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Original Article

A dynamic requirement for community interactions during *Xenopus* myogenesis

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ABSTRACT The community effect is an interaction among a group of many nearby cells that is necessary for them to maintain tissue-specific gene expression and differentiate co-ordinately. A community interaction is required for the muscle precursor cells of the *Xenopus* embryo to develop into terminally differentiated muscle, but exactly when and where the community effect acts during myogenesis has not been determined. Here, we ask whether dependence on the community effect varies with the developmental age of the muscle precursor cells. We find that dependence on the community signal changes with time through the muscle precursor cell population. During neurulation muscle precursor cells that are still in the vicinity of the blastopore and that are fated to form posterior muscle continue to require interactions with their neighbours, while differentiation of the anterior paraxial mesoderm, which gastrulated earlier, is independent of cell contact at this time. Thus the time during which a particular sub-population of muscle precursor cells requires a community interaction is related to their final destination along the anterior-posterior axis. In addition we show that this later acting community interaction around the blastopore involves FGF signalling.

KEY WORDS: *Community effect, FGF, XMyoD, muscle, Xenopus*

Introduction

The community effect describes an intercellular signalling interaction that takes place among a group of tissue precursor cells and is necessary for the cells to differentiate and express genes characteristic of that tissue. All the cells in the group secrete the community factor, and each cell must receive an above-threshold amount of this factor from its neighbours in order to differentiate. The community effect was first demonstrated when it was shown that groups of more than one hundred *Xenopus* muscle precursor cells were able to differentiate, while smaller groups and single cells could not (Gurdon *et al.*, 1993). We have recently shown that embryonic Fibroblast Growth Factor (eFGF; Isaacs *et al.*, 1992) is able to mediate the community effect in *Xenopus* myogenesis, and indeed that it is likely to be the endogenous community factor (Standley *et al.*, 2001). In particular, eFGF is able to support expression of the myogenic transcription factors *XMyoD* (Hopwood *et al.*, 1989) and *XMyf5* (Hopwood *et al.*, 1991) in single muscle precursor cells dissected from the dorsolateral mesoderm of early gastrulae, effectively substituting for the endogenous community factor. Community effects have also been identified in the differentiation of the *Xenopus* notochord (Weston *et al.*, 1994) and endoderm (Yasuo and Lemaire, 1999), in mammalian somitogenesis (Cossu *et al.*, 1995), *Drosophila* neurogenesis (Stuttem and Campos-Ortega, 1991), in *C. elegans* sex determination (Hunter and Wood, 1992; Kuwabara *et al.*, 1992) and in mouse coat pigment patterning (Aubin-Houzelstein *et al.*, 1998). The community effect thus appears to be a fundamental mechanism in development, promoting and co-ordinating cell differentiation within forming tissues.

During *Xenopus* gastrulation, the mesoderm involutes over the blastopore lip, bringing the prospective somite beneath the ectoderm. After involution, the axial and paraxial mesoderm converge and extend to elongate the body axis. By the end of gastrulation (stage 13) the prospective anterior somitic mesoderm has come to lie beneath the neural plate on the anterior dorsal side of the embryo. At this time the prospective posterior somitic mesoderm has not yet extended, instead forming a thick ring around the lateroventral margin of the closed blastopore. During neurula stages these cells stream around the blastopore and give rise to posterior somites (Keller, 1976; Gurdon *et al.*, 1992; Keller, 2000). Early work indicated that the community effect acts during mid-gastrula stages (Gurdon *et al.*, 1993). However, since myogenesis is a progressive process it was not clear exactly when particular muscle precursor cells experi-

Abbreviations used in this paper: BSA, bovine serum albumin; (e)FGF, (embryonic) Fibroblast Growth Factor; FGF-R, Fibroblast Growth Factor Receptor.

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ence community interactions during normal development. One possibility is that all the muscle precursor cells experience the community effect in the mid-gastrula, after which time the entire group are committed to muscle differentiation. However, previous work has shown that at the end of gastrulation some single muscle precursor cells are committed while others are not (Kato and Gurdon, 1993), suggesting that not all the muscle precursor cells have experienced the community effect. In this work we directly test whether muscle precursor cells require community interactions at discrete times and places along the anterior-posterior axis during myogenesis. We find that as late as mid-neurula stages posterior muscle precursor cells require continuing community interactions while the more mature anterior cells are independent. Thus, there is a spatiotemporal relationship between dependence on the community effect and the generation of the musculature in an anterior to posterior direction. Furthermore, we show that FGF signalling is a critical component of this continuing community interaction around the blastopore.

Results

Anterior and Posterior Mesoderm from Neurulae both give rise to Terminally Differentiated Muscle

The cells that form the anterior somites of the tadpole are those that involute around the blastopore early during gastrulation, while the cells that make up the more posterior somites involute later, throughout neurula and into tailbud stages (Keller, 1976). Our aim was to ask whether the requirement for community interactions changes in time and space during myogenesis. Before testing this directly, we analysed anterior and posterior tissue pieces dissected from stage 15 neurulae (Fig. 1A), to confirm that both dissections recovered muscle-forming tissue. Anterior and posterior explants both included cells positive for XMyoD protein, when assayed by immunohistochemistry at stage 15, immediately after dissection, or after culture to stage 20 (Fig. 1B). The percentage of XMyoD-positive cells in a typical set of anterior tissue pieces did not change appreciably between stages 15 and 20. The percentage of XMyoD-positive cells in the posterior pieces increased during this period, probably through cell division. When cultured intact until stage 28 and assayed with the 12/101 antibody (Kintner and Brockes, 1984), both anterior and posterior explants proved positive for this marker of terminal muscle differentiation (Fig. 1C). Consistent with morphological descriptions (Keller, 1976), both pieces contain cells that normally express myogenic genes and give rise to terminally differentiated muscle.

Community Interactions persist in Posterior Paraxial Mesoderm

We next asked whether cells from anterior and posterior presomitic mesoderm are subject to a community effect during neurula stages. Anterior and posterior tissue pieces were dissected from stage 15 neurulae as before. They were cultured to stage 20 either as dispersed cells or as intact tissue pieces, so that their dependence on community interactions for myogenic gene expression could be compared. The difference when the cells were assayed for XMyoD protein was striking (Fig. 2 A,B). The cells from the anterior pieces were able to maintain expression of XMyoD protein even when dispersed, while, surprisingly, those from the posterior tissue were not (seen in 7/7 independent experiments). Therefore at stage 15 posterior cells are still dependent on interactions with their neighbours, while more anterior cells are independent of each other and can maintain their muscle status even when isolated. The community effect is therefore an ongoing process, rather than one restricted to mid-gastrula embryos. Furthermore, an uncommitted population of muscle precursor cells exists around the blastopore at least until neurula stages, and these cells still require community interactions if they are to realise their ultimate myogenic fate. Importantly, posterior dispersed cells can maintain XMyoD expression when they are immediately reaggregated (data not shown; Fig. 4A), demonstrating that the mechanical process of cell disaggregation was not responsible for the loss of XMyoD from the dispersed posterior mesoderm cells.

This result sheds light on the earlier observation that a proportion of muscle precursor cells are independent of their neighbours by stage 13, that is, they can continue to differentiate as muscle even when transplanted singly to an ectopic location in the embryo (Kato and Gurdon, 1993). It is likely that those cells that were independent by stage 13 in the transplantation experiments were

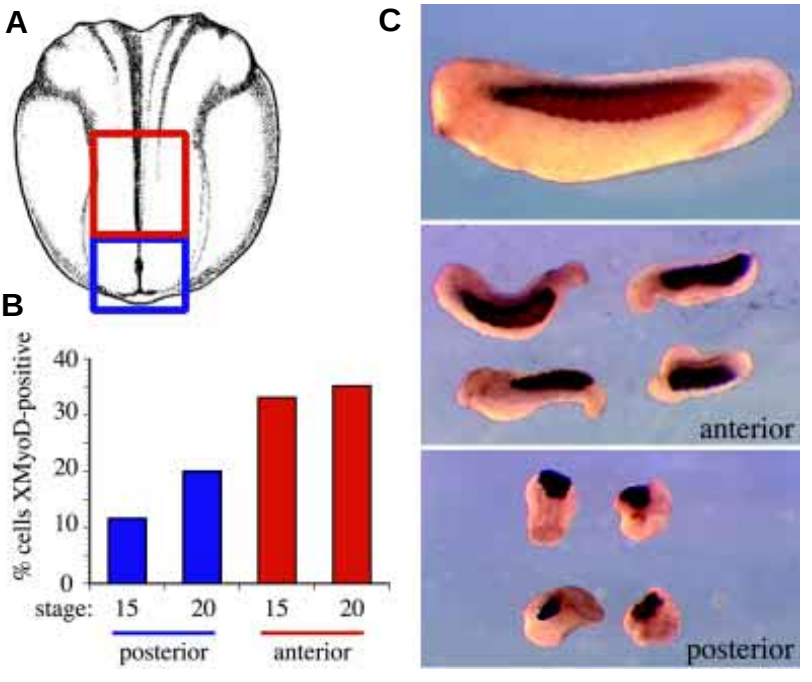


Fig. 1. Anterior and posterior paraxial mesoderm contain muscle precursor cells that give rise to terminally differentiated muscle. (A) *Posterior dorsal view of stage 15 embryo, with anterior and posterior (circumblastoporal) tissue dissections outlined in red and blue respectively. Drawing from Nieuwkoop and Faber (1967).* **(B)** *Quantified data showing the percentage of anterior and posterior cells expressing XMyoD when analysed by immunohistochemistry at stages 15 and 20. The pieces were cultured intact throughout, and only disaggregated immediately prior to fixation, so that individual cells could be counted.* **(C)** *Whole embryo, anterior and posterior tissue pieces assayed with the 12/101 antibody at stage 28. All dissected pieces contain terminally differentiated muscle.*

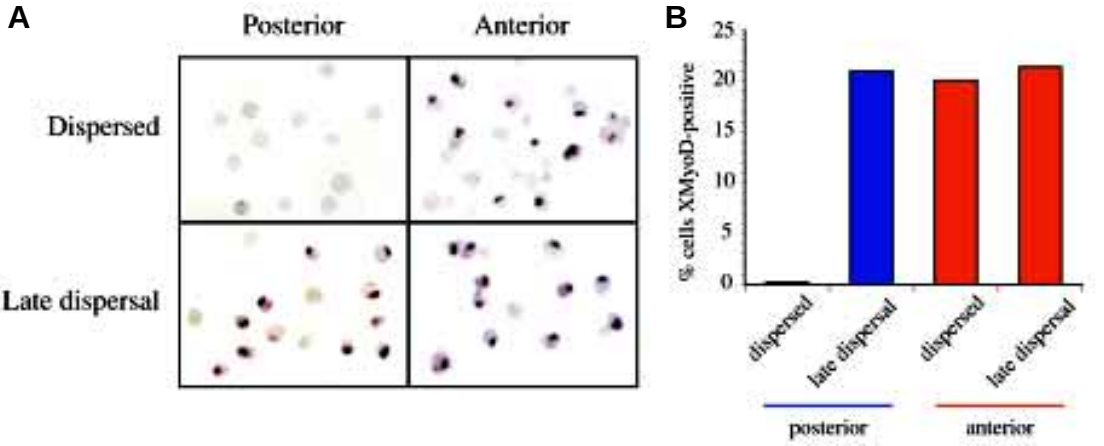


Fig. 2. Community interactions persist in posterior mesoderm. **(A)** *Bright field views of cells dissected at stage 15 and assayed at stage 20 with an antibody against XMyoD. On the left are posterior cells, on the right are anterior cells. The top row shows cells that were cultured dispersed from stage 15, below are late dispersal controls (dispersed at stage 20, immediately prior to fixation). Anterior cells can maintain expression of XMyoD when dispersed from stage 15, while posterior cells cannot. Thus community interactions persist in the posterior paraxial mesoderm after anterior cells have become independent of their neighbours.* **(B)** *Quantified data from one representative assay.*

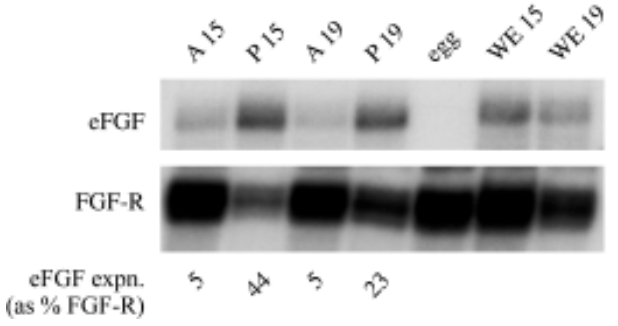
from the anterior paraxial mesoderm, while those that were still dependent on community interactions were from the more posterior region.

FGF Signalling is required for the Community Interaction in the Posterior Paraxial Mesoderm

We have recently shown that eFGF is likely to be the endogenous myogenic community factor during mid-gastrula stages (Standley *et al.*, 2001). Our next question was whether FGF signalling could also mediate the community effect in the posterior circumblastoporal mesoderm.

The published expression pattern of *eFGF* indicates that it is found in the circumblastoporal mesoderm during neurulation (Isaacs *et al.*, 1995). RNase protection analysis confirmed that *eFGF* is expressed in our posterior mesoderm explants during neurulation (Fig. 3). The posterior pieces express *eFGF* at a higher level than the anterior pieces during this time, consistent with the proposal that posterior muscle precursor cells require FGF signalling during neurula stages.

Next we asked whether FGF signalling is necessary for muscle gene expression in posterior mesoderm tissue. We used the tyrosine kinase inhibitor SU5402 (Mohammadi *et al.*, 1997; Standley *et al.*, 2001) to block activity of the FGF receptor, and hence FGF signalling, in posterior cell reaggregates. Anterior and posterior explants dissected at stage 15 were disaggregated, the inhibitor added or not, and the cells immediately reaggregated for culture, bringing the cells back into contact with one another. At stage 20, the reaggregates were analysed for XMyoD expression by immunohistochemistry. Uninhibited posterior cell reaggregates expressed XMyoD (Fig. 4A). In reaggregates treated with 15 μM SU5402 the percentage of cells that expressed XMyoD was greatly reduced (Fig. 4A), suggesting that FGF signalling is necessary for XMyoD expression in these cells.



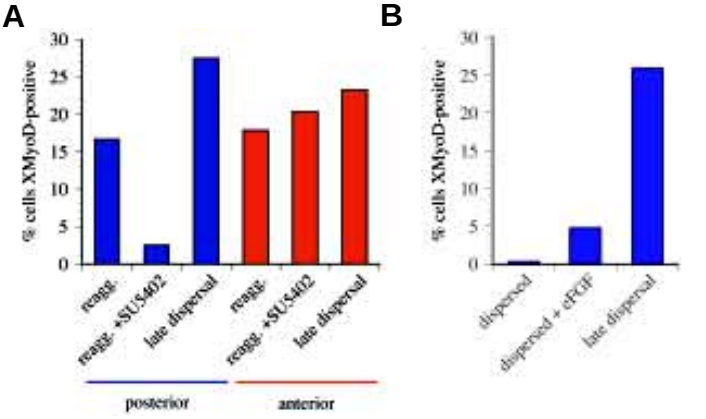


Fig. 3. (Left) *eFGF* is expressed in the posterior mesoderm between stages 15 and 19. *Anterior (A) and posterior (P) explants were analysed by RNase protection. The expression level of* eFGF *is shown as a percentage of the cognate* FGF-R *loading control value beneath each lane. WE 15/20; whole embryo control, stage 15/20. Posterior pieces express a higher level of* eFGF *than anterior pieces.* eFGF *is present in the posterior mesoderm at the stage when these cells are still dependent on a community interaction if they are to express myogenic genes.*

Fig. 4. (Right) FGF signalling is required for posterior cells to express XMyoD. (A) *Posterior cell reaggregates express XMyoD. The percentage of XMyoD-expressing cells is reduced on treatment with 15 μM SU5402. Quantified data from one experiment analysed by immunohistochemistry. Posterior cells are subject to a community effect for XMyoD expression, and FGF signalling is an essential component of this community interaction.* **(B)** *The percentage of dispersed cells expressing XMyoD is increased on addition of 100 ng/ml eFGF protein to the culture medium, but does not reach control levels. Quantified data from one experiment analysed by immunohistochemistry.*

XMyoD expression in the anterior cells was unaffected by SU5402 treatment, as predicted if these cells are independent of an FGF-induced community interaction. We concluded that the loss of community interactions and XMyoD expression in dispersed posterior mesoderm cells is due, at least in part, to the lack of FGF signalling. FGF signalling is an essential component of the community interaction persisting in the posterior paraxial mesoderm.

Finally we tested whether the addition of eFGF protein to the culture medium of dispersed posterior cells is sufficient to maintain XMyoD expression in dispersed posterior mesoderm cells. Intact posterior pieces were used as late dispersal controls, to determine the percentage of cells that would have differentiated as muscle if they had been left in normal group contact. We consistently observed a modest increase in the percentage of cells expressing XMyoD on eFGF treatment, rising from less than 1% to 13% of the late dispersal control value (averaged from five independent experiments; a representative set of results is shown in Fig. 4B). While addition of eFGF did not completely recover XMyoD expression in the dispersed posterior mesoderm cells, the results in Fig. 4A and 4B taken together clearly show that FGF signalling is involved in this later community interaction among the posterior muscle precursor cells.

Discussion

In *Xenopus* many aspects of muscle development progress in a wave from anterior to posterior. For example, segmentation of the somites commences in the anterior paraxial mesoderm, and it is the oldest somite that is the first to undergo the ninety-degree rotation that each somite makes after segmentation (Hamilton, 1969; Youn and Malacinski, 1981). Gene expression also reveals this anterior-to-posterior wave, myogenic genes being down-regulated as markers of terminal muscle differentiation are up-regulated. Our work here shows that there is a dynamic anterior-to-posterior requirement for the myogenic community effect, which had not been apparent from earlier studies. While naive muscle precursor cells are in the vicinity of the blastopore they depend on community interactions; when they move anteriorly and begin to differentiate they become independent of their neighbours for continued expression of myogenic genes. The cells that involute later and contribute to the more posterior somites depend on a community signal until a later developmental stage than the more anterior cells. Presumptive muscle cells only become independent of the community effect once they have reached their final destination in the paraxial mesoderm, where they differentiate into fully-formed somites. We have also shown that this aspect of the community effect is likely to be mediated at least in part by FGF signalling. In the future it will be interesting to determine the cues that allow muscle precursor cells to become independent of the FGF-mediated community effect as they move anteriorly.

Materials and Methods

Embryological Techniques

Xenopus embryos were obtained by *in vitro* fertilisation, reared in 0.1X Modified Barth Saline (MBS; Gurdon, 1977) and dejellied in 2% cysteine-HCl. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Dissections were carried out in 1X MBS. Anterior and posterior pieces were dissected from stage 14-16 neurulae as indicated in Fig. 1A. Intact pieces were cultured in 1X MBS, 0.1% BSA (Sigma), 1 μg/ml gentamicin (Sigma). To disaggregate the cells, the pieces were incubated for 20 minutes in 1X Ca^{2+}- and Mg^{2+}-free MBS with 0.1% BSA and 0.5 mM EDTA, and then transferred to polyhema-treated Eppendorf tubes containing the same solution and pipetted gently to complete disaggregation. The tubes were centrifuged briefly to pellet the cells, the excess medium discarded, and the cells dispersed into agarose-lined dishes for culture in normal (Ca^{2+}-containing) 1X MBS, 0.1% BSA, 1 μg/ml gentamicin, plus 100 ng/ml eFGF protein. *Xenopus* eFGF protein was a gift from Drs H. Isaacs and M. E. Pownall, University of York, UK. To make reaggregates, cells were centrifuged in medium containing 2.2 mM (3X the normal MBS concentration) of Ca^{2+}. SU5402 was purchased from Calbiochem (Cat. no. 572630) and dissolved in DMSO. SU5402 was added at a final concentration of 15 μM to dispersed cells prior to their reaggregation, and the reaggregates then incubated continuously in inhibitor-containing medium. At the end of the culture period, the dispersed cells were collected from the dishes. Cells to be analysed by RNase protection were spun down in Eppendorf tubes and frozen; those to be analysed by immunohistochemistry were transferred to glass slides pre-treated with poly-L-lysine (Sigma), and fixed for 30 minutes in MEMFA (Hemmati-Brivanlou *et al.*, 1990), followed by storage in methanol at -20°C. Reaggregates were disaggregated and the cells fixed immediately to slides as above for immunohistochemical analysis (Fig. 4B).

RNase Protection Assays

mRNA was prepared and RNase protections were performed as described previously (Ryan *et al.*, 1996). Plasmid templates were linearised and antisense RNA probes transcribed as follows: *eFGF* (Isaacs *et al.*, 1992; EcoRI, T7 polymerase), *FGF-R* (Lemaire and Gurdon, 1994; BamHI, T7 polymerase). Quantitation was carried out using a Fujifilm Phosphor Imager and MacBAS 2.5 software.

Immunohistochemistry

Cells fixed to slides were rehydrated through an ethanol series and blocked for 30 minutes in 1X PBS, 0.2% (mass/volume) BSA, 0.1% Triton X-100, 10% goat serum, then treated for 2 hours with D7F2 (anti-XMyoD; Hopwood *et al.*, 1992), diluted 1:4 in blocking solution. This was followed by rabbit anti-mouse IgG for 1 hour (1:200; ICN) and finally APAAP for 1 hour (1:50; Serotech), with half-hour washes in 1X PBS, 0.2% BSA, 0.1% Triton X-100 after each antibody layer. All antibody steps were carried out at room temperature in a humid chamber. Immunolabelling was visualised by NBT-BCIP (Roche). The nuclei were stained with 5 μg/ml Hoechst 33258 (Sigma) for 30 minutes, and the slides mounted in PBS-90% glycerol. At least 500 cells were counted on every slide and scored as being positive or negative for XMyoD protein.

Embryos and dissected tissues for 12/101 whole-mount immunohistochemistry were fixed for 2 hours in MEMFA and stored in methanol at -20°C. They were rehydrated through an ethanol series, washed in maleic acid buffer (MAB; 100 mM maleic acid pH 7.5, 150 mM NaCl) and blocked for 2 hours in MAB, 2% blocking reagent (Roche), 20% goat serum. The samples were treated with 12/101 antibody diluted 1:200 in blocking solution for 4 hours, then washed 7 times in MAB, including an overnight wash at 4°C. The samples were blocked again, and then treated with anti-mouse conjugated to alkaline phosphatase for 4 hours (1:100; Roche). After washing, the immunolabelling was visualised by NBT-BCIP (Roche), and the samples re-fixed in MEMFA.

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