Epiblast cells that express MyoD recruit pluripotent cells to the skeletal muscle lineage

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Embryonic stem cells are derived from the epiblast. A subpopulation of epiblast cells expresses MyoD mRNA and the G8 antigen in vivo. G8 positive ($G8^{pos}$) and G8 negative ($G8^{neg}$) populations were isolated by magnetic cell sorting. Nearly all $G8^{pos}$ cells switched from E- to N-cadherin and differentiated into skeletal muscle in culture. $G8^{neg}$ cells were impaired in their ability to switch cadherins and few formed skeletal muscle. Medium conditioned by $G8^{pos}$ cells stimulated skeletal myogenesis and N-cadherin synthesis in $G8^{neg}$ cultures. The effect of conditioned medium from $G8^{pos}$ cultures was inhibited by bone morphogenetic protein (BMP) 4. Treatment of $G8^{neg}$ cells with a soluble form of the BMP receptor-IA or Noggin promoted N-cadherin synthesis and skeletal myogenesis. These results demonstrate that MyoD-positive epiblast cells recruit pluripotent cells to the skeletal muscle lineage. The mechanism of recruitment involves blocking the BMP signaling pathway.

Introduction

Embryonic stem cells (ES cells) are pluripotent cells derived from the epiblast (Smith, 2001). Defining the molecular mechanisms involved in regulating the pathways of differentiation of these pluripotent cells is critical for understanding tissue formation in the embryo and for using stem cells to repair damaged tissues in the adult. Various methods have been devised to promote mammalian ES cell differentiation including culturing them as three-dimensional aggregates, plating them on different extracellular matrices at high or low density, and supplementing the medium with growth factors (O'Shea, 2001).

We use an in vitro system comprised of epiblast cells from the chick embryo to dissect the steps involved in commitment and differentiation. Chick epiblast cells differentiate when cultured at high density in medium that lacks exogenous growth factors (George-Weinstein et al., 1996a). Although some neurons, notochord, and cardiac muscle cells emerge under these conditions, the preferred pathway of differentiation is skeletal myogenesis (George-Weinstein et al., 1996a).

The precise factors that direct the majority of chick epiblast cells to the skeletal muscle lineage in vitro are unknown; however, a change in the expression of cell–cell adhesion molecules appears to be required for terminal stages of differentiation. Cultured epiblast cells recapitulate the switch in expression from E- to N-cadherin that occurs in vivo when they enter the primitive streak and mesoderm (Edelman et al., 1983; Hatta and Takeichi, 1986; George-Weinstein et al., 1997). Although both E- and N-cadherin support the synthesis of the skeletal muscle specific transcription factor MyoD (Holt et al., 1994; George-Weinstein et al., 1997), adhesions via N-cadherin are necessary for the accumulation of sarcomeric proteins (George-Weinstein et al., 1997).

Alterations in cell adhesion also accompany skeletal myogenesis in vivo. Muscle differentiation is initiated in the embryonic somites, structures that form as a result of compaction and epithelialization of mesoderm cells (Christ and Ordahl, 1995; Pourquie, 2001). Members of the Wnt family produced in the neural tube and presomitic mesoderm,

Figure 1. **Characteristics of G8^{pos} and G8^{neg} epiblast cells in vivo and in vitro.** The stage 4 embryo was labeled with the G8 mAb and a secondary antibody conjugated with Alexa 488, followed by in situ hybridization with Cy3 labeled dendrimers containing a recognition sequence for MyoD mRNA. (A) Nuclei were stained with bis-benzamide (blue). Cells coexpressed the G8 antigen (green) and MyoD mRNA (red). The G8^{pos} and G8^{neg} populations were isolated from stages 3 to 5 epiblasts. After 5 d in culture, cells were stained with antibodies to the skeletal muscle specific 12101 antigen, cardiac muscle specific troponin T (CTpn), neurofilament associated antigen (Nf), and type II collagen (Col). G8^{pos} cells differentiated into skeletal muscle when plated at high (B) or low density (C). Some G8^{neg} cells differentiated into cardiac muscle (D), neurons (E), and chondroblasts (F). G8^{neg} cells formed skeletal muscle when grown in conditioned medium from G8^{pos} cultures (G). Noggin (H) and soluble BMP receptor-IA (I) also stimulated skeletal myogenesis in G8^{neg} cultures. Bar, 10 μm.
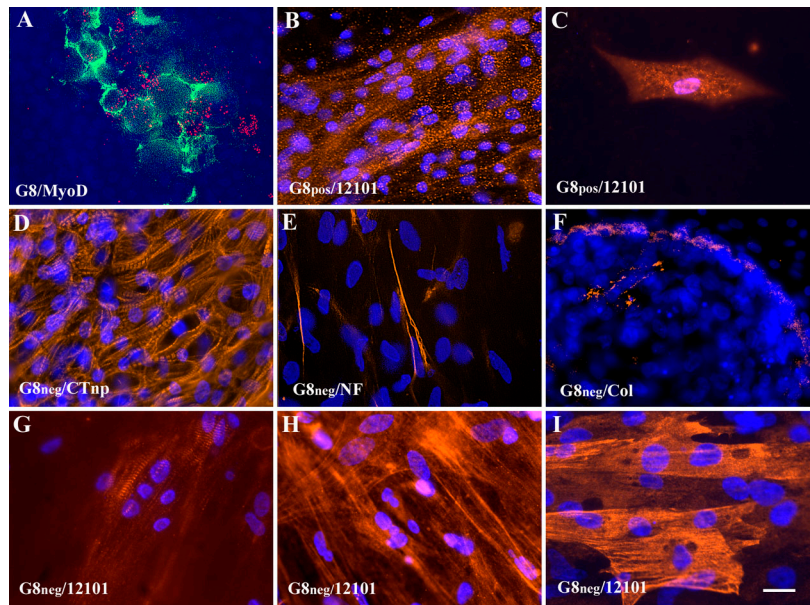
and sonic hedgehog released from the notochord, promote the expression of members of the MyoD family and the onset of differentiation in somites (Pownall et al., 2002). Wnts may play a dual role in regulating myogenesis in somites because they enhance the stability of β-catenin, a protein that serves as both a transcription factor and a link between cadherins and the actin cytoskeleton (Gumbiner, 2000). Somite cells from N-cadherin null mice are able to undergo myogenesis; however, they appear to express another cadherin (Radice et al., 1997).

Although skeletal myogenesis is regulated by factors in the environment of somites, MyoD mRNA is present in a small number of cells in the epiblast before somite formation (Gerhart et al., 2000). The following experiments were designed to explore the significance of this early expression of MyoD in the epiblast and the potential of these cells to regulate the fate of pluripotent cells. These experiments demonstrate that epiblast cells expressing MyoD mRNA in vivo differentiate into skeletal muscle in vitro. The MyoD positive (MyoD^{pos}) cells promote the expression of N-cadherin in pluripotent cells and their recruitment to the skeletal muscle lineage. The recruitment process involves blocking the bone morphogenetic protein (BMP) signaling pathway.

Results

Myogenesis in cultures of G8 positive (G8^{pos}) and G8 negative (G8^{neg}) epiblast cells

The epiblast of the chick embryo is an epithelium that expresses E-cadherin and gives rise to all three germ layers (Edelman et al., 1983; Bellairs, 1986; Hatta and Takeichi, 1986). A subpopulation of these cells expresses MyoD mRNA (Gerhart et al., 2000). When epiblasts are isolated from the mesoderm and hypoblast, dissociated, and cultured in serum-free medium on a substrate of gelatin and fibronectin, most cells switch from expression of E- to N-cadherin and differentiate into skeletal muscle (George-Weinstein et al., 1996a, 1997). To understand the role of epiblast cells that express MyoD

mRNA in these processes, MyoD^{pos} and MyoD-negative populations were isolated and cultured separately. The ability to purify living epiblast cells with MyoD is based on the expression of an as yet unidentified cell surface antigen that is recognized by the G8 mAb. In fetal organs, the G8 mAb selectively binds to cells that express MyoD mRNA (Gerhart et al., 2001). This is also the case for stages 3–5 epiblasts (Fig. 1 A).

Magnetic sorting of epiblast cells resulted in the isolation of the G8^{pos} and G8^{neg} populations, each with a purity of >97%, as determined by labeling with a fluorescent secondary antibody directly after sorting. After 5 d in culture, nearly all of the G8^{pos} cells contained MyoD protein and differentiated into skeletal muscle (Table I and Fig. 1 B). By contrast, only a small percentage of cells in the G8^{neg} cultures synthesized detectable levels of MyoD protein and 12101, a skeletal muscle specific marker for differentiation (Kitner and Brockes, 1984; Table I). The number of cells that stained with MF20, a mAb that recognizes sarcomeric myosin in both skeletal and cardiac muscle (Bader et al., 1982),

Table I. **Skeletal myogenesis in unsorted, G8^{pos}, G8^{neg}, and epiblast cultures**

	Percent positive		
	MyoD	**12101**	**Myosin**
Unsorted	82 ± 10 (10)	67 ± 8 (31)	80 ± 12 (16)
G8^{pos} cells	98 ± 3 (5)	95 ± 6 (16)	94 ± 4 (7)
G8^{neg} cells	7 ± 4 (21)	4 ± 4 (26)	33 ± 9 (16)

Stages 3–5 epiblast cells were labeled with the G8 mAb, separated into G8^{pos} and G8^{neg} populations by magnetic cell sorting, and cultured for 5 d. Unsorted cells were also plated. Cells were labeled with mAbs to MyoD, the skeletal muscle specific 12101 antigen, or sarcomeric myosin heavy chain present in both skeletal and cardiac muscle. Percent positive = (No. of fluorescent cells ÷ total cells) × 100. Values are the mean ± SD. The number of cultures scored is indicated in parentheses. A minimum of 200 cells was scored per culture. Nearly all cells in G8^{pos} cultures differentiated into skeletal muscle, whereas few cells formed skeletal muscle in G8^{neg} cultures. The higher percentage of myosin positive cells compared with 12101 positive cells reflects the presence of cardiomyocytes (see Table II).

Table II. **Differentiation of epiblast cells into chondroblasts, neurons, and cardiac muscle**

	Chondroblasts	Neurons	Cardiac muscle
	%	%	%
Unsorted	<1 (8)	4 ± 1 (10)	22 ± 10 (9)
G8^{pos} cells	ND	ND	1 ± 1 (7)
G8^{neg} cells	<1 (7)	5 ± 3 (6)	50 ± 7 (7)

Epiblast cells were sorted into G8^{pos} and G8^{neg} populations and cultured for 5 d. Cells were stained with the CIICI mAb to type II collagen (chondroblasts), 3A10 mAb to neurofilament-associated protein (neurons), and an mAb to cardiac muscle–specific troponin T (cardiac muscle). Percent = (No. of fluorescent cells ÷ total cells) × 100. Values are the mean ± SD. The number of cultures scored is indicated in parentheses. A minimum of 200 cells was scored per culture. The percentage of cardiomyocytes was higher in G8^{neg} cultures than in unsorted cultures which contain G8^{pos} cells (P ≤ 0.025).

Table III. **Differentiation of G8^{neg} cells in the presence of medium conditioned by G8^{pos} cells, Noggin, BMP-4, and soluble BMP receptor**

	Percent skeletal muscle
Untreated	2 ± 2 (22)
G8^{neg}-conditioned medium	2 ± 1 (4)
Unsorted conditioned medium	6 ± 2 (5)
G8^{pos}-conditioned medium	49 ± 12 (18)
Noggin	50 ± 9 (10)
G8^{pos}-conditioned medium + BMP-4	2 ± 2 (10)
Soluble BMP receptor-IA	69 ± 9 (8)
HGF/SF	6 ± 2 (4)

Medium conditioned for 48 h by unsorted, G8^{pos}, or G8^{neg} cells was added to 48-h G8^{neg} cultures. 50 ng/ml Noggin, 80 ng/ml of soluble BMP receptor-IA, and 25 ng/ml HGF/SF also were added to 48-h G8^{neg} cultures. 40 ng/ml BMP-4 was added to G8^{neg} cultures in G8^{pos}-conditioned medium. Cells were stained with the 12101 mAb on the fifth day in culture. Percent skeletal muscle = (No. of fluorescent cells ÷ total cells) × 100. Values are the mean ± SD. The number of cultures scored is indicated in parentheses. A minimum of 200 cells was scored per culture. Medium from G8^{pos} cultures, Noggin, and soluble BMP receptor-IA stimulated myogenesis in G8^{neg} cultures. BMP-4 blocked the stimulatory effect of G8^{pos}-conditioned medium.

was also reduced in G8^{neg} cultures; however, this population was larger than that expressing 12101 (Table I). The significance of this finding is addressed in the next section.

After 2 wk in culture the percentage of G8^{neg} cells with 12101 remained low (3% ± 2, $n = 6$). Therefore, in the absence of cells that express MyoD (and the G8 antigen) in vivo, most epiblast cells do not differentiate into skeletal muscle in vitro. When G8^{neg} cells were recombined with G8^{pos} cells, the percentage of differentiated skeletal muscle was similar to that found in unsorted cultures (62% ± 6, $n = 4$). This indicates that the cell sorting procedure did not impair the innate ability of the G8^{neg} population to undergo skeletal myogenesis and demonstrates the validity of using unsorted cells as controls in subsequent experiments.

Differentiation of nonskeletal muscle cell types in epiblast cultures

The ability of epiblast cells to differentiate into nonskeletal muscle cell types in the absence of the MyoD/G8^{pos} cells was investigated with cell type–specific antibodies. Although the majority of cells in unsorted cultures formed skeletal muscle, some chondroblasts, cardiac muscle cells, and neurons began to emerge after the first day in culture (George-Weinstein et al., 1996a; Tables I and II). The sum of the percentages of these four cell types equaled >90%, indicating that in unsorted cultures few undifferentiated cells or those of other lineages were present by the fifth day in culture.

Sorted cells also were examined for markers of other lineages (Table II; Fig. 1, D–F). The number of chondroblasts and neurons were similar in unsorted and G8^{neg} cultures, suggesting that enhanced recruitment of cells to the chondrogenic and neurogenic lineages did not occur in the absence of the G8^{pos} cells. The percentage of cells labeled with the cardiac troponin T antibody was similar to that of the MF20/sarcomeric myosin–positive population (Tables I and II). Therefore, most muscle cells in G8^{neg} cultures were cardiomyocytes and not skeletal muscle. The percentage of cardiomyocytes in G8^{neg} cultures was larger than that in unsorted cultures (P ≤ 0.025). However, all three populations combined accounted for only 55% of the cells in these cultures suggesting that nearly half of the cells in G8^{neg} cultures were not recruited to the chondrogenic, cardiomyogenic, or neurogenic lineage even in the absence of G8^{pos} cells. This may

reflect suboptimal culture conditions for the differentiation of these cell types and/or the absence or ineffectiveness of cells capable of recruiting pluripotent cells to these lineages.

The effect of medium conditioned by G8^{pos} cells on G8^{neg} cells

The effects of the G8^{pos} cells on G8^{neg} cells in mixed cultures were at least partially mediated by soluble factors because transfer of conditioned medium from 48-h G8^{pos} cultures stimulated skeletal myogenesis in G8^{neg} cultures (Table III and Fig. 1 G). Medium from unsorted 48-h cultures had a slight stimulatory effect on muscle differentiation in G8^{neg} cultures. When conditioned medium from G8^{pos} cultures was added to G8^{neg} cells plated at low density, only a small percentage of cells not in contact with other cells synthesized detectable levels of the 12101 antigen (10 ± 4, $n = 4$). Therefore, cell–cell contacts facilitate the effect of conditioned medium on differentiation. By contrast, most G8^{pos} cells plated at low density were stained with the 12101 mAb (92% ± 2, $n = 5$; Fig. 1 C). This demonstrates that the differentiation of epiblast cells expressing MyoD mRNA before removal from the embryo is independent of direct cell–cell interactions.

Effects of noggin, BMP-4, and soluble BMP receptor-IA on the differentiation of G8^{neg} cells

In an attempt to identify the factors produced by G8^{pos} cells that stimulated myogenesis in G8^{neg} cultures, conditioned medium was analyzed by silver staining. Bands of similar molecular weight were present in G8^{pos}- and G8^{neg}-conditioned media indicating that the factors were present in amounts below the level of detection by this method. A second approach was taken to identify the factors that involved addition of growth factors to G8^{neg} cultures.

We had shown previously that treatment of epiblast cells from pregastrulating embryos with hepatocyte growth factor/scatter factor (HGF/SF) resulted in a moderate increase in muscle differentiation (DeLuca et al., 1999). G8^{neg} cells from
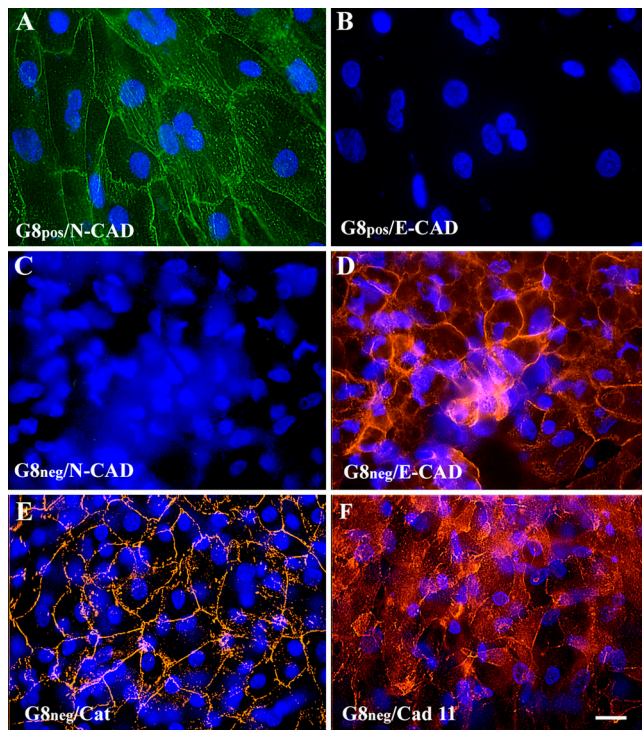
Figure 2. **Immunofluorescence localization of cadherins and β-catenin in G8^pos and G8^neg cultures.** G8^pos and G8^neg cells were isolated from stages 3 to 5 epiblasts, cultured for 5 d, and double labeled with antibodies to N- and E-cadherin (A–D), or single labeled with antibodies to cadherin 11 and β-catenin. Most G8^pos cells expressed N- but not E-cadherin (A and B). G8^neg cultures contained cells with E- but not N-cadherin (C and D). G8^neg cells stained with antibodies to β-catenin (E) and cadherin 11 (F). Bar, 10 μm.

gastrulating embryos did not form skeletal muscle in response to HGF/SF, Wnt-1 (George-Weinstein et al., 1998), or Wnt-3A; however, they did respond to Noggin (Table III and Fig. 1 H). A dose response experiment revealed that 50 ng of human recombinant Noggin per milliliter of medium produced maximal stimulation of myogenesis.

Because Noggin is an inhibitor of BMPs (for review see Balemans and Van Hul, 2002), and BMP-4 is a regulator of myogenesis in the somites (Pourquie et al., 1996; Dietrich et al., 1998; Sela-Donenfeld and Kalcheim, 2002), BMP-4 was added to G8^pos-conditioned medium at the time of its addition to G8^neg cultures. BMP-4 blocked the stimulatory effect of G8^pos-conditioned medium on myogenesis (Table III). Maximal inhibition was produced with a dose of 40 ng/ml.

Further evidence for involvement of BMPs in the inhibition of G8^neg differentiation is that a soluble form of the BMP receptor-IA, a known competitor of the endogenous receptor for ligand binding, stimulated myogenesis in G8^neg cultures (Table III and Fig. 1 I). The optimal dose for this effect was 80 ng/ml. These results suggest that BMP signaling represses myogenesis in G8^neg cultures and G8^pos cells produce a BMP antagonist.

Expression of cadherins in G8^pos and G8^neg epiblast cultures

Previous experiments demonstrated that epiblast cells switch from E- to N-cadherin as they ingress through the primitive

streak (Edelman et al., 1983; Hatta and Takeichi, 1986; unpublished data), and down-regulation of E-cadherin and adhesion via N-cadherin are required for epiblast cells to form skeletal muscle in vitro (George-Weinstein et al., 1997). 3 h after plating, most G8^pos cells continued to express E-cadherin (67 ± 17, $n = 5$); however, some were synthesizing N-cadherin (21 ± 11, $n = 5$). After 24 h in culture, most G8^pos were stained with antibodies to both N-cadherin (70 ± 19, $n = 6$) and E-cadherin (74 ± 12, $n = 6$). By the fifth day, most cells in G8^pos cultures expressed N-cadherin, but not E-cadherin (Table IV; Fig. 2, A and B).

G8^neg cells were impaired in their ability to switch from E- to N-cadherin (Table IV; Fig. 2, C and D). Although there was some variability of cadherin expression in G8^neg cultures between experiments, the percentage of N-cadherin positive (N-cadherin^pos) cells was significantly smaller ($P \leq 0.0005$) and the E-cadherin positive population significantly larger ($P \leq 0.005$) than that observed in unsorted and G8^pos cultures. Some cells in G8^neg cultures expressed neither N- nor E-cadherin (Table IV); however, β-catenin was localized to the membrane of all cells and most cells expressed cadherin 11 (Fig. 2, E and F).

The percentage of MyoD^pos cells in unsorted cultures was similar to the number of cells expressing N- but not E-cadherin (Tables I and IV). This was not the case in G8^neg cultures where the percentages of cells with MyoD and the 12101 antigen were smaller than the N-cadherin^pos/E-cadherin negative population (Tables I and IV). Therefore, a switch in expression of cadherins alone does not promote myogenesis. However, when G8^neg cells were cultured in medium conditioned by G8^pos cells or treated with Noggin or soluble BMP receptor-IA, the increase in muscle differentiation was accompanied by an increase in the percentage of cells with N-cadherin and a decrease in the population of cells expressing both cadherins (Tables III and IV). These results suggest that G8^pos cells produce an antagonist of the BMP pathway that promotes the expression of N-cadherin while stimulating skeletal myogenesis.

Discussion

The epiblast is a pluripotent tissue that gives rise to the three germ layers of the embryo (Bellairs, 1986; O'Shea, 2001; Smith, 2001). Undifferentiated cells that express MyoD mRNA are embedded within the epiblast epithelium (Gerhart et al., 2000). This study revealed that these MyoD^pos cells form skeletal muscle when removed from the embryo, differentiate in the absence of cell–cell contacts, promote a switch in the expression of E- to N-cadherin in pluripotent cells, and recruit them to the skeletal muscle lineage. These findings enhance our knowledge of the behavior of epiblast cells in vitro and have important implications for understanding the regulation of ES cell fate.

Skeletal myogenesis is the preferred pathway of differentiation when the entire population of chick epiblast cells is grown in vitro (George-Weinstein et al., 1996a). This phenomenon is dependent on several factors including separating the epiblast from the mesoderm, disrupting cell–cell contacts within the epithelium, plating cells on a substrate of denatured collagen and fibronectin to promote their attach-

Table IV. **Expression of cadherins in cultures of unsorted, G8^{pos}, and G8^{neg} epiblast cells**

	% total with N-cad	N-cad^{pos} without E-cad	% total with E-cad	E-cad^{pos} without N-cad	% total with N-cad and E-cad	% total without N-or E-cad
Unsorted n = 8	87 ± 5	83 ± 4	15 ± 3	11 ± 6	5 ± 3	2 ± 5
G8^{pos} cells n = 7	87 ± 4	85 ± 3	14 ± 3	12 ± 3	1 ± 1	1 ± 1
G8^{neg} cells n = 16	45 ± 23	34 ± 25	39 ± 21	29 ± 26	10 ± 8	26 ± 30
G8^{neg} cells with unsorted CM n = 4	45 ± 9	95 ± 2	56 ± 10	96 ± 1	2 ± 1	0
G8^{pos} CM n = 6	65 ± 8	94 ± 7	39 ± 10	92 ± 11	4 ± 5	0
Noggin n = 6	63 ± 13	98 ± 2	35 ± 15	98 ± 2	1 ± 1	2 ± 2
Sol. BMP receptor n = 7	62 ± 7	98 ± 2	37 ± 5	97 ± 3	1 ± 1	2 ± 2

Stages 3–5 epiblast cells were labeled with the G8 mAb, separated by magnetic cell sorting, and cultured for 5 d in DMEM/F12 medium. G8^{neg} cells were also cultured in conditioned medium (CM) by unsorted or G8^{pos} cells, or DMEM/F12 containing 50 ng/ml Noggin or 80 ng/ml soluble BMP receptor-IA. Cells were double labeled with a rabbit polyclonal antibody to N-cadherin and a mouse mAb to E-cadherin, and species-specific fluorescent secondary antibodies. Percentage of total cells = (No. of cells with N-cadherin, E-cadherin, both cadherins, or neither cadherin ÷ total cells) × 100. N-Cadherin^{pos} without E-cadherin = (No. of N-cadherin^{pos} cells without E-cadherin ÷ No. of cells with N-cadherin) × 100. E-Cadherin^{pos} without N-cadherin = (No. E-cadherin^{pos} cells without N-cadherin ÷ No. of cells with E-cadherin) × 100.   Values are the mean ± SD. The number of cultures scored is indicated in parentheses. A minimum of 200 cells was scored per culture. Most cells in G8^{pos} cultures expressed N- but not E-cadherin. Cadherin switching was reduced in G8^{neg} cultures. Conditioned medium from G8^{pos} cultures, Noggin, and soluble BMP receptor-IA stimulated N-cadherin expression in G8^{neg} cultures.

ment and survival, and culturing them in serum-free medium (George-Weinstein et al., 1996a). In addition, most unsorted epiblast cells must be plated at high density in order to differentiate (George-Weinstein et al., 1996b). This reflects a requirement for cadherin-mediated adhesions to support the synthesis of MyoD and sarcomeric myosin (George-Weinstein et al., 1997). Our conclusion from these earlier experiments was that the emergence of skeletal muscle as the dominant phenotype in epiblast cultures was the result of a "community effect" (Gurdon, 1988) in which equivalent cells were induced to differentiate along the same pathway (George-Weinstein et al., 1996a). In these cultures, the community effect was mediated by cell–cell adhesions via N-cadherin (George-Weinstein et al., 1997).

We have now determined the epiblast is a heterogeneous tissue comprised of both pluripotent cells and those that are biased, and possibly committed, to differentiate into skeletal muscle. Furthermore, it would seem that the single most important factor for the widespread induction of skeletal myogenesis in epiblast cultures is the release of factors from cells that are preprogrammed to form skeletal muscle within the embryo. Despite the fact that the culture conditions overwhelmingly support skeletal myogenesis, most pluripotent cells cannot form skeletal muscle in the absence of epiblast cells that express MyoD mRNA and the G8 antigen in vivo.

The MyoD^{pos}/G8^{pos} epiblast cells also affect cadherin expression. In unsorted and G8^{pos} cultures, most cells down-regulated E-cadherin and up-regulated N-cadherin during skeletal muscle differentiation. In addition, previous experiments with unsorted epiblast cells revealed that both E- and N-cadherin supported MyoD protein synthesis; however, only those cells with N-cadherin but not E-cadherin differentiated (George-Weinstein et al., 1997). The G8^{neg} cells

differed from unsorted and G8^{pos} cells in three ways. First, G8^{neg} cells were impaired in their ability to switch cadherins. Second, neither cadherin appeared to support the synthesis of MyoD protein in more than a minor population of G8^{neg} cells. Finally, the percentage of differentiated skeletal muscle cells was significantly less than the percentage of N-cadherin^{pos}/E-cadherin negative cells. The lack of skeletal myogenesis could reflect, in part, reduced homotypic N-cadherin–mediated adhesions due to the presence of cells that continued to express E-cadherin alone or both cadherins.

The precise factors produced by MyoD^{pos}/G8^{pos} cells that stimulate myogenesis and N-cadherin expressions remain to be identified; however, the effect of the G8^{pos} cells appears to involve antagonism of the BMP signaling pathway. Noggin, an inhibitor of BMPs-4, -6, and -7, and growth and differentiation factor-5 (for review see Balemans and Van Hul, 2002), stimulated N-cadherin synthesis and skeletal myogenesis when added to G8^{neg} cultures. Furthermore, BMP-4 blocked the stimulatory effect of G8^{pos}-conditioned medium, and a soluble form of BMP receptor-IA known to compete with the endogenous receptor for ligand binding, increased muscle differentiation in G8^{neg} cultures in the absence of G8^{pos}-conditioned medium. Releasing the G8^{neg} cells from the inhibitory effects of BMPs may be sufficient to trigger myogenesis, although it is possible that these cells produce stimulatory factors that are also required for skeletal muscle differentiation.

These experiments provide additional evidence that skeletal myogenesis is the preferred pathway of chick epiblast cells under these culture conditions. They also complement experiments of the regulation of muscle differentiation in the somite. Expression of Noggin in the presomitic and somitic mesoderm releases cells from BMP-mediated repression of

MyoD and Myf5 activation (Pourquie et al., 1996; Hirsinger et al., 1997; Marcelle et al., 1997; Dietrich et al., 1998; Reshef et al., 1998; Amthor et al., 1999; Sela-Donenfeld and Kalcheim, 2002; Linker et al., 2003). Noggin is also involved in the down-regulation of E-cadherin during epithelial bud formation in combination with Wnt3a (Jamora et al., 2003). At this time it is unclear whether G8^{pos} cells directly affect cadherin synthesis, or if the expression of N-cadherin follows the recruitment of G8^{neg} cells to the skeletal muscle lineage.

The results of our cell sorting experiments support the theory proposed decades ago by Holtzer and colleagues (Holtzer et al., 1983) that founder cells for the myogenic lineage exist in the early embryo (Choi et al., 1989). Testing the role of these cells in vivo could be accomplished by ablating the MyoD population at early times in development. There are several possible outcomes of this type of experiment including a complete or partial disruption of muscle differentiation, or no effect due to a stimulation of myogenic transcription factor expression in other cells. Molecules downstream in the sonic hedgehog pathway can directly regulate the promoter of the MyoD family member, myf5 (Gustafsson et al., 2002), suggesting that signaling from the notochord may compensate for the loss of MyoD^{pos} epiblast cells. Alternatively, release of factors from the original population of MyoD expressing cells could prime surrounding pluripotent cells to respond to molecules produced by the notochord and neural tube. In this regard, a subpopulation of cells within the presomitic mesoderm secretes Wnt5b which stimulates MyoD expression (Linker et al., 2003). It is possible that the Wnt5b producing mesoderm cells are derived from cells that express MyoD within the epiblast.

Apart from any role that the MyoD^{pos} epiblast cells may play in regulating muscle differentiation in vivo, their ability to recruit pluripotent cells to the myogenic lineage in vitro has important implications in the field of stem cell biology. We suggested previously that implanting a mixture of committed and pluripotent stem cells into diseased tissues might facilitate regeneration (Gerhart et al., 2001). Our results now suggest that exposure of stem cells to committed precursors or the factors they produce before implantation may preprogram them to follow the desired pathway in vivo. Asakura et al. (2002) demonstrated that neonatal myoblasts can induce stem cells derived from adult muscle to undergo skeletal myogenesis; however, the rate of conversion of these stem cells to the muscle lineage was low ($\sim$4%). Furthermore, conditioned medium from the neonatal myoblast cultures did not stimulate the stem cells to differentiate. This suggests that stem cells from adult muscle are more resistant to conversion to the myogenic lineage than pluripotent epiblast cells and/or neonatal myoblasts are less effective recruiters than MyoD^{pos} epiblast cells. A third possibility is that the culture conditions for the neonatal myoblasts and adult stem cells were less permissive for the recruitment process than the epiblast culture system. Modifications in the method for pretreating adult stem cells with committed cells may enhance the extent of their conversion to the myogenic lineage and their regenerative potential in vivo.

An equally important consideration for the use of stem cells therapeutically is the role of the community effect in facilitating differentiation (Gurdon, 1988; Cossu et al., 1995). The molecular mechanism for the community effect involves cadherin-mediated adhesion (Holt et al., 1994; George-Weinstein et al., 1997). Although the G8^{pos} cells release soluble factors that stimulate skeletal myogenesis in G8^{neg} cultures, the factors are more effective when G8^{neg} cells are plated at high density. Ensuring that the appropriate cadherin is expressed in the stem cell population may be a critical component of a strategy to regenerate skeletal muscle and other tissues.

Materials and methods

Double labeling of embryos with 3DNA™ dendrimers and the G8 antibody

Previous experiments revealed that MyoD mRNA and the G8 antigen are expressed in the same cells in fetal organs (Gerhart et al., 2001). To test whether G8 is a marker for embryonic cells expressing MyoD, double labeling was performed with the G8 mAb and Cy3-labeled 3DNA™ dendrimers (Wang et al., 1998; Genisphere, Inc.), having a cDNA sequence for MyoD mRNA ligated to their outer arms as described previously (Gerhart et al., 2001). The antisense oligonucleotide 5'-TTCTCAAGAGCAAAT-ACTCACCATTTGGTGATTCCGTGTAGTAGCTGCTG-3' was derived from chicken MyoD (Dechesne et al., 1994). The antibody was applied to living stages 3–5 (Hamburger and Hamilton, 1951) White Leghorn chick embryos (CBT Farms; B&E Farms), followed by fixation in 2% formaldehyde and incubation in a goat anti–mouse IgM secondary antibody conjugated with Alexa 488 (Molecular Probes). After permeabilizing cells with Triton X-100 and pepsin, in situ hybridization with Cy3-labeled dendrimers was performed and nuclei were counterstained with bis-benzamide (Gerhart et al., 2000, 2001). Embryos were observed with an epifluorescence microscope (model Eclipse E800; Nikon, purchased from Optical Apparatus). Photomicrographs were produced with the Evolution QE video camera (Media Cybernetics) and Image-Pro Plus image analysis software (Phase 3 Imaging Systems).

Magnetic cell sorting

Epiblasts were isolated from stages 3 to 5 embryos and dissociated in 0.25% trypsin-EDTA (Invitrogen) at 37°C for 10 min (George-Weinstein et al., 1996a). Cells were centrifuged and resuspended in Dulbecco's minimal essential medium (DMEM) containing 5% FBS, 5% horse serum, 100 U of penicillin and streptomycin (Invitrogen Corp.), and 5% chick embryo extract (Myo medium). The G8 mAb was added to the cell suspension at a concentration of 2 μg/ml. After incubation at 37°C for 45 min, cells were centrifuged and resuspended in 80 μl PBS. 20 μl of rat anti–mouse IgM microbeads (Miltenyi Biotec) were added for 15 min at RT. Cells were centrifuged, resuspended in 500 μl PBS, and added to the MiniMACS column attached to the magnetic sorting separation unit (Miltenyi Biotec). G8^{neg} cells were eluted by rinsing the column with PBS. The column was then removed from the separation unit and the G8^{pos} cells eluted in PBS with gentle plunging. The purity of the sorts was determined by labeling an aliquot of the positive and negative populations with a fluorescent secondary antibody, and calculating the percentage of the total cells that was fluorescent. G8^{pos} and G8^{neg} cells were centrifuged, resuspended in Myo medium, and placed in culture.

Preparation of epiblast cell cultures

Sorted and unsorted epiblast cells were cultured under conditions shown previously to promote myogenesis (George-Weinstein et al., 1994, 1996a). 20,000 cells in 15 μl Myo medium were plated in the center of 35-mm tissue culture dishes coated with gelatin and human serum fibronectin (Invitrogen). After the cells had attached to the dish (90 min), they were flooded with 1 ml serum and hormone-free DMEM/F12 medium (Invitrogen). Low density cultures were prepared by plating 40,000 cells in 1 ml of Myo medium over the entire surface of a coated 35-mm dish. 90 min after plating, the medium was replaced with DMEM/F12.

G8^{neg} cells were treated with conditioned medium by removing their medium after 48 h in culture and replacing it with 48-h medium obtained from G8^{pos}, other G8^{neg}, or unsorted cultures. The medium was centrifuged to remove cells before its addition to the G8^{neg} cultures. Medium in 48-h G8^{neg} cultures was replaced with 1 ml DMEM/F12 medium containing recombinant human Noggin (PeproTech), soluble BMP receptor-IA/Fc chi-

meric protein, HGF/SF, or 1 ml G8[pos]-conditioned medium containing recombinant human BMP-4 (all purchased from Sigma-Aldrich).

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, 1996a). Labeling with antibodies to the cadherins required fixation in methanol. The skeletal muscle specific 12101 mAb (Kitner and Brockes, 1984), MF20 mAb to sarcomeric myosin heavy chain (Bader et al., 1982), mAb to LCAM/E-cadherin (Gallin et al., 1983), CIIC1 mAb to type II collagen (Holmdahl et al., 1986), and 3A10 mAb to neurofilament associated antigen (Furley et al., 1990) were obtained from the Developmental Studies Hybridoma Bank. The AB-1 mAb to cardiac muscle specific troponin T (Neomarkers) stained cardiomyocytes in vivo but did not bind skeletal muscle cells in fetal pectoralis muscle cultures or G8[pos] cultures (Table IV; Fig. 1 E). The mAb 5B2H5 to cadherin 11 was purchased from Zymed Laboratories. A rabbit polyclonal antiserum to N-cadherin was generated as described previously (George-Weinstein et al., 1997). Secondary antibodies were affinity purified, goat anti–mouse F(ab')2 fragments conjugated with rhodamine or Cy3 (Jackson ImmunoResearch Laboratories), and goat anti–rabbit IgG conjugated with Cy2 (CHEMICON International, Inc.). Cells that had been prelabeled with the G8 IgM mAb before being placed in culture were later stained with IgG primary mAbs and a goat anti–mouse IgG secondary antibody conjugated with rhodamine (CHEMICON International, Inc.). Nuclei were counterstained with bis-benzamide. The anti-IgG secondary antibody did not recognize the G8 IgM mAb.

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