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Single-cell transplantation determines the time when *Xenopus* muscle precursor cells acquire a capacity for autonomous differentiation

(muscle cell differentiation/*Xenopus* embryos)

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ABSTRACT **We have used a single-cell transplantation technique to find out whether there is a stage in development when a single cell can reach and maintain its differentiated state in the absence of its neighbors. Muscle precursor cells from early, mid-, and late gastrula stages of *Xenopus laevis* embryos were isolated and transplanted singly into the ventral region of late gastrula hosts. Single cells from late gastrulae differentiated into muscle when surrounded by nonmuscle cells. Similar cells from early or mid-gastrulae did not, unless they were transplanted as a group of adjacent cells taken from the same region of an embryo. These results show that single embryonic cells in a tissue can complete their differentiation without interacting with their normal neighbors and that, in the case of *Xenopus* muscle precursor cells, they acquire this capacity at the late gastrula stage. Our results also suggest that, in addition to mesoderm induction, further cell interactions during gastrulation are required for *Xenopus* muscle cell differentiation.**

It is becoming increasingly clear that the process of cell differentiation in vertebrates as well as invertebrates is controlled by both cell-autonomous and environmental mechanisms (1–3). For example, it was once thought that the fates of cells in some invertebrates such as *Caenorhabditis elegans* and ascidians are determined by the patterns of cell division and are independent of influences from surrounding cells (3, 4). However, recent results show that some of the cells in these two species are also formed as a result of cell interactions (4, 5).

In vertebrates, emphasis has always been put on the roles of cell interactions. To date numerous examples of cell interactions are known to be important in development (2, 6, 7). One example is mesoderm induction in amphibian embryos (6–8), and at present this is one of the best-characterized inductive interactions. However, even in the case of amphibian mesoderm formation, it is not yet clear precisely when and where cell interactions are required. It may be that the receipt of the mesoderm-inducing signals during blastula stages is sufficient for the subsequent differentiation of induced cells, and they may acquire the capacity for autonomous differentiation once this step is completed. Alternatively, since animal cells are always surrounded by other cells in a tissue, it is possible that cells continue to require signals from their neighbors to establish and maintain their differentiated state throughout embryogenesis and perhaps in adult life. Moreover, in the case of cells in a solid tissue such as muscle where they are often in contact with cells of the same kind as themselves, signals from their own type of cells may be especially important to maintain clear boundaries and homogeneity within tissues.

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To guide analysis at the molecular level, it is important, in any developmental system, to determine the precise requirement for cell interactions. One of the best ways to do this is to change the location of individual cells so that they are exposed to a new environment (1). The transfer of a single cell is especially important because if groups of cells are transplanted together, they can still interact with their original neighbors. If, at the time of manipulation, a single cell is no longer dependent on any interactions with its neighboring cells, it should be able to differentiate even in a foreign environment.

In this work, therefore, we have used a single-cell transplantation technique to analyze muscle cell differentiation in *Xenopus laevis* embryos. When single muscle precursor cells from late gastrulae are transplanted into the blastocoel of late gastrula hosts, they differentiate into mature muscle in the ventral trunk region of host embryos. In contrast, muscle precursor cells from early or mid-gastrulae do not differentiate into muscle under the same conditions, though they do so when they are transplanted as a group of cells. These results show that after the late gastrula stage *Xenopus* muscle precursor cells can complete their differentiation in the absence of their original neighbors. They also suggest that, in addition to mesoderm induction, further cell interactions during gastrulation are required for *Xenopus* muscle cell differentiation.

MATERIALS AND METHODS

Culture of Embryos and Single-Cell Transplantation. Embryos were obtained by *in vitro* fertilization and cultured in 1/10 MBS (9). They were staged according to Nieuwkoop and Faber (10). Donor embryos were injected with rhodamine-conjugated lysinated dextran (RLDx; Molecular Probes D-1817, 5 mg/ml in water) in both blastomeres at the two-cell stage to label all the cells. When injected embryos reached the stage for analysis, tissue pieces containing muscle precursor cells were isolated and dissociated in Ca^{2+}- and Mg^{2+}-free MBS containing 0.5% bovine serum albumin. For the late gastrula stage, muscle precursor cells were isolated from either side of the anterior two-thirds of the notochord, and dissection of the tissue pieces was done in MBS containing collagenase at 3 mg/ml. Pipettes used for transplantation were hand pulled from glass capillary tubes (BDH) and coated with 0.1% poly(2-hydroxyethylmethacrylate) (Aldrich) in ethanol/acetone (1:1, vol/vol) to prevent sticking of cells. Tips of pipettes had an inner diameter of 100–150 μm and were polished by flaming. Host embryos were transferred to small holes in 1% agarose-coated dishes containing MBS, after removal of the vitelline membrane, and were maintained with the ventral side up. Dissociated cells were sucked individually into a pipette and expelled or dropped

Abbreviation: RLDx, rhodamine-conjugated lysinated dextran.

into the blastocoel of host embryos through a hole made in the ectoderm. Host embryos were left in MBS for 1 hr to allow healing, then transferred to 1/10 MBS and cultured until control embryos became tadpoles (stage 28–30).

Antibody Staining. Cultured embryos were fixed in MEMFA (11) for about 2 hr, kept in methanol overnight, and bleached in 10% (wt/vol) hydrogen peroxide in 70% (vol/vol) methanol overnight for whole-mount viewing. For whole-mount viewing, embryos were dehydrated and cleared in 2:1 (vol/vol) benzyl benzoate/benzyl alcohol. Embryos with intact labeled cells were selected and embedded in Paraplast. Antibody staining of paraffin sections (10 μm) was carried out with alkaline phosphatase–anti-alkaline phosphatase complex (APAAP; Boehringer Mannheim) according to ref. 12. Substrates for color reactions were 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT). The antibody 12/101 (13), as well as antibody MF20 [which recognizes the myosin heavy chain (14)], first stains embryos weakly around stage 18, and the staining becomes stronger as development proceeds. The antigen of 12/101 antibody has not been identified, but the antibody is highly specific to axial muscle cells in *Xenopus* embryos. Dissociated cells were washed in Ca^{2+}- and Mg^{2+}-free MBS twice to remove bovine serum albumin and fixed on a polylysine-coated slide with MEMFA. They were then stained with antibody against *Xenopus* MyoD protein (XMyoD), which labels the nuclei of future muscle cells (12).

Labeling Transplanted Cells with [^{3}H]Uridine. For the labeling of single transplanted cells with [^{3}H]uridine, single cells were isolated from dorsolateral pieces at mid-gastrula stage and transplanted to the blastocoel of late gastrula hosts as described in Fig. 2*d*. After overnight culture in 1/10 MBS, when control embryos reached about stage 27, [5,6-^{3}H]uridine (Amersham) solution (30 nl) was injected to the middle trunk region and embryos were left for 2 hr. They were fixed with MEMFA and processed for antibody stain-

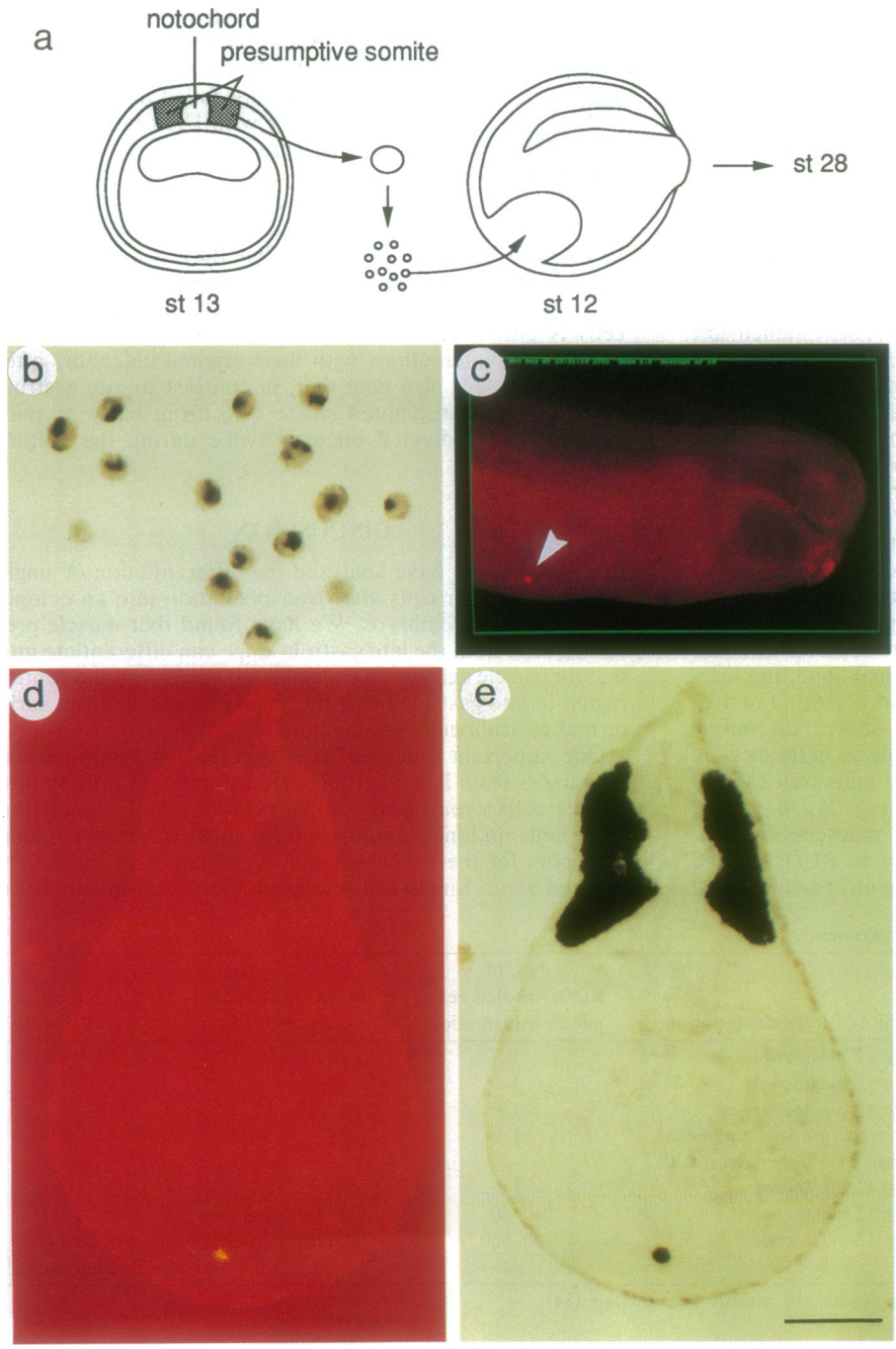

FIG. 1. Single-cell transplantation of muscle precursor cells from late gastrulae. (*a*) Procedure for single cell transplantation. st, Stage. (*b*) Dissociated presumptive somite cells stained with anti-XMyoD antibody after isolation and fixation at stage 13. (*c*) Confocal whole-mount image of a host embryo with an RLDx-labeled transplanted cell in the ventral trunk region (arrowhead). (*d*) Cross section of a host embryo with an RLDx-labeled transplanted cell in the endoderm region. (*e*) Same section as *d* stained with the anti-muscle antibody 12/101 (13). The transplanted cell, as well as host muscle cells, shows strong staining. (Scale bar = 100 μm for *b*, 550 μm for *c*, and 200 μm for *d* and *e*.)

ing. The results of antibody staining were recorded and the slides were processed for autoradiography. After 15 days of exposure, they were developed, stained with Hoechst 33258 (10 μg/ml in phosphate-buffered saline) for 3 hr, washed, and mounted with 90% (vol/vol) glycerol.

RESULTS

In the first experiment, we have used muscle precursor cells (those fated for axial muscle) from late gastrulae [stage 13 (10)] because this is the first stage when such cells can be easily isolated. They were isolated from either side of the notochord and dissociated (Fig. 1*a*). When stained with anti-XMyoD antibody (12), more than 80% of them showed nuclear staining (Fig. 1*b*). They were transplanted individually into the blastocoel of late gastrula hosts, which were cultured until they became tadpoles (stage 28) (Fig. 1*a*). After fixation, host embryos were examined under a fluorescence microscope. About 90% of the host embryos contained transplanted cells in the ventral trunk region (Fig. 1*c*). Sections were stained with the muscle-specific antibody 12/101 (13), a late marker of axial muscle, and more than 80% of the transplanted cells showed positive staining (Fig. 1 *d* and *e*; Table 1, Exp. 1). None of the positive cells had divided, a result expected because in normal embryos more than 90% of muscle precursor cells do not undergo cell division after late gastrula stages (E. Hirsinger and K.K., unpublished observation). These positive cells also showed round morphology and did not elongate, but this could be due to the lack of other muscle cells to form correct tissue organization. An antibody against the myosin heavy chain, MF20 (14), another late marker for muscle, also stained 80% of the transplanted cells (Table 1, Exp. 2). In normal embryos, both of these antibodies label muscle cells starting from late neurula stages and do not stain gastrula cells. It should also be noted that, in *Xenopus* embryos, axial muscle cells mature without undergoing cell fusion (15). Therefore, we conclude that single muscle precursor cells from late gastrulae can differentiate into mature muscle even when surrounded by nonmuscle cells in an ectopic position.

We next asked whether muscle precursor cells from earlier stages behave in the same way as the late gastrula cells just described (Fig. 2). As a source of muscle precursor cells from the early gastrula (stage 10¼), we used small pieces of tissue from the dorsolateral region of the marginal zone, the location of future somite cells [Fig. 2 *a* and *c* (16)]. For two reasons, we believe they are a good source of muscle precursor cells. First, when dorsolateral pieces of tissue were cultured as explants *in vitro* and assayed with anti-XMyoD (12) or 12/101 antibody (13) as shown in Fig. 2*b*, 30–60% of cells always became positive for these markers. Second, when a dorsolateral piece of tissue from an RLDx-labeled embryo was transplanted into the same region of an unlabeled embryo, 30–50% of labeled cells always differentiated into muscle. For mid-gastrula donors (stage 11½), we used cells from dorsolateral pieces of tissue isolated at stage 10¼ and cultured as tissue explants until stage 11½ (Fig. 2*d*). We believe the conclusions with cells from explants are applicable to normal embryos because, when these explants are cultured to later stages without dissociation (Fig. 2*b*), the time course of expression of XMyoD protein and the 12/101 marker in the explants was the same as in control embryos.

When single cells from early or mid-gastrula stages were transplanted into the blastocoel of late gastrulae (Fig. 2 *c* and *d*), none of them showed positive staining with 12/101 antibody (Table 1, Exps. 3 and 5; Fig. 3 *a* and *b*). Since the original cell population contained at least 30% muscle precursor cells, we must have transplanted muscle precursor cells. Moreover, when single cells from the mid-gastrula stage were reaggregated with dorsolateral cells of the same age and cultured *in vitro* (Fig. 2*d*), some of them expressed muscle markers (Table 1, Exp. 4), showing that they can differentiate as muscle if they are provided with cells from the original region. They also differentiated into muscle when transplanted as a large group of cells (Figs. 2*d* and 3 *d–f* and Table 1, Exp. 7), though small groups failed to do so (Table 1, Exp. 6). Labeling experiments with [^{3}H]uridine showed that transplanted single cells were synthetically active and viable (Fig. 3*c*).

We conclude that single muscle precursor cells from early or mid-gastrula stages do not differentiate into muscle when surrounded by nonmuscle cells, but they can do so when surrounded by normal neighbors. These results show that the requirement of further cell interactions for muscle precursor cells is during gastrulation and that cells no longer need specific cell interactions with their original neighbors after gastrulation. We also note that, in contrast to late gastrula cells, all the transplanted single cells from early or mid-gastrula stages divided once or twice during the culture period.

DISCUSSION

In this work, we have analyzed the differentiation of single muscle precursor cells after transplantation into an ectopic position of host embryos. We have found that muscle precursor cells from the late gastrula stage can differentiate into mature muscle cells when transplanted singly into the ventral region of late gastrula hosts but that similar cells from early or mid-gastrula embryos cannot.

One important finding of this work is that single muscle precursors from late gastrulae can differentiate into mature muscle cells even in an ectopic position. This shows that these cells no longer require interactions with their original neighbors for the execution and maintenance of the differentiated state. Single-cell transplantation experiments have

Table 1. Summary of cell transplantation experiments

Exp.	Donor cell source	Site of transplantation	No. of RLDx-labeled cells per transplantation	No. of transplanted cells analyzed	No. of 12/101$^+$ cells
1	Stage 13 (late gastrula)	Stage 12 blastocoel	1	28	23
2	Stage 13	Stage 12 blastocoel	1	11	9*
3	Stage 10¼ (early gastrula)	Stage 12 blastocoel	1	27	0
4	Stage 11½ (mid-gastrula)	Reaggregation with unlabeled stage 11½ dorsolateral cells	1	35	8
5	Stage 11½	Stage 12 blastocoel	1	53	0
6	Stage 11½	Stage 12 blastocoel	40–50	225 (in 5 groups)	0
7	Stage 11½	Stage 12 blastocoel	300–500	2400 (in 6 groups)	Positive cells in each group†

*Cells were analyzed with MF20, an antibody against the myosin heavy chain (14).
†Each group contained 20–40% positive cells.

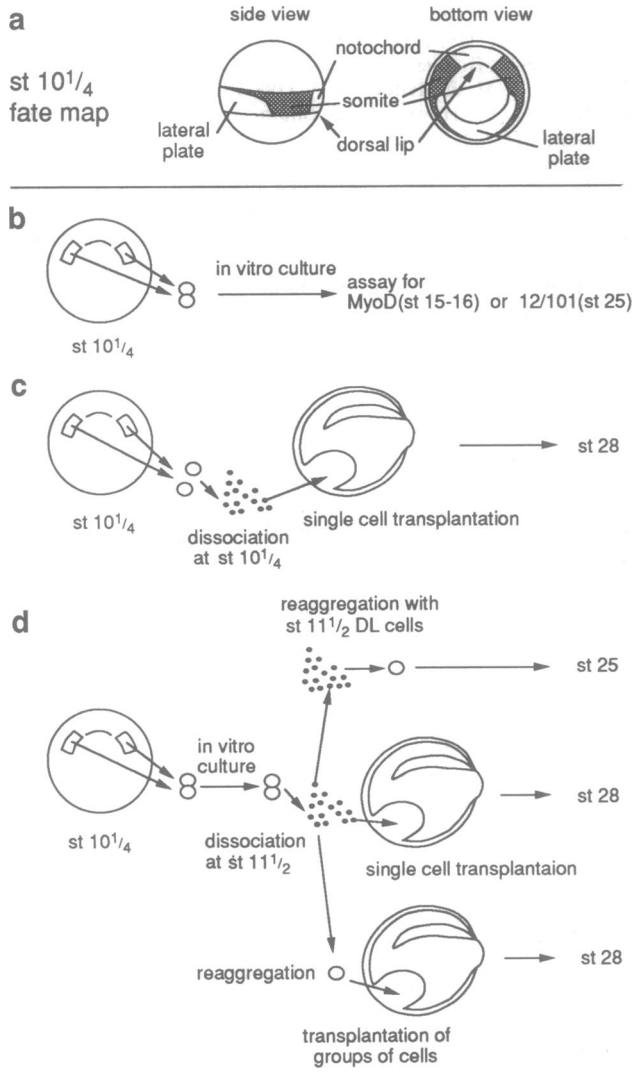

FIG. 2. Design of single-cell transplantation experiments using early and mid-gastrula cells. (*a*) Fate map of an early gastrula [stage 10¼ (16)]. At this stage, mesoderm cells are in a ring-like configuration in the marginal zone. Within this ring, presumptive somite cells are adjacent to the presumptive notochord region, which lies just above the dorsal lip. (*b*) *In vitro* culture of dorsolateral (DL) pieces. (*c*) Transplantation of early gastrula cells. DL pieces were dissected out from donor embryos at stage 10¼ (early gastrula) and immediately dissociated. Cells were then individually transplanted to the blastocoel of late gastrula hosts. (*d*) Transplantation of mid-gastrula cells. DL pieces were dissected out from RLDx-labeled early gastrulae and cultured *in vitro* in MBS. When control embryos reached the mid-gastrula stage (stage 11½), cells were dissociated and used for transplantation. For the reaggregation with DL cells, a single RLDx-labeled cell was mixed with 300–500 unlabeled DL cells in an agarose-coated well of a Terasaki plate containing MBS. Reaggregates were cultured until control stage 25 and assayed with 12/101 antibody. To transplant a group of cells, dissociated cells were transferred to a microculture well containing MBS to allow reaggregation. After about 30 min, they were transplanted into the blastocoel of host embryos.

been reported in *Xenopus*, mice, and *Drosophila* (for example, see refs. 17–21), and in a few cases progeny of transplanted cells differentiated according to the original fate even in a different cellular environment (20, 21). But these transplanted cells always divided so that progeny could interact each other. There are many reports on the clonal culture of single progenitor cells *in vitro*, including myoblasts, neural crest cells, and hematopoietic cells (for example, see refs. 22–24). But again these single progenitor cells always divided

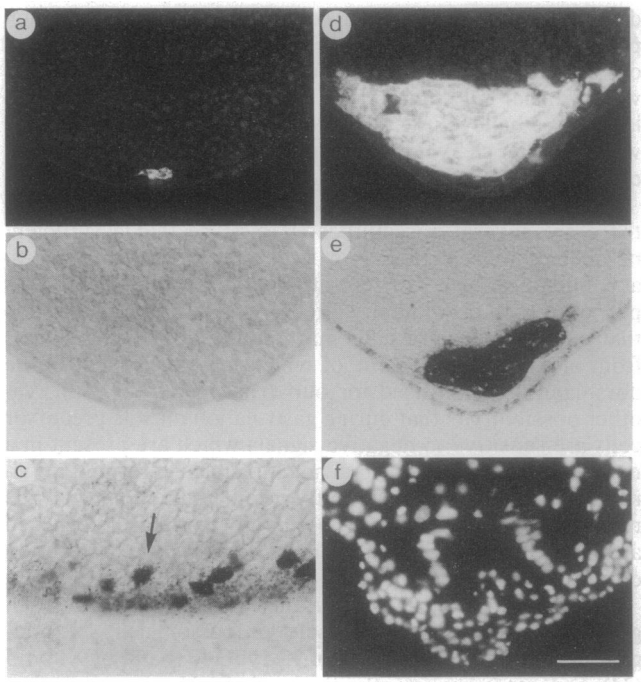

FIG. 3. Mid-gastrula muscle precursor cells differentiate into muscle when transplanted as a group of cells but not as single cells. (*a*) Example of a single transplanted cell (labeled with RLDx) in the ventral trunk region of a host embryo. The cell is negative for the staining with the muscle-specific antibody 12/101 (*b*), though the labeling with [^{3}H]uridine shows that it is synthetically active (*c*). The arrow in *c* shows the nucleus of the labeled cell. Nuclei of host cells (without arrows) are similarly labeled. Since the labeling time is short, the majority of the labeled RNAs are in nuclei. (*d*) Section of a host embryo with a group of RLDx-labeled cells. (*e*) Same section as *d* stained with 12/101, showing positive cells. Positive cells in groups are often elongated and form bundles, as can be seen in the arrangement of nuclei in a section stained with Hoechst 33258 (*f*). In *f* two bundles of several elongated muscle cells can be recognized by the positions of nuclei. (Scale bar = 100 μm for *a*, *b*, and *d–f* and 60 μm for *c*.)

so that their progeny could interact each other, thus making interpretation of the effect of environment difficult. In our experiments transplanted muscle precursor cells remained single and yet they differentiated. Therefore we believe our result is one of the first clear demonstrations of the differentiation of *single cells* without cell division in a foreign environment.

It should be noted that, in single-cell transplantation experiments carried out in the past, the identification of differentiated cell types usually had to rely on the positions and/or morphology of the transplanted cells. In this work, we have used molecular markers to analyze muscle differentiation. This enabled us to identify the differentiation of cells unambiguously without relying on their morphology and position. We believe it is important to use specific molecular markers because it will also facilitate further analysis of cell interactions at the molecular level.

The second important finding of our work is that single muscle precursor cells from the mid-gastrula stage do not differentiate into muscle when surrounded by nonmuscle cells, but they can do so when surrounded by cells from the same region. Muscle cells are formed as a result of mesoderm induction (6–8), but by the early gastrula stage most of the cells are already induced (7, 8), and by mid-gastrula stage they can no longer respond to natural inducers (25–28). Therefore our results show that, in addition to the receipt of the mesoderm-inducing signals during blastula stages, further cell interactions during gastrulation are essential for cells to

differentiate into muscle. This confirms previous work which proposed the requirement for additional steps (29–32), but our results are important because they show that the period when further cell interactions are required is during gastrulation. We believe it is necessary to study gastrula stages as well as earlier cleavage stages for the complete understanding of mesoderm formation.

As to the nature of the interactions during gastrulation, there are several possibilities. It may be that a sufficient number of muscle precursor cells have to be together to accumulate a high concentration of diffusible factors, as proposed in the community effect (32, 38). It is also possible that muscle precursor cells may require dorsalizing signals from organizer cells, some of which may be present in the dorsolateral region used for our experiments (29, 30). Another possibility is that during gastrulation muscle precursor cells are sensitive to negative signals which are present in a nonmuscle environment but not in a muscle-forming environment. In this case, inhibitory cell interactions may be required to modulate the proper number of muscle cells. In any case, this work has enabled us to specify the time period during which the cell interactions are needed.

Finally, we can draw important conclusions about myogenic gene expression. Muscle precursor cells of mid-gastrulae contain transcripts of XMyoD (33) and of XMyf-5 (34), and, more importantly, the level of XMyoD protein first becomes detectable at this stage of development (12). Since auto- and cross-activation of myogenic genes (34–36) have been proposed as mechanisms for stable muscle cell commitment (35), one might expect that cells at the mid-gastrula stage could differentiate autonomously into muscle. Our results show that this is not the case and that the mid-gastrula level of XMyoD and XMyf-5 expression is insufficient for autonomous differentiation. This therefore demonstrates that, even in normal embryonic muscle precursor cells, the possession of XMyoD protein is not sufficient for full myogenesis, a conclusion based so far only on RNA analyses of cells that are not normal muscle precursors (36, 37). We conclude that cell interactions during gastrulation are required for the stabilization of myogenic gene expression and for subsequent muscle cell differentiation.

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