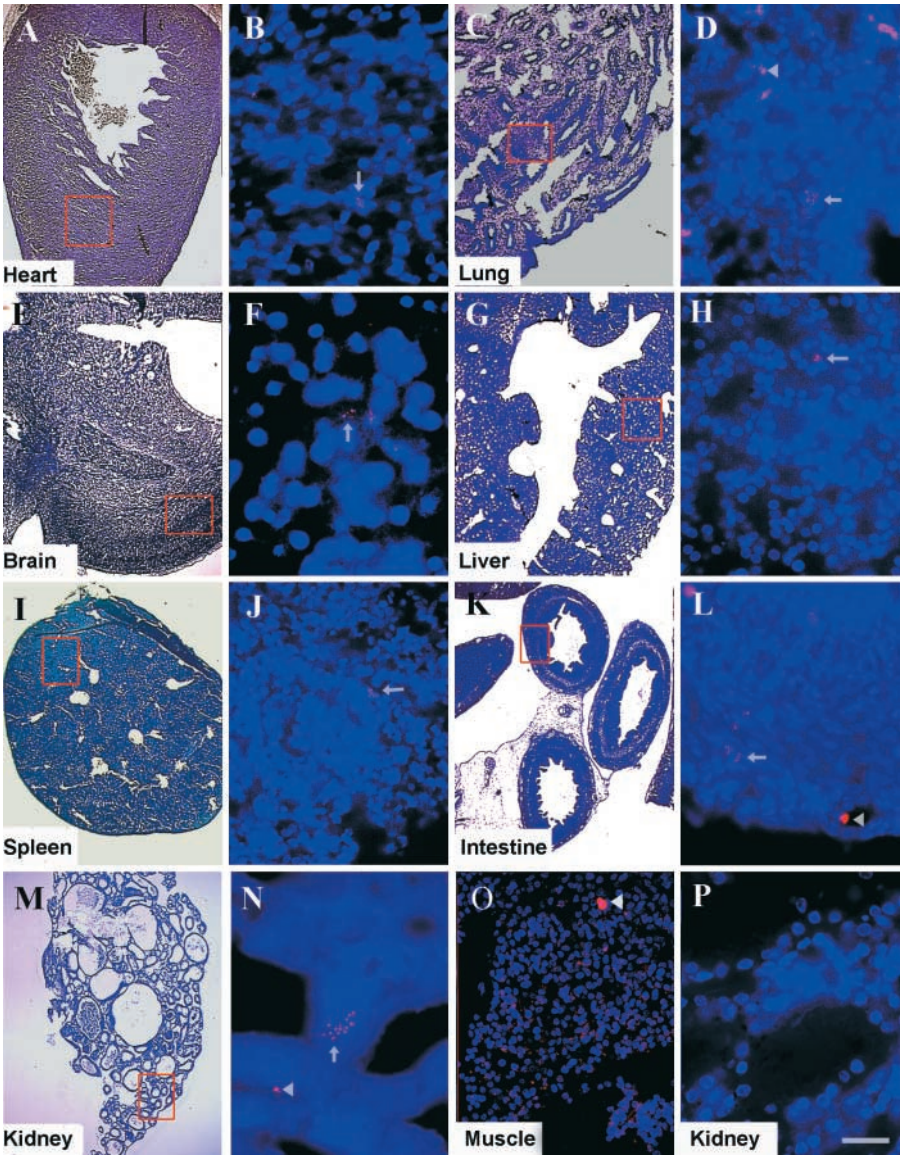


Figure 2. **Localization of MyoD mRNA in fetal organs.** In situ hybridizations were performed on sections of fetal organs. Low magnification images of the sections are shown to the left of each series. Fluorescence photomicrographs are the merged images of dendrimers in red and bis-benzamide labeled nuclei in blue. A few MyoD-positive cells were found in sections through each organ (arrows). Erythrocytes were autofluorescent (arrowheads). Pectoralis muscle was abundantly labeled with MyoD dendrimers (O). Dendrimers to myosin (P) did not label these sections. Bar, 135 μm in the fluorescence photomicrographs; 9 μm in the bright field images.

Determination of whether MyoD mRNA was translated into protein was carried out by fixing cells immediately after organ dissociation and centrifuging them onto slides. This was done because the antibody to MyoD protein did not label pectoralis muscle cells after embedding in paraffin. MyoD protein was not detected in cells from the intestine, kidney, heart, liver, lung, and brain (unpublished data).

## Myogenesis in cultures prepared from fetal organs

The ability of cells from fetal organs to undergo skeletal myogenesis was tested by culturing them under conditions previously designed to support the differentiation of embryonic epiblast and paraxial mesoderm cells (George-Weinstein et al., 1994, 1996). When plated on a substrate of gelatin and fibronectin, >90% of cells from all of the organs

Table I. **Myogenesis in fetal organ cultures**

| | % Muscle: 2 d | % Skeletal muscle: 2 d | % Multinucleated myotubes: 10 d |
|---|---|---|---|
| Intestine | 15 ± 3 (10) | 16 ± 3 (9) | 45 ± 6 (6) |
| Kidney | 17 ± 8 (8) | 15 ± 7 (13) | 52 ± 4 (8) |
| Lung | 12 ± 6 (15) | 17 ± 6 (7) | 35 ± 2 (8) |
| Spleen | 9 ± 4 (8) | 9 ± 5 (8) | 35 ± 4 (9) |
| Liver | 2 ± 1 (12) | <1 | 43 ± 6 (8) |
| Heart | 86 ± 10 (6) | 17 ± 10 (8) | 46 ± 4 (4) |

Cells were fixed after 2 or 10 d in culture and stained with the MF20 antibody to sarcomeric myosin (Muscle) and the 12101 or skeletal troponin T antibodies (Skeletal muscle). The percentage of muscle and the percentage of skeletal muscle = number of MF20, 12101, or skeletal troponin T cells/total number of cells × 100. The percentage of muscle cells that formed multinucleated myotubes = number of nuclei in MF20 or 12101 positive multinucleated cells/total number of nuclei in MF20 and 12101 positive multinucleated and single nucleated cells × 100. The number of cultures scored is indicated in parentheses. A minimum of 200 cells were scored per culture. Results are the mean ± SD.

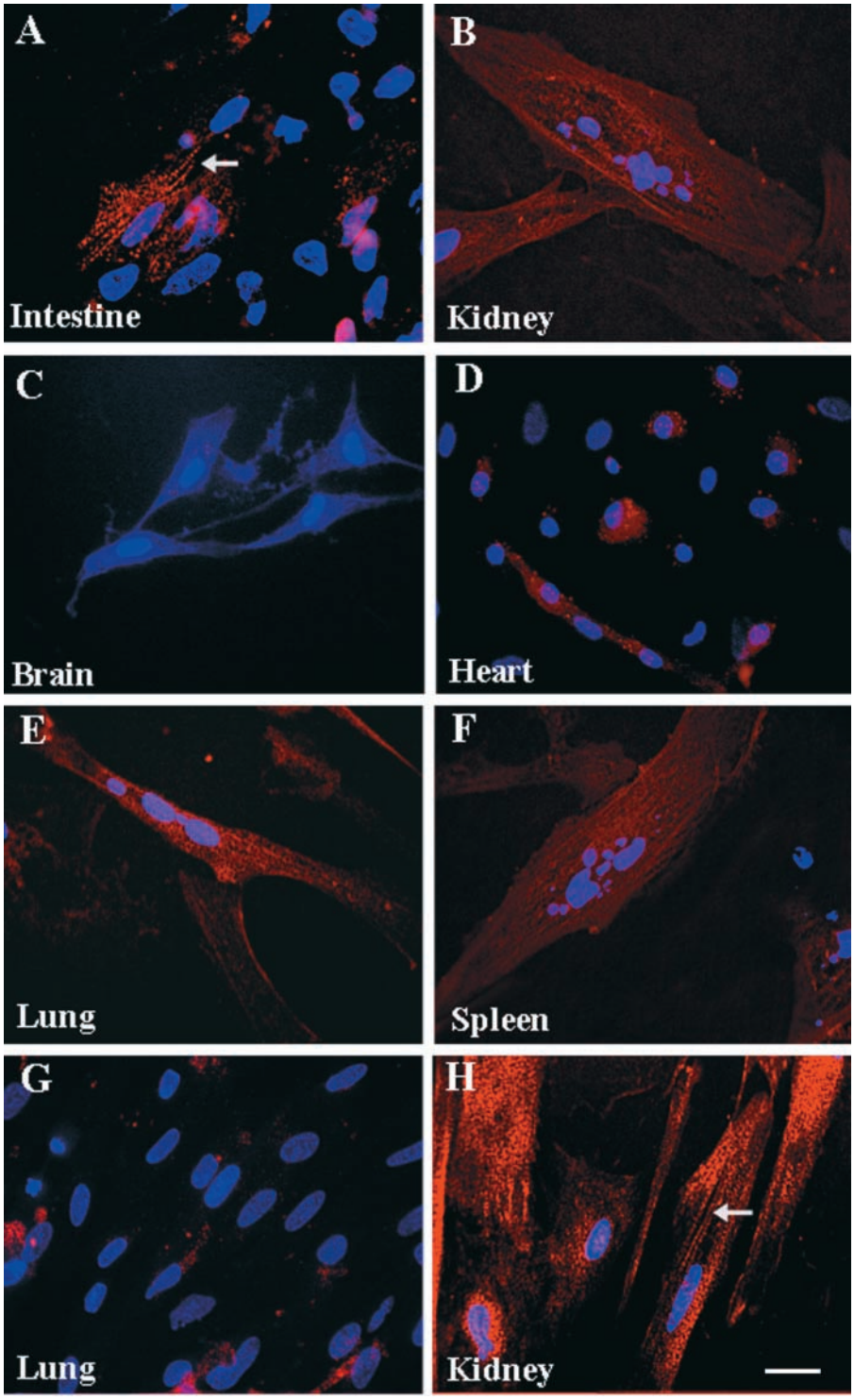


Figure 3. **Myogenesis in cultures of cells prepared from fetal organs.** Fetal organs were dissociated and the cells plated at high density. Cells were stained with the skeletal muscle specific 12101 (A–C, E and F) or troponin T (D) antibody, and an antibody to α-actinin (G and H). Striations can be seen in A and H (arrows). Multinucleated myotubes are shown in B and D–F. Bar, 9 μm.

except the brain attached to the dish. The plating efficiency for brain cells was <5%, reflecting less than optimal culture conditions. Therefore, only qualitative analyses were performed with cultures of brain cells.

Cells were stained with antibodies to sarcomeric myosin, α-actinin, and the skeletal muscle–specific 12101 antigen and troponin T (Fig. 3 and Table I). The specificity of the 12101 and troponin T antibodies was tested in cryosections of days 10 and 11 chick embryo pectoralis muscle and heart. Abundant staining was observed in pectoralis muscle, whereas neither antibody labeled cardiac muscle.

All of the organs gave rise to varying amounts of skeletal muscle (Table I and Fig. 3). The percentage of muscle cells began to decline after 48 h (unpublished data), presumably due to the continued growth of nonmuscle cell types. Consistent with the RT-PCR and in situ hybridization results, very few muscle cells were observed in cultures of liver cells. The spleen produced more muscle than would have been expected from the other types of analyses. In some cells, a striated pattern was observed after staining with the myosin, 12101, and α-actinin antibodies suggesting sarcomere formation (Fig. 3). Multinucleated myotubes were observed in older cultures

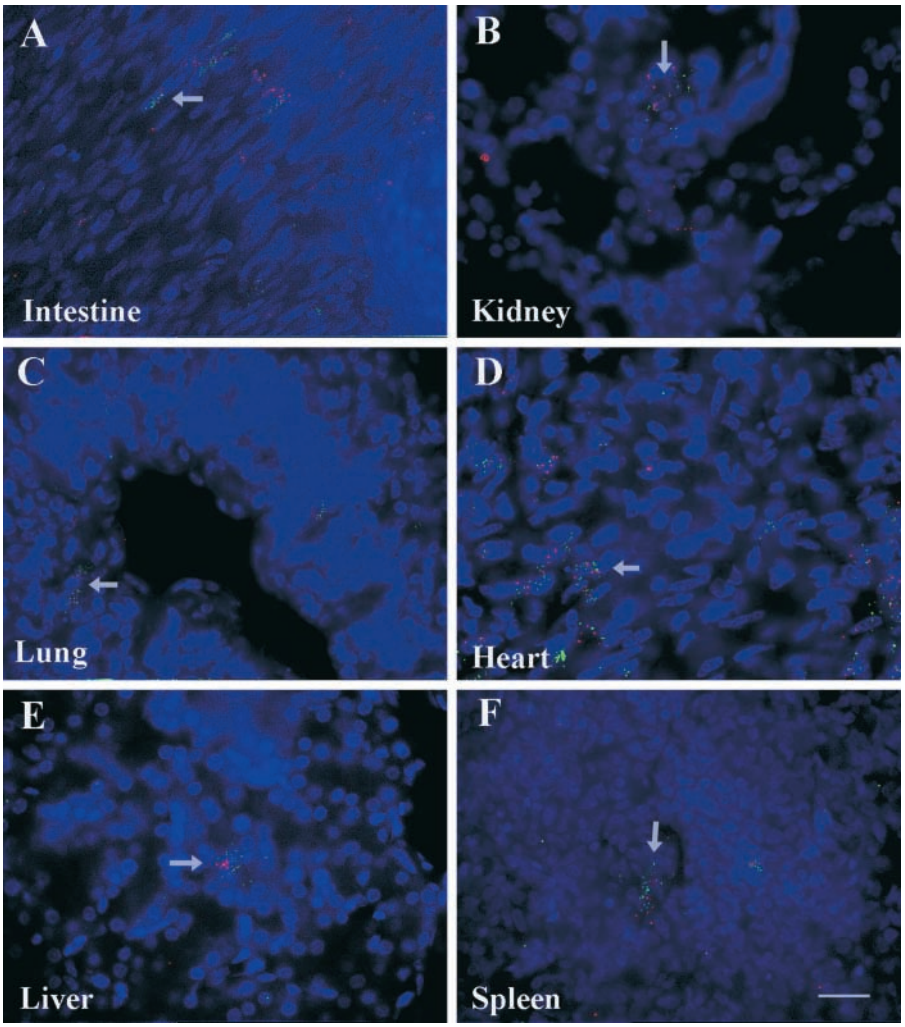


Figure 4. **Colocalization of the G8 antigen with MyoD mRNA in vivo.** Sections were labeled with the G8 antibody and a secondary antibody conjugated with Alexa 488 (green). In situ hybridization was then performed with MyoD dendrimers (red). G8 colocalized with MyoD in most cells.

(Figure 3 and Table I). The percentage of myotubes was relatively high considering the heterogeneity of the cultures.

## Analysis of the potential of MyoD-positive cells to differentiate into skeletal muscle

Two methods were employed to determine whether the cells that formed muscle in culture arose from those that express MyoD in vivo. In both types of experiments, the G8 monoclonal antibody was used to mark the MyoD-positive cells.

Table II. **Differentiation of G8-positive cells in vitro**

| | % G8-positive cells with myosin | % Myosin-positive cells with G8 |
|---|---|---|
| Intestine | 92 ± 3 (8) | 93 ± 4 (8) |
| Kidney | 42 ± 9(4) | 90 ± 3 (4) |
| Spleen | 31 ± 7 (4) | 94 ± 5 (4) |
| Lung | 46 ± 9 (10) | 92 ± 5 (10) |
| Heart | 90 ± 7 (4) | 28 ± 4 (4) |
| Liver | 43 ± 8 (7) | 100 (7) |

Cells were prelabeled with G8, cultured for 48 h, and stained with fluorescent antibodies to IgM, the MF20 antibody to sarcomeric myosin, and fluorescent antibodies to IgG. The percentage of G8-positive cells with myosin = cells positive for both G8 and myosin/total number of G8-positive cells × 100. The percentage of myosin-positive cells with G8 = cells positive for both molecules/total number of myosin-positive cells × 100. The number of cultures scored is indicated in parentheses. A minimum of 200 cells were scored per culture. Results are the mean ± SD.

This antibody was generated by immunizing mice with somite and segmental plate mesoderm cells from stages 12–14 embryos. Details of the pattern of expression of the G8 antigen during embryogenesis will be presented elsewhere. In brief, G8 binds to myoblasts and myotubes in pectoralis muscle cultures, a subpopulation of segmental plate cells and the dermomyotome in vivo, and colocalizes with MyoD dendrimers in the epiblast.

Double label experiments were performed to test whether the G8 antibody would bind to cells that express MyoD mRNA in fetal organs. Most cells that expressed MyoD were labeled with the G8 antibody in sections from the intestine, kidney, heart, lung, spleen, and liver (Fig. 4). A few cells with weak labeling for MyoD did not stain with G8. This may be due to the fact that the antigen was below the level of detection with antibody or that the cells were sectioned, as MyoD protein was detected in 100% of the G8-positive cells in culture (see below).

The G8 antibody was used to mark living cells that express MyoD. Organs were dissociated, labeled in suspension with G8, and then placed in culture. Cells were stained with an IgM-specific secondary antibody conjugated with Alexa 488 to tag G8, the MF20 IgG antibody to sarcomeric myosin, and an IgG specific secondary antibody conjugated with rhodamine. All G8-positive cells contained MyoD protein, and all cells with MyoD were stained for G8 (Fig. 5, A, D,

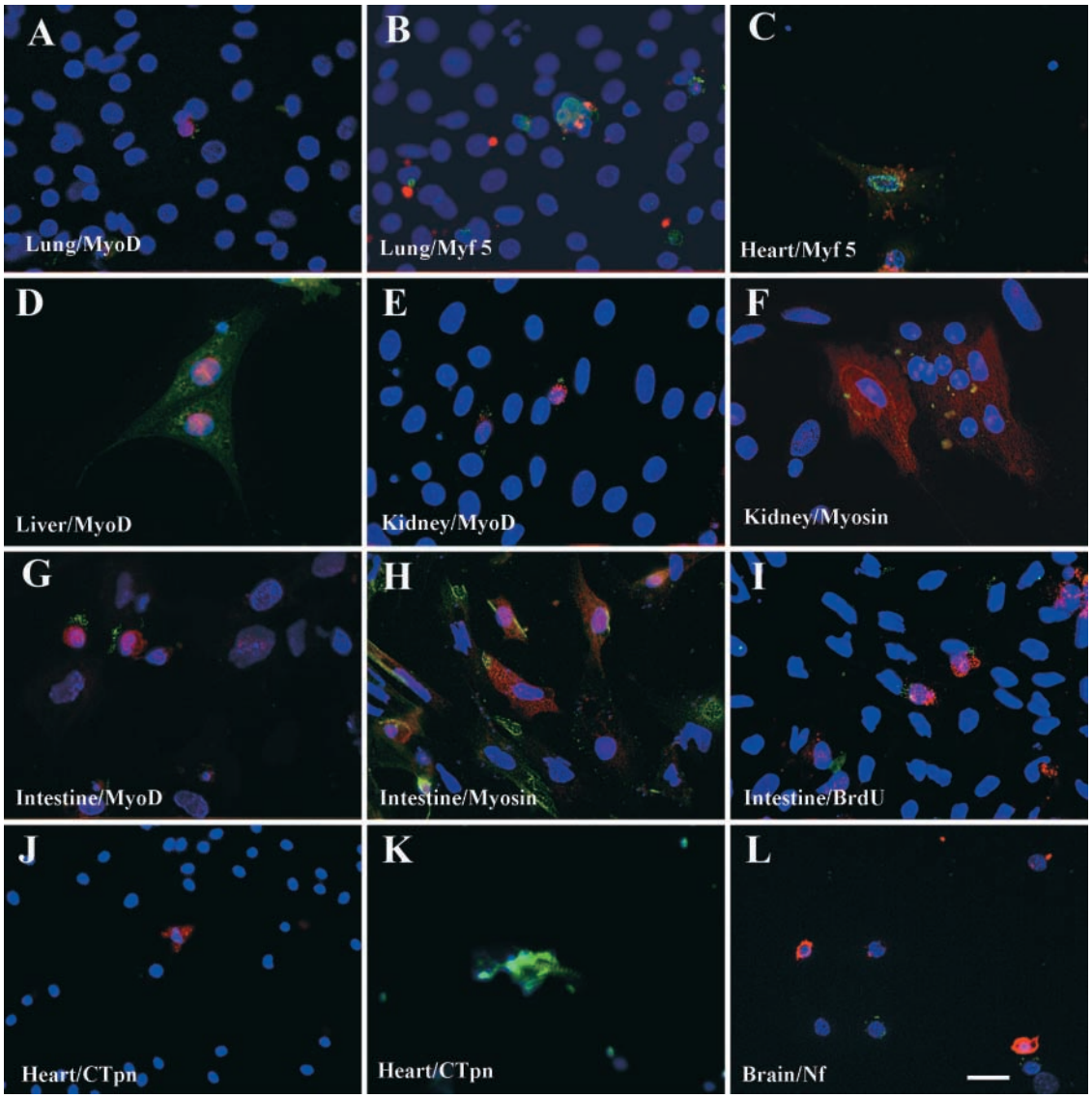


Figure 5. **Immunofluorescence localization of cell type–specific proteins and BrdU in G8-positive cells in vitro.** Cells from fetal organs were prelabeled with the G8 antibody, cultured for 48 h, and then stained with a secondary antibody to G8 and antibodies to MyoD, Myf5, or sarcomeric myosin. Cells from the lung, liver, kidney, and intestine that were labeled with G8 (green) had MyoD and myosin (red). Double labeling for G8 (red) and Myf5 (green) was seen in lung and heart cells. G8-positive intestine cells (green) incorporated BrdU (red) indicating replication. In a second type of experiment, cells from the heart and brain were fixed directly after removal from the fetus, centrifuged onto slides, and stained with G8 (red in J; green in L) and antibodies to cardiac troponin I (green) and neurofilament protein (red), respectively. Cells labeled with G8 did not have detectable levels of cardiac troponin (J) or neurofilament protein (L). Cells with cardiac troponin (K) and neurofilament protein (L) were not labeled with G8. Bar, 9 μm.

E, and G). Most of the G8-positive cells (~95%) also expressed Myf5 protein, and all cells with Myf5 had G8 (Fig. 5, B and C).

Some of the cells that had bound G8 directly after removal from the fetus differentiated into skeletal muscle (Table II and Fig. 5, F and H). Approximately 90% of the myosin positive cells that formed from all organs except the heart were also positive for G8, suggesting that most, if not all, of the differentiated muscle arose from cells that expressed MyoD in vivo. The low percentage of myosin-positive cells with G8 in heart cultures was expected because the MF20 antibody recognizes the myosin heavy chain in both skeletal and cardiac muscle.

Because the intensity of G8 fluorescence varied from cell to cell, it was possible that some cells lost the G8 signal as a result of metabolism or dilution from replication. Intestine cultures were labeled with BrdU to determine whether cells with G8 were capable of proliferating. After 4 h of incubation in BrdU, 11 ± 5% ($n$ = 8) of the intestine cells that bound G8 were determined to be in the S phase of the cell cycle (Fig. 5 I).

## Analysis of the expression of nonskeletal muscle proteins in G8-positive cells

Cells from the heart and brain with the potential to differentiate into skeletal muscle were examined for the presence of cardiac muscle–specific troponin I and neurofilament protein, respectively. Because cytoplasmic staining of a single MyoD-positive cell surrounded by cardiac myocytes or neu-

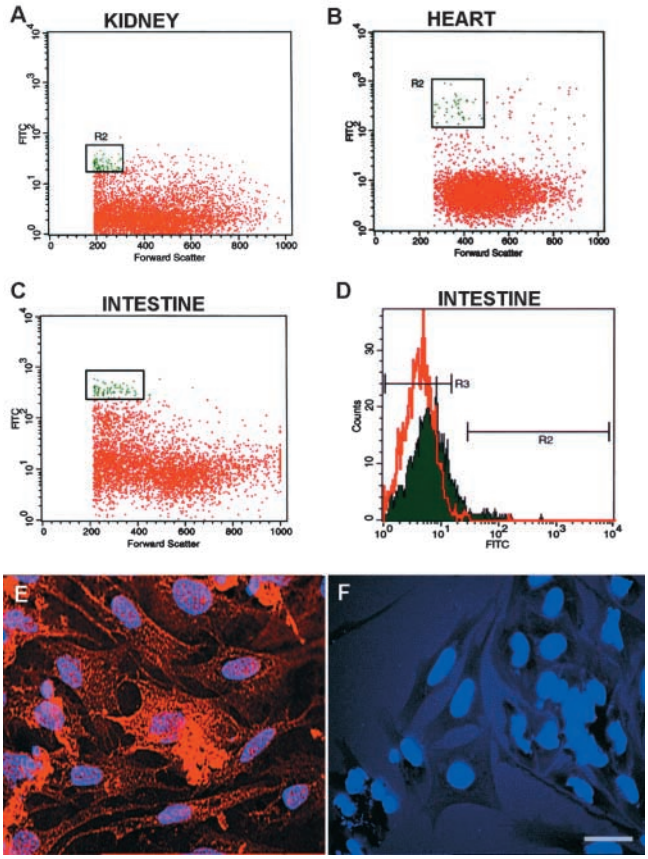


Figure 6. **Flow cytometry and FACS® of fetal cells.** Fetal heart, kidney, and intestine cells were labeled with the G8 antibody and fluorescein-conjugated secondary antibody. Profiles of fluorescence intensity versus forward light scatter (cell size) were compared in cells labeled with G8 (green) and those labeled with the secondary antibody alone (red) (A–C). G8-positive (R2 population) and negative intestine cells (R3 population) were sorted (D), placed in culture for 2 d, and then stained with the 12101 antibody. Most of the G8-positive cells differentiated into skeletal muscle (E), whereas very few myosin positive cells were observed in the G8-negative cultures (F). Bar, 9 μm.

rons would be difficult to unambiguously resolve in paraffin sections, this experiment was carried out with freshly dissociated and fixed cells centrifuged onto glass slides. The specificity of the cardiac troponin I antiserum was tested by labeling cryosections of pectoralis muscle and the heart. The antiserum-stained cardiac tissue but not skeletal muscle.

None of the G8-positive cells contained detectable levels of cardiac troponin I or neurofilament protein (Fig. 5, J and L). These proteins were observed in cells lacking G8 (Fig. 5, K and L). This suggests that cells that express MyoD in the heart and brain do not synthesize cardiac or neuronal proteins.

### Isolation of G8/MyoD-positive cells by FACS®

FACS® was used to isolate those cells that expressed MyoD in vivo after labeling with the G8 antibody. Profiles of forward light scatter, a relative measure of cell size, versus G8 fluorescence intensity, were similar for cells of the intestine, kidney, and heart (Fig. 6). After gating for dead cells and debris, the G8-positive cells were the smallest in preparations from all three organs. The total cells labeled with the G8 antibody was ~0.5%, or 1.2% after gating.

G8 labeled and unlabeled intestine cells were sorted and placed in culture. Greater than 90% of the G8-positive cells differentiated into muscle within the first 48 h in culture (% = 92 ± 5, $n$ = 9). Less than 1% of the cells in G8-negative cultures contained detectable levels of sarcomeric myosin ($n$ = 10). Staining with the 12101 antibody revealed that the muscle that formed in G8-positive cultures was skeletal muscle (Fig. 6). These experiments suggest that the muscle that emerged in unsorted cultures arose from cells that expressed MyoD and G8 in vivo.

## Discussion

Cells that express MyoD have been found in a variety of fully differentiated fetal organs derived from all three germ layers. This was predicted based on the fact that MyoD-positive cells are present in regions of the chick epiblast that give rise to the ectoderm, medial and lateral mesoderm, and endoderm (Gerhart et al., 2000). Slightly later in development, a few cells with MyoD were observed throughout the embryo, including the neural tube. Myf5 also was detected in nonsomitic tissues of the chick embryo (Kiefer and Hauschka, 2001), and in transgenic mice in which the lacZ reporter gene was targeted into the myf5 locus (Tajbakhsh et al., 1994).

Several questions arise from these findings. First, can cells that express MyoD in ectopic locations differentiate into skeletal muscle in the appropriate environment? The G8 antibody has enabled us to test this directly, as G8 colocalizes with MyoD in fetal organs and presumably is expressed in the same cells. Cells that bound G8 directly after organ dissociation synthesized sarcomeric myosin and the skeletal muscle–specific 12101 antigen in vitro. The percentage of this population that differentiated increased dramatically when the G8-positive cells from the intestine were isolated by FACS® before placement in culture. This probably reflects a "community effect," a phenomenon in which cells of similar potential communicate with one another to promote differentiation (Gurdon, 1988). However, in unsorted cultures, some of the cells with myosin were completely surrounded by nonmuscle cells, and therefore did not require contact with other myogenic cells. These experiments demonstrate that cells expressing MyoD in ectopic locations in vivo have retained the capacity to form muscle and are poised to do so when placed in a permissive environment.

Do the MyoD-positive cells of fetal chick organs resemble any cell type previously identified? Myofibroblasts are present in many organs, and under certain conditions express genes typical of smooth and/or skeletal muscle cells (for review see Mayer and Leinwand, 1997). Sarcomeric myosin was detected in myofibroblast stellate cells of the liver in vivo (Mayer and Leinwand, 1997). Cell lines derived from liver stellate cells and myofibroblasts of the kidney called mesangial cells express several sarcomeric proteins in vitro; however, they do not fuse to form multinucleated myotubes or assemble sarcomeres (Mayer and Leinwand, 1997). By contrast, the MyoD-positive cells in fetal organs do not express sarcomeric myosin or myogenin in vivo, but do form myotubes and appear to assemble sarcomeres in vitro. Furthermore, MyoD-expressing cells were found in the tubules of the kidney, and were not associated with renal corpuscles where mesangial cells are located (Johnson et al., 1992).

Therefore, the cells that express MyoD in organs lacking skeletal muscle may be distinct from the myofibroblast.

MyoD-positive cells in fetal organs also appear to differ from fibroblasts (Chaudhari et al., 1989; Gibson et al., 1995, Salvatori et al., 1995), bone marrow cells (Wakitani et al., 1995; Prockop, 1997; Ferrari et al., 1998; Bittner et al., 1999; Gussoni et al., 1999), cells from the embryonic dorsal aorta (DeAngelis et al., 1999), and neuronal stem cells (Galli et al., 2000) that can differentiate into skeletal muscle in vitro and fuse with myofibers in vivo. RT-PCR analyses of the dorsal aorta and neural stem cells failed to detect the expression of myogenic transcription factors (DeAngelis et al., 1999; Galli et al., 2000). This, and the fact that they are able to differentiate into other cell types, has led to the conclusion that they are less mature and have greater plasticity than myoblasts that do express MyoD and Myf5.

The MyoD-positive cells located in fetal organs most resemble the myoblast. Both cell types express myogenic transcription factors, replicate, and form striated myotubes. Myoblasts can be considered a stem cell in that they replicate and self-renew; however, their potential to form other tissues has not been equivocally demonstrated. Over two decades ago it was suggested that myoblasts may be at least bipotential, as replicating cells from fetal muscle resembling myoblasts at the ultrastructural level formed bone when cultured on demineralized bone matrix (Nathanson and Hay, 1989). More recent studies demonstrated that undifferentiated cells isolated from adult muscle are heterogeneous (Schultz and Jaryszak, 1985; Barrofio et al., 1995; Rantenen et al., 1995; Schultz, 1996; Yoshida et al., 1998; Beauchamp et al., 1999; Lee et al., 2000), and at least some have the capacity to undergo myogenesis, osteogenesis, and/or hematopoiesis (Gussoni et al., 1999; Jackson et al., 1999; Lee et al., 2000). These cells appear to differ from the majority of myogenic cells in adult muscle that have limited proliferative and regenerative capacity (Beauchamp et al., 1999). Most precursors in adult muscle also express CD34, Myf5, and M-cadherin, and therefore, may be committed to the myogenic lineage (Beauchamp et al., 2000).

The MyoD-positive/myosin negative cells located in fetal organs lacking skeletal muscle also appear to be stably committed to the myogenic lineage. This is based on several pieces of evidence. First, most of the cells prelabeled with the G8 antibody that differentiated in unsorted cultures were surrounded by nonmuscle cells, thereby demonstrating independence from a community effect. Second, when the MyoD/G8-positive cells were isolated from the intestine by FACS®, practically all of them differentiated rapidly. Third, the foreign environment in which these cells reside in vivo, surrounded by differentiated epithelial, connective tissue, or smooth and cardiac muscle cells, has not repressed the expression of MyoD. Lastly, in the heart and brain, the MyoD/G8-positive cells do not appear to express cardiac and neuronal proteins. Although obviously biased towards a myogenic fate, it is possible that if placed in a more challenging environment, for example, in developing tissues of younger embryos, multipotentiality would be revealed. This experiment is now possible, as the MyoD-positive cells can be purified by sorting the with G8 antibody.

Related to the issue of commitment is the origin of this subpopulation of cells. Cells with MyoD have been localized to regions of the epiblast that give rise to all three germ layers of the embryo (Gerhart et al., 2000). A day later, small numbers of cells expressing MyoD were found outside of the somites, the source of most skeletal muscle of the body. Preliminary studies in which the G8 antibody was used to label epiblast cells in whole embryo culture suggest that this population does indeed become incorporated into a variety of tissues during gastrulation. Whether they survive until fetal stages and continue to express MyoD remains to be determined. If fetal cells with MyoD do originate in the epiblast, then one would expect them to retain their myogenic identity when challenged in an embryonic environment that promotes the differentiation of nonskeletal muscle cell types.

The biological relevance of finding potentially stably programmed myoblasts in ectopic locations is a matter of speculation. Although apparently quiescent in a foreign environment, it seems possible that damage to a tissue with resulting inflammation could activate their proliferation. Another possible scenario is that they may be vulnerable to transformation. Rhabdomyosarcomas are malignant tumors comprised of cells expressing skeletal muscle proteins. Interestingly, the primary tumors most often are found in sites other than skeletal muscle such as the genitourinary tracts, orbit of the eye, and glands (for review see Dagher and Helman, 1999; Merlino and Helman, 1999). It has been postulated that these neoplasias arise from myogenic precursor cells, although their origin has not been identified. It seems possible that those rhabdomyosarcomas histologically and genetically characterized as embryonal tumors could arise from ectopically located myoblasts that have now been identified based on the expression of MyoD. On a more optimistic note, recent advances in the isolation of stem cells from the adult has generated tremendous hope for the treatment of a variety of diseases. Although multipotential cells may have a greater capacity for proliferation and self-renewal than committed cells, communication between the two is critical for tissue development in the embryo (for review see Gurdon, 1992; Horvitz and Herskowitz, 1992; Schnabel, 1995). Implantation of a mixture of these cell types may improve the efficiency of tissue regeneration.

## Materials and methods

### RT-PCR

The following organs were removed from day 15 White Leghorn chick fetuses: pectoralis muscle, intestine, kidney, heart, spleen, lung, brain, spinal cord, liver, the entire eye, lens, tendon, skin, and adipose tissue. RNA was extracted and RT-PCR performed as described previously (George-Weinstein et al., 1996). Primer pairs for MyoD were nucleotides 620–639, 5′-CGT GAG CAG GAG GAT GCA TA-3′ and 864–883, 5′-GGG ACA TGT GGA GTT GTC TG-3′ (Lin et al., 1989); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) nucleotides 680–699, 5′-AGT CAT CCC TGA GCT GAA TG-3′ and 990–1009, 5′-AGG ATC AAG TCC ACA ACA CG-3′ (Dugaiczyk et al., 1983); myogenin nucleotides 435–453, 5′AGC CTC AAC CAG CAG GAG C-3′ and 694-713, 5′-TGC GCC AGC TCA GTT TTG GA-3′ (Fujisawa-Sehara et al., 1990); and myosin heavy chain nucleotides 392–411, 5′-GAT CCA GCT GAG CCA TGC CA-3′ and 1008–989, 5′-GCT TCT GCT CAG CAT CAA CC-3′ (Kavinsky et al., 1983). Reaction products were separated on 6% polyacrylamide gels and Phosphorous-32 incorporation visualized by autoradiography.

### In situ hybridization

The intestine, kidney, heart, spleen, lung, brain, liver, and pectoralis muscle were removed from day 17 chick fetuses, fixed in 4% formaldehyde for 18 h, and embedded in paraffin. 10-mm sections were applied to teflon printed slides (Electron Microscopy Sciences) coated with 0.2% gelatin. The in situ hybridization procedure was carried out with 3DNA Starfish™ reagents, as described in Gerhart et al. (2000). In brief, tissues were permeabilized in Triton X-100 and pepsin. Sections were incubated in buffer containing Cy3 labeled DNA dendrimers (Nilsen et al., 1997; Vogelbacker et al., 1997; Wang et al., 1998) to which cDNA sequences for specific mRNAs plus 7 bases complementary to the dendrimer had been ligated to the outer dendrimer arms (Genisphere, Inc.). The dendrimers contained the following antisense sequences for mRNAs: chicken MyoD (Dechesne et al., 1994), 5′-TTC TCA AGA GCA AAT ACT CAC CAT TTG GTG ATT CCG TGT AGT AGC TGC TG-3′; chicken embryonic fast myosin (Freyer and Robbins, 1983), 5′-CAG GAG GTG CTG CAG GTC CTT CAC CGT CTG GTC CAG GTT CTT CTT CAT CCT CTC TCC AGG-3′; and chicken GAPDH (Dugaiczyk et al., 1983), 5′-ATC AAG TCC ACA ACA CGG TTG CTG TAT CCA AAC TCA TTG TCA TAC CAG GAA-3′. Dendrimers lacking a specific recognition sequence were used as a negative control for background fluorescence. Nuclei were counterstained with bis-benzamide. Sections were observed with a Nikon Eclipse E800 epifluorescence microscope and photomicrogaphs produced with the Optronics DEI 750 video camera and Image-Pro Plus image analysis software (Phase 3 Imaging Systems).

The following number of in situ hybridizations were performed with MyoD dendrimers: 15 intestine sections from 3 fetuses, 27 kidney sections from 5 fetuses, 24 heart sections from 5 fetuses, 15 lung sections from 3 fetuses, 9 spleen sections from 2 fetuses, 15 liver sections from 3 fetuses, and 5 brain sections from 1 fetus.

### Cell culture

Organs from day 17 fetuses were incubated in 0.25% trypsin-EDTA for 10–20 min and passed through an 18–22-gauge needle to dissociate the tissues. Cells were cultured under conditions that were previously determined to promote the differentiation of embryonic epiblast and paraxial mesoderm cells (George-Weinstein et al., 1994, 1996). Twenty thousand cells in 15 μl of DME containing 5% fetal bovine serum, 5% horse serum, 5% chick embryo extract, and 100 units of penicillin and streptomycin (myo medium) were plated onto tissue culture dishes coated with gelatin and human serum fibronectin (GIBCO/BRL). Dishes were flooded with 1.5 ml serum and hormone-free DME/F12 medium (GIBCO/BRL) 45 min after plating.

### Generation of the G8 monoclonal antibody

The G8 monoclonal antibody was generated by immunizing female BALB/c mice three times with $10^6$ cells from the posterior 12 pairs of somites and the segmental plate mesoderm cells from stages 12–14 embryos (Hamburger and Hamilton, 1951). Cells were fixed in 2% formaldehyde before intraperitoneal injection. Immune lymphocytes were fused with SP2/0-AG14 myeloma cells and plated in HAT selection media (Kohler and Milstein, 1976). Monoclonal antibody–producing clones were selected based on their immunoreactivity against cryosections from stage 14 embryos. Hybridomas producing antibodies that bound to subpopulations of cells were cloned and retested for reactivity in cryosections. Antibody subclass characterization was determined using the MonoAb ID-mouse kit (Zymed). The G8 monoclonal antibody was determined to be an IgM, kappa. Ascites fluid was produced by the method of Hoogenraad et al. (1983), treated with 5% sodium dextran sulfate and 11.1% calcium choloride to remove lipoproteins (Walton and Scott, 1964), immunoglobulin precipitated by 50% saturation with ammonium sulfate, dialyzed against calcium and magnesium-free Dulbecco's PBS, and filter sterilized. Immunizations, fusion, subcloning, subclass determination, and production of ascites fluid were carried out in the University of Illinois' Hybridoma Laboratory in the Biotechnology/Cell Science Center (Urbana, IL). The antigenic determinant of the G8 antibody has yet to be identified and characterized.

### Immunofluorescence localization

Cells were fixed in 2% formaldehyde, permeabilized with 0.5% Triton X-100, and labeled with primary and fluorescent secondary antibodies as described previously (George-Weinstein et al., 1994). The MF20 monoclonal antibody to myosin heavy chain (Bader et al., 1982), the skeletal muscle specific 12101 monoclonal antibody (Kitner and Brockes, 1984), and the 2H3 monoclonal antibody to neurofilament protein (Dodd et al., 1988) were obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA. The F5D3 monoclonal antibody to skeletal muscle specific troponin T (Shimizu and Shimada, 1985) and polyclonal rabbit antiserum to cardiac muscle specific troponin I (Toyota and Shimada, 1981) were gifts from Dr. N. Toyota (Chiba University, Chiba, Japan). Monoclonal antibodies α-actinin (A7811) and MyoD (NCL-MyoD1) were purchased from Sigma-Aldrich and Novocastra Labs., respectively. Myf5 polyclonal antiserum was supplied by Dr. Bruce Paterson (National Institutes of Health, Bethesda, MD). Secondary antibodies were affinity-purified, goat anti–mouse or anti–rabbit F(ab′)2 fragments conjugated with rhodamine, Cy3, or fluorescein (Jackson ImmunoResearch Laboratories). Nuclei were counterstained with bis-benzamide.

Double labeling with the G8 antibody and MyoD dendrimers was carried out in paraffin sections of day 17 fetal organs. The antibody was applied first, followed by a goat anti–mouse IgM secondary antibody conjugated with Alexa 488 (Molecular Probes). In situ hybridization with Cy3-labeled MyoD dendrimers then was performed as described above. Nuclei were counterstained with bis-benzamide.

The G8 antibody was used to label living cells that expressed MyoD and to determine whether those cells could differentiate into skeletal muscle in vitro. Organs were dissociated in 0.25% trypsin-EDTA, centrifuged, and resuspended in myo medium. Cells were incubated in G8 diluted 1:40 for 1 h, centrifuged, and resuspended in myo medium. Cells were plated as described above and fixed 24 or 48 h later. G8 was labeled with the IgM-specific secondary antibody conjugated with Alexa 488, MF20 IgG, and an IgG specific secondary antibody conjugated with rhodamine (Chemicon).

Determination of whether cells that expressed the G8 antigen in vivo contained MyoD protein was carried out by dissociating organs directly after their removal from the fetus, fixing cells as described above, then centrifuging them onto gelatin-coated glass slides. Cells were stained with the G8 antibody, the anti-IgM antibody conjugated with Alexa 488, the antibody to MyoD, and anti–mouse IgGs conjugated with Cy3. This procedure was also used to analyze G8-positive cells from the heart and brain for the expression of cardiac troponin I and neurofilament protein, respectively.

### Analysis of replication of G8-positive cells

Intestine cells were prelabeled with the G8 antibody and placed in culture as described above. 10 μM bromodeoxyuridine (BrdU) was added to the medium the day after plating. 4 h later the cells were rinsed, fixed, and stained with the IgM-specific secondary antibody conjugated with Alexa 488 to label G8, an IgG to BrdU (Roche Diagnostics), and rhodamine-conjugated anti-IgGs. Nuclei were counterstained with bis-benzamide.

### Flow cytometry and FACS®

Day 17 intestines, kidneys, and hearts were dissociated as described above and divided into three aliquots. One aliquot was incubated with the G8 antibody diluted 1:20–1:40 for 1 h at 37°C, centrifuged, and resuspended in myo medium containing goat anti–mouse IgM antibodies conjugated with fluorescein for 1 h at 37°C. The second aliquot of cells was incubated with only secondary antibody (control for background fluorescence) and the third aliquot received no antibodies (control for autofluorescence). Cells were centrifuged, resuspended in PBS containing 0.5% bovine serum albumin and 100 units of penicillin and streptopmycin, and filtered through a cell strainer (Falcon). Profiles of fluorescence intensity, forward light scatter, and side scatter were obtained with the FACStar Plus cell sorter (Becton Dickinson). After gating to eliminate dead cells and debris, G8-positive and negative intestine cells were sorted. Cells were placed in culture, fixed after 48 h, and stained with the MF20 and 12101 antibodies and IgG-specific secondary antibody conjugated with rhodamine. Cell sorting was carried out at the University of Pennsylvania's Flow Cytometry and Cell Sorting Shared Resources Facility (Philadelphia, PA). Analyses of fluorescence intensity and forward light scatter were consistent in eight experiments. Cell sorting results were from three different experiments. Multiple cultures were prepared with the positive and negative cells from each experiment.

We thank Dr. Karen Knudsen for critically reading the manuscript, Drs. Robert Fogel and James Kadushin for insightful discussions, William Murphy, Richard Schretzenmair, and Hank Pletcher for assistance with flow cytometry and cell sorting, Steven DeLuca, David Beckmann, Brigit Hearon, and Jordanna Perlman for technical assistance, and Genisphere, Inc. for supplying the dendrimers. RT-PCR experiments were conducted in the laboratory of the late Dr. Harold Weintraub.

This work was supported by the National Institutes of Health (HD36650-01) to M. George-Weinstein.

Submitted: 31 May 2001
Revised: 21 September 2001
Accepted: 21 September 2001

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