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Short Contribution

The community effect in *Xenopus* myogenesis is promoted by dorsalizing factors

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ABSTRACT The community effect describes a process required for *Xenopus* muscle progenitor cells to progress to the expression of myogenic genes. Past work has suggested that this effect is dependent on secreted factors released by dorsolateral or dorsal lip cells. We show here that known dorsalizing molecules as well as other natural dorsal lip factors contribute to, but do not wholly account for, the community effect. We conclude that, in addition to dorsalizing molecules, the community effect requires factors or conditions peculiar to the dorsolateral mesoderm, a region of the embryo that contains muscle progenitor cells.

KEY WORDS: *community effect, MyoD, muscle, dorsalizing molecules, Xenopus*

A community effect is needed by *Xenopus* mesoderm cells to enable them to progress to muscle or notochord differentiation (Gurdon *et al.*, 1993b; Weston *et al.*, 1994). Cell recombination experiments have led to the idea that lateral mesoderm cells secrete a diffusible factor that initiates expression of muscle specific genes when it exceeds a certain threshold concentration (Gurdon *et al.*, 1993a). A community effect has been described for muscle formation in mammalian cells (Skerjanc *et al.*, 1994; Cossu *et al.*, 1995). A similar phenomenon may also be involved in the progression of tumorigenicity in carcinoma cells (Jouanneau *et al.*, 1994).

To progress towards identification of the presumed community factor, we now describe experiments that ask whether secreted dorsalizing molecules known to have myogenic activities in *Xenopus* gastrulae are responsible, either wholly or in part, for the community effect leading to muscle. We conclude that such molecules contribute to, but do not wholly account for, the community effect, and that this must also require factors or conditions provided by lateral mesoderm cells themselves.

We first tested the proposition that the community factors responsible for myogenesis in the dorsolateral mesoderm of early gastrulae are the same as the known dorsalizing factors that are synthesized in, and secreted by, cells of the Nieuwkoop center or Spemann organizer. A plausible explanation of the community effect is that diffusible molecules emanating from these regions would reach a certain concentration in dorsolateral mesoderm cells which would respond by activating muscle-specific genes. The Spemann organizer is a source of the diffusible molecules *noggin* (Smith and Harland, 1992), *chordin* (Sasai *et al.*, 1994), and *Xnr-3* (Smith *et al.*, 1995). We have also tested the homeobox gene *Siamois* (Lemaire *et al.*, 1995), which is the source of a dorsalizing signal pathway likely to be different from that of *noggin* and *chordin* (Carnac *et al.*, 1996).

To investigate the dorsalizing capacities of these molecules, muscle precursor cells were taken from dorsolateral regions of *Xenopus* embryos previously lineage labeled with the red fluorescent compound RLDx (Fig. 1). Dorsolateral tissues were dissociated in Ca-free medium and single muscle precursor cells, or small reaggregates of them, were placed between two populations of ectoderm (animal cap) cells previously injected with different amounts of *noggin* or *Siamois* mRNA (Gurdon *et al.*, 1993b). These "sandwiches" were then cultured until tadpole stages and the presence of muscle cells determined by immunostaining, using a monoclonal antibody which detects the muscle specific protein MyoD (Hopwood *et al.*, 1992). When small aggregates of (or single) muscle precursor cells (under 70 cells/aggregate) were placed between two uninjected animal caps, no MyoD staining was detected, a result previously described by Gurdon *et al.* (1993b). In contrast, *Siamois* or *noggin*-expressing animal caps induced muscle cell differentiation in aggregates of only 10 cells, as seen by the co-localization of RLDx and MyoD staining (Fig. 2). However, MyoD staining was never detected in single muscle precursor cells even if animal caps expressed both *Siamois* and *noggin* (Fig. 3). Nuclei stained with Hoechst show no sign of DNA damage indicating that single cells and reaggregates are equally viable. Using the same assay, we also tested the dorsalizing activity of Xnr-3, *chordin*, and *Xwnt-8* (Sokol *et al.*, 1991) a glycoprotein which induces *Siamois* (Carnac *et al.*, 1996). *Xnr-3*-expressing animal caps do not activate muscle differentiation whatever the size of aggregates. *Chordin* and *Xwnt-8*-expressing animal caps present a myogenic activity similar to that of *Siamois* and *noggin*-expressing animal caps (Table 1). We conclude that Spemann or Nieuwkoop organizer molecules can activate the myogenic program in aggregates of more than 10 adjacent lateral mesoderm cells. Therefore they can contribute to, but do not wholly account for, the community effect.

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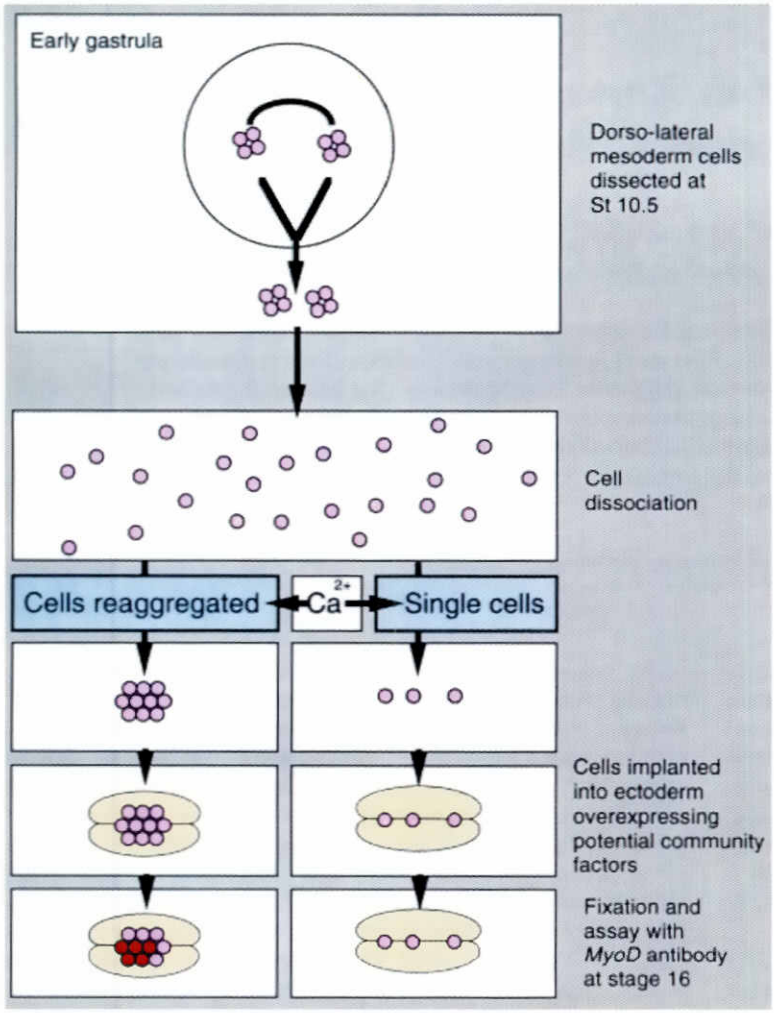

Fig. 1. Diagram to illustrate the experimental procedures used. *Dissection, cell dissociation, reaggregation and sandwich construction took less than one hour. Sandwiches were cultured for 18 h at 18°C, until control embryos reached stage 18. The procedure for dissection of dorsolateral mesoderm tissue from Stage $10^{1/2}$ embryos is described by Gurdon et al. (1993b).*

We then asked whether the community factor is subject to the same inhibitory influence of the ventralizing factor *Bmp-4* (Dale *et al.*, 1992; Jones *et al.*, 1992) that has been shown to counteract known dorsalizing activities such as those of *noggin* (Zimmerman *et al.*, 1996), *chordin* (Piccolo *et al.*, 1996), and *Siamois* (G. Carnac, unpublished results). To address this issue, we combined RLDx-labeled dorsolateral early gastrula cells with *Bmp-4*-expressing animal caps. We found that *Bmp-4* completely inhibits MyoD gene expression in reaggregates of 100-200 implanted cells (not shown). As expected, large aggregates of dorsolateral cells activate MyoD gene expression when cultured alone or when combined with uninjected animal caps. Overexpression of a Bmp-4 dominant negative receptor in implanted dorsolateral cells did not give meaningful results, because this caused cell dissociation and obvious signs of toxicity (data not shown). We conclude that *Bmp-4* can exert a strong anti-myogenic effect by directly preventing the community effect in muscle progenitor cells.

These results lead to the question whether other myogenic factors secreted by the dorsal cells, but so far unidentified, might constitute the community factor(s) able to induce single dorsolateral cells to enter the muscle pathway. To determine the myogenic properties of such unidentified factors, we placed test dorsolateral (muscle precursor) or other cells, singly or in reaggregated groups, in a sandwich between two dorsal lip tissues. As above, test cells were lineage labeled with RLDx. We first tested ventral mesodermal cells which were dissected from the ventral region of early gastrula *Xenopus* embryos. The normal fate of these cells is to form ventral tissue derivatives but they can be easily differentiated into muscle when combined with a dorsal lip (Lettice and Slack, 1993). We found that dorsal lips can induce large groups of ventral mesoderm cells to become muscle. But these cells, tested singly or in small groups of less than 20 cells, did not express MyoD (Fig. 4A). Furthermore, the percentage of MyoD positive cells increased with the size of the group beyond 20 cells, as expected of the community effect. Since ventral mesodermal cell are not normally fated to become muscle and might be therefore less sensitive to myogenic signals, we repeated the same design of experiment with dorsolateral early gastrula cells that include normal muscle progenitors. We were, again, able to see MyoD positive cells in groups containing as few as 15 cells but never in smaller groups or in single cells (Fig. 4B).

Conclusion

We have provided evidence that known dorsalizing molecules or natural myogenic signals secreted by dorsal cells can contribute to the community effect. However, such molecules are not a complete source of the community factors necessary for single muscle progenitor cells to progress to muscle differentiation. It is conceivable, though we think improbable, that peculiar combinations of such molecules could reproduce the community effect. We therefore propose that other factors or conditions are required for mesoderm cells to differentiate as muscle, and that the synthesis of these is a special characteristic of dorsolateral mesoderm cells.

TABLE 1

GENES TESTED FOR DORSALIZING ACTIVITY IN SANDWICH EXPERIMENTS

Animal caps expressing	Size of aggregates required to detect MyoD
rRNA	>100 cells
noggin	> 10 cells
Siamois	> 10 cells
Xwnt-8	> 20 cells
chordin	> 20 cells
gsc	>100 cells

Noggin, Siamois, Xwnt-8, and *chordin,* but not *goosecoid,* can reduce the number of dorsolateral cells required in a reaggregate for MyoD to be expressed. For *Xwnt-8, chordin* and *goosecoid,* 423, 526, and 387 cells were scored in four sandwiches per condition.

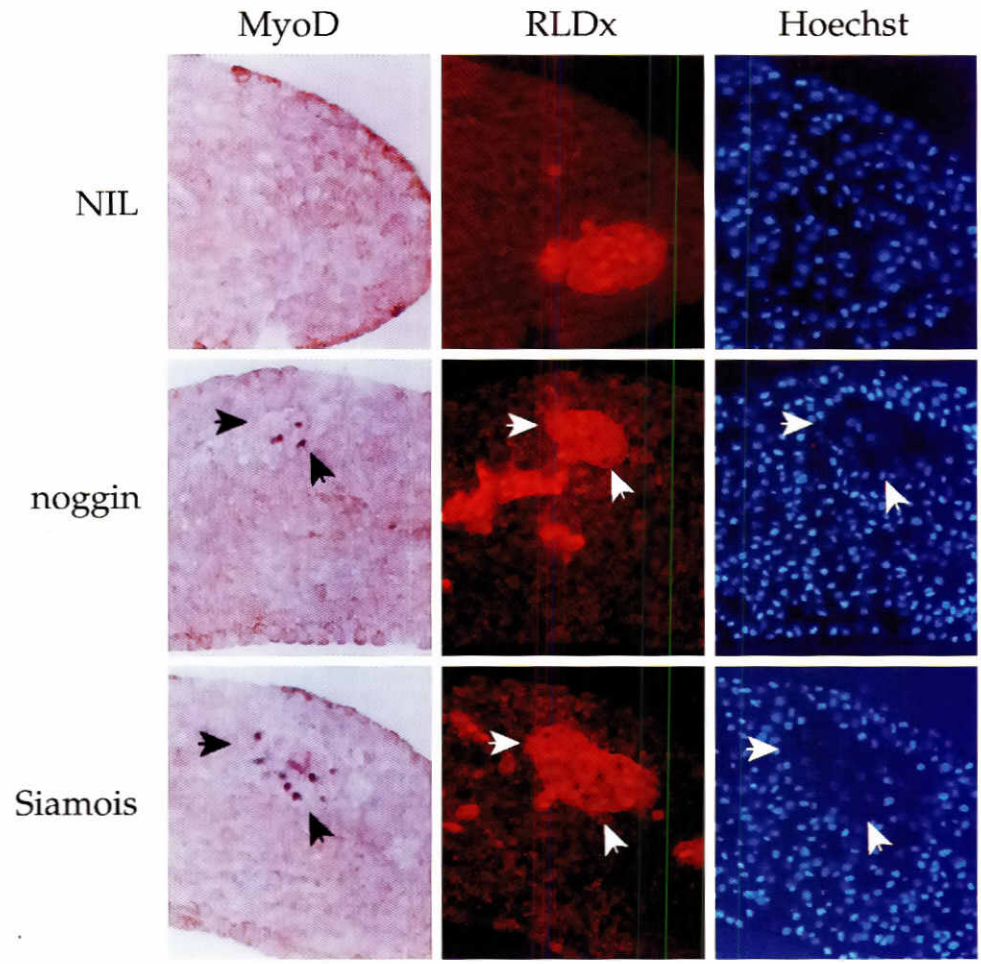

Fig. 2. Dorsalizing molecules contribute to the community effect in *Xenopus* myogenesis. *RLDx-labeled dorsolateral mesoderm cells of gastrulae were implanted singly, or in groups of different sizes, into blastula animal cap sandwiches, and were immunostained for MyoD at the equivalent of stage 18 (see Gurdon et al., 1993b, for methods). The animal cap sandwich tissues derived from blastulae were injected with 100 pg mRNA for noggin or Siamois mRNAs; NIL, animal caps not injected with mRNA. Sections are of sandwiches with small (10-50 cell) reaggregates, viewed for MyoD antibody stain* **(left)**, *RLDx* **(middle)**, *or Hoechst* **(right)**. *MyoD-positive nuclei are seen in the noggin and Siamois injected samples.*

Experimental Procedures

Embryo injections

Embryos were *in vitro* fertilized, dejellied and cultivated in 10% MBS, and 50-100 pg of mRNA for *Siamois, noggin, goosecoid, Xnr-3, chordin, Xwnt-8, Bmp-4* were prepared and injected as previously described (Carnac *et al.*, 1996). 4.6 nl of Rhodamine lysinated dextran (RLDx; 5 mg/ml in water, Molecular Probes) was injected into both blastomeres of 2-cell embryos.

Immunostaining

Tissue explants were fixed in MEMFA for 2 h and kept (overnight or longer) in methanol at -20°C. 10 μM sections were cut from tissue explants or whole embryos embedded in Histoplast:beeswax (98:2). Immunostainings were performed using the monoclonal antibody D7F2 (recognizing the muscle-specific protein MyoD; Hopwood *et al.*, 1992). Incubations, washes and color reactions (using NBT-BCIP; Boehringer MA) were performed according to Hopwood *et al.* (1992). When indicated, the nuclei were stained with Hoechst 33258 (Boehringer, 2 μg/ml) for 40 min.

Embryonic cell manipulation and sandwich formation

Dorsolateral regions (composed mostly of muscle precursor cells) of *Xenopus* embryos previously lineage labeled with RLDx were dissected at the early gastrula stage (stage 10.25) and then dissociated in Ca-free medium. Cells were reaggregated in medium containing CaCl (1 mM) as

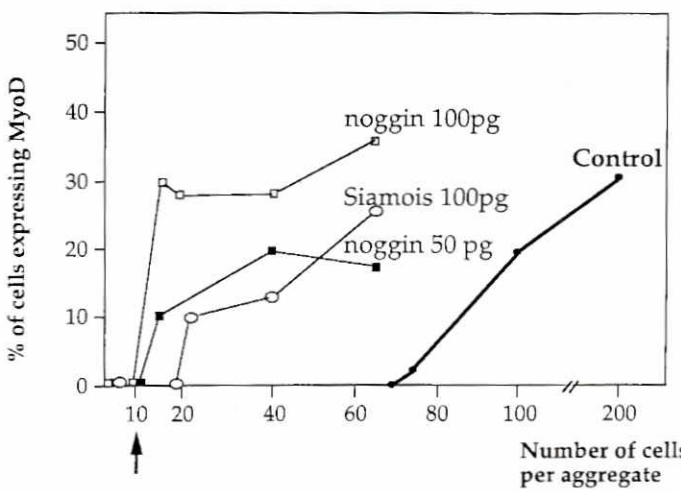

Fig. 3. *Siamois* and *noggin* reduce the size of a reaggregate required for the community effect. *In dorsolateral cell reaggregates of 200 or more cells, inserted into uninjected sandwiches, up to 40% of cells will express MyoD. In noggin and Siamois injected sandwiches, at least 15 adjacent dorsolateral cells are required for MyoD expression. 965, 800 and 525 cells were scored, and 6 sandwiches were tested per condition.*

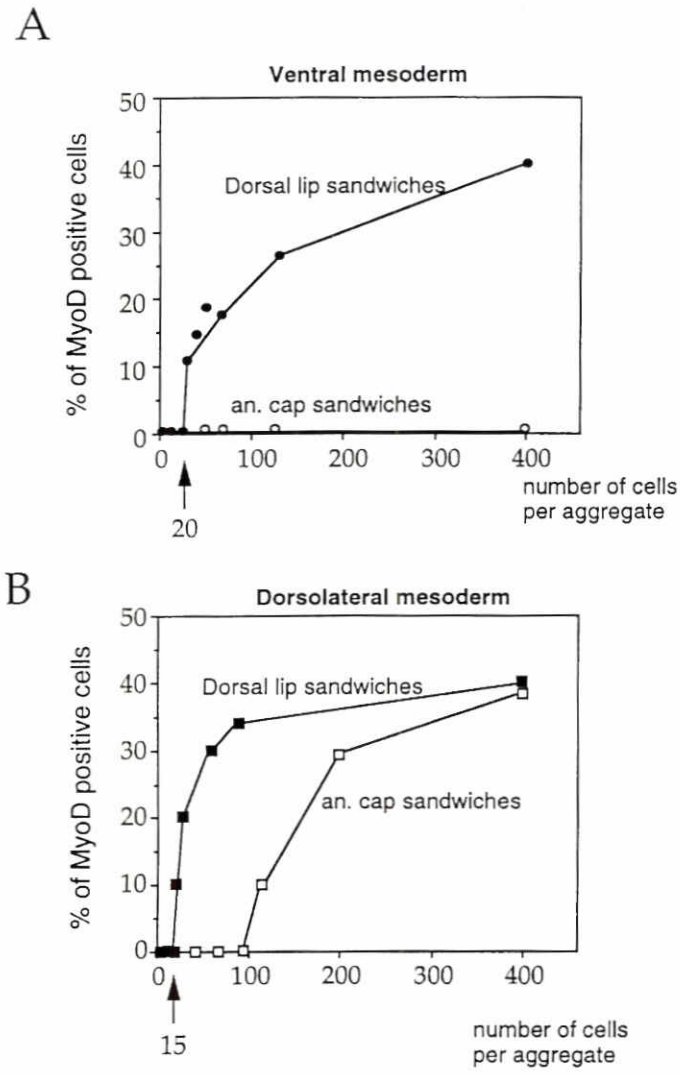

Fig. 4. Sandwiches of dorsal lip tissue can induce small groups of implanted mesoderm cells from ventral (A) or dorsolateral (B) regions to express MyoD. *At least 15 cells must be enclosed in a reaggregate for some of them to express MyoD in dorsal lip sandwiches* **(A and B)**. *Animal cap sandwiches are totally unable to induce MyoD. As a result, ventral mesoderm cells are not induced to express MyoD at all in animal cap sandwiches* **(A)**; *over 100 dorsolateral cells must be reaggregated and placed in uninjected animal cap sandwiches if they are to become positive for MyoD* **(B)**.

previously described (Gurdon *et al.*, 1993b) and enclosed between two stage 9 animal caps overexpressing *Siamois, noggin, gsc, Xnr-3, chordin, Bmp-4 or Xwnt-8* (Fig. 1). For experiments described in Figure 4, test dorsolateral or ventral cells were placed, singly or in reaggregate groups, between two dorsal lip tissues. All sandwiches were cultured at 18°C in 1x MBS with 0.1% BSA until sibling control embryos reached stage 18.

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