eFGF and its mode of action in the community effect during *Xenopus* myogenesis

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SUMMARY

The community effect is an interaction among a group of many nearby precursor cells, necessary for them to maintain tissue-specific gene expression and differentiate co-ordinately. During *Xenopus* myogenesis, the muscle precursor cells must be in group contact throughout gastrulation in order to develop into terminally differentiated muscle. The molecular basis of this community interaction has not to date been elucidated. We have developed an assay for testing potential community factors, in which isolated muscle precursor cells are treated with a candidate protein and cultured in dispersion. We have tested a number of candidate factors and we find that only eFGF protein is able to mediate a community effect, stimulating stable muscle-specific gene expression in demonstrably single muscle precursor cells. In contrast, Xwnt8, bFGF, BMP4 and TGFβ2 do not show this capacity. We show that eFGF is expressed in the muscle precursor cells at the right time to mediate the community effect. Moreover, the time when the muscle precursor cells are sensitive to eFGF corresponds to the period of the endogenous community effect. Finally, we demonstrate that FGF signalling is essential for endogenous community interactions. We conclude that eFGF is likely to mediate the community effect in *Xenopus* myogenesis.

Key words: Community effect, Muscle, XMyoD, XMyf5, eFGF, Xwnt8, *Xenopus*

INTRODUCTION

In *Xenopus*, the mesoderm is induced in the equatorial region of the blastula by signals from the vegetal pole. Combinations of signalling molecules pattern the mesoderm during gastrulation. Ventralising molecules such as bone morphogenetic protein 4 (BMP4) (Dale et al., 1992; Jones et al., 1992) and Xwnt8 (Christian et al., 1991) are expressed throughout the ventral and lateral mesoderm. They promote cell fates such as blood and mesenchyme, and their activities are modulated by dorsalising antagonists secreted from the Spemann organiser, such as noggin (Smith and Harland, 1992), chordin (Sasai et al., 1994), follistatin (Hemmati-Brivanlou et al., 1994) and Frzb (Leyns et al., 1997; Wang et al., 1997), and by other antagonists from the most ventral marginal zone, such as sizzled (Salic et al., 1997). The nodal-related proteins 1 and 2 additionally promote dorsal development (Jones et al., 1995). Together, these signals establish a gradient of mesodermal character along the dorsoventral axis. In addition to these patterning signals, essential community interactions have been identified within the populations of somite (Gurdon et al., 1993b) and notochord (Weston et al., 1994) precursor cells. The notochord develops from the most dorsal mesoderm, and the bulk of the somites from the dorsolateral region (Keller, 1976). Both somite and notochord precursor cells need to be in contact with each other during gastrulation, if they are to differentiate and stably express tissue-specific genes. This reflects a requirement by each cell to receive a signal from surrounding cells of its own kind.

The importance of the community effect in normal development was first demonstrated when it was shown that groups of more than 100 muscle precursor cells were able to differentiate, while smaller groups and single cells could not (Gurdon et al., 1993b). This suggested that some muscle-promoting influence was being transmitted from cell to cell. The community effect appears to be a fundamental mechanism in development, promoting coordinate cell differentiation within a tissue, and enhancing demarcation between tissues (Gurdon et al., 1993a). Interactions within a population of induced cells could act to increase the uniformity of their response to the earlier patterning molecules, and hence the distinction between responding and non-responding cells. After gastrulation, cells lose their community dependence, such that isolated muscle precursor cells can continue to develop as muscle, even if transplanted to an ectopic region of the embryo (Kato and Gurdon, 1993). The combination of positive, negative and community signals set up the final pattern of the mesoderm by the end of gastrulation.

We aim to characterise the molecular basis of the community interaction in *Xenopus* muscle development. The community factor is proposed to have the following properties: it will be expressed in a region that includes the muscle precursor cells...
(that is, in the dorsolateral mesoderm) from the start of gastrulation, and it will be able to maintain stable expression of muscle-specific genes in these cells when they are experimentally separated from their normal neighbours. The community factor could be secreted and freely diffusible, or associated with the cell membrane, but is known not to require cytoplasmic connections between cells (Gurdon et al., 1993b). Factors produced by the Spemann organiser have previously been shown to promote muscle development, but are unable to account for the community effect (Carnac and Gurdon, 1997).

We have developed an assay system that allows us to evaluate whether candidate factors are able to function as a community signal. In this system, muscle precursor cells are dissociated, treated with the candidate factor, and cultured in dispersion until control whole embryos have formed neurulae. The cells are thus not in contact with their neighbours during the crucial mid-gastrula stage when the community effect is known to take place (Gurdon et al., 1993b). The cells are then assayed for expression of muscle-specific genes. If the cells express such genes, this means that the factor with which they were treated was able to compensate for the lack of cell contact and substitute for the normal community signal. This factor is therefore likely to have a role in mediating the endogenous community effect during normal development.

We have tested a number of candidate factors in the community assay, including fibroblast growth factor (FGF), BMP4 and transforming growth factor β (TGFβ) proteins, and also components of the Xwnt8 signalling pathway, all of which have previously been suggested to have a role in mesoderm maintenance or muscle differentiation. Xwnt8, Xnr1 and Xnr2 can convert ventral mesoderm to muscle (Hoppeler et al., 1996; Jones et al., 1995), BMP4 is essential for XMyf5 expression (Dosch et al., 1997), and FGFs have been shown to maintain early mesoderm gene expression (Isaacs, 1997; Isaacs et al., 1995, 1996; Slack et al., 1996). Any of these factors could potentially also be involved in the community effect, acting to stabilise myogenic gene expression within the dorsolateral region.

Of these candidate factors, we find that only treatment with embryonic FGF (eFGF; Isaacs et al., 1992) is sufficient to allow dispersed muscle precursor cells to maintain expression of the muscle-specific genes XMyoD (Hopwood et al., 1989) and XMyf5 (Hopwood et al., 1991). eFGF can therefore behave as a community factor, substituting for the endogenous community signal that each cell would normally receive from its neighbours. We show that eFGF has signalling properties that are indistinguishable from the endogenous community signal, and, furthermore, that blocking FGF signalling in the muscle precursor cells inhibits community signalling. We conclude that eFGF is therefore likely to be the myogenic community factor. We find that the mode of action of eFGF in the community effect takes place on a timescale that is entirely different from that of the TGFβ factor activin in mesoderm induction.

**MATERIALS AND METHODS**

**Embryological techniques**

*Xenopus* embryos were obtained by in vitro fertilisation, reared in 0.1× modified Barth saline (MBS; Gurdon, 1977) and dejellied in 2% cysteine-HCl. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Embryos were transferred to 1× MBS, 4% Ficoll (Pharmacia) for micro-injection. The dorsal blastomeres were identified by differences in size and pigmentation at the four-cell stage; and a volume of 4.6 nl was injected sub-equatorially into both dorsal cells using a Drummond Nanoinjector variable microinjector (Drummond).

Dissections were carried out in 1× MBS. Dorsaloral pieces were taken from stage 10.25 gastrulae as described previously (Kato and Gurdon, 1993). Intact pieces were cultured in 1× MBS, 0.1% bovine serum albumin (BSA; Sigma), 1 µg/ml gentamicin (Sigma). To disaggregate the cells, the pieces were incubated for 20 minutes in 1× Ca2+- and Mg2+-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 (Ca2+-containing) 1× MBS, 0.1% BSA, 1 µg/ml gentamicin, plus the appropriate dose of eFGF or other growth factors. eFGF protein was a gift from Drs H. Isaacs and M. E. Pownall, University of York, UK; other factors were purchased from R&D Systems. Washing dispersed cells to remove unbound eFGF was carried out by rinsing the cells in three changes of Ca2+-free medium before the cells were returned to normal 1× MBS for continued culture. To make reaggregates, the cells were centrifuged in medium containing 2.2 mM (three times the normal MBS concentration) of Ca2+. SU5402 was purchased from Calbiochem (Cat. no. 572630) and dissolved in DMSO. The FGFR1 alpha (IIIC)/Fc chimera was purchased from R&D Systems (Cat. no. 658-FR) and reconstituted in 1× MBS, 0.1% BSA. In each case, the inhibitors were added to dispersed cells prior to their reaggregation, and the reaggregates then incubated continuously in inhibitor-containing medium.

Typically, the total culture period of the experiment was 4.5-5 hours, the exact length of time taken for the cells to reach stage 13 varying with ambient temperature (21-24°C). 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.

**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: XMyoD (K. Ryan, unpublished; EcoRI, T7 polymerase); XMyf5 (K. Ryan, unpublished; PmeI, T7 polymerase); chordin (Ryan et al., 1996; EcoRI, T3 polymerase); FGF (Lemaire and Gurdon, 1994; BamHI, T7 polymerase), XSox17β (K. Ryan, unpublished; BamHI, T7 polymerase), eFGF (Isaacs et al., 1992; EcoRI, T7 polymerase). Quantitation was carried out using a Fujifilm Phosphor Imager and MacBAS 2.5 software.

**Immunohistochemistry**

Cells on slides were rehydrated through an ethanol series and blocked for 30 minutes in 1× PBS, 0.2% (w/v) BSA, 0.1% Triton X-100, 10% goat serum. Slides were treated for 2 hours with D7F2 (anti-XMyoD; Hopwood et al., 1992), diluted 1:4 in blocking solution, or for 1 hour with 12/101 diluted 1:200 (Kintner and Brockes, 1984), followed by rabbit anti-mouse IgG for 1 hour (1:200; ICN) and finally APAAP for 1 hour (1:50; Senotech), with half-hour washes in 1× PBS, 0.2% BSA, 0.1% Triton X-100 after each 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 Hoechst 33258 (5 μg/ml) for 30 minutes, and the slides mounted in PBS-90% glycerol.
RESULTS

Our aim was to identify the community factor on the basis of its function: the ability to promote stable muscle-specific gene expression in single muscle precursor cells, when supplied experimentally at a sufficiently high dose. Several signalling factors were tested for a potential role in the community effect, including Xwnt8, BMP4, FGFs and TGFβs.

Xwnt8 is a candidate community factor

Xwnt8 (Christian et al., 1991) is a secreted glycoprotein signalling factor that is present and active in the dorsolateral mesoderm during the period when the community effect takes place (Leysn et al., 1997; Salic et al., 1997; Wang et al., 1997). Xwnt8 can regulate expression of the myogenic gene XMyoD (Hopwood et al., 1989). Hoppler et al. showed that a dominant negative Xwnt8 abolished expression of XMyoD in the gastrula, and reduced or eliminated further somite development (Hoppler et al., 1996). Conversely, when an active Xwnt8 was targeted to the dorsal marginal zone, the XMyoD expression domain was expanded. These results suggested that Xwnt8 could also be involved in the community effect.

Xwnt8 and its downstream signalling pathway components were tested for community activity

Wnts are not readily available in protein form, so in this case we used DNA injections to overexpress Xwnt8. DNA, rather than mRNA, was used so that the gene would be expressed only after the mid-blastula transition (MBT), thus avoiding the complicating effects of early Wnt signalling (Smith and Harland, 1991; Sokol et al., 1991). Although expression of DNA constructs is mosaic (Vize et al., 1991), it was predicted that a community effect would still be discernible, even if from only a percentage of the cells.

An Xwnt8 DNA expression plasmid, pCSKA-Xwnt8 (Christian and Moon, 1993), was injected at a range of doses (20-100 pg/blastomere) into the future muscle region (Fig. 1A). This might activate the Xwnt8 signalling pathway in the muscle precursor cells in an autocrine manner, by-passing the need for an Xwnt8 signal from neighbouring cells. As this autocrine induction might not occur, two downstream components of the Wnt signal transduction pathway were also tested in the same assay, dishevelled (50-200 pg/blastomere pCS2-Xdsh) (Sokol, 1996) and β-catenin (20-200 pg/blastomere pCS2-pt-β-catenin) (Yost et al., 1996), to stimulate the Wnt pathway intracellularly.

Dorsolateral mesodermal tissue, containing muscle precursor cells, was dissected from injected embryos at early gastrulation (stage 10.25). The cells were disaggregated in Ca2+- and Mg2+-free medium, and cultured singly until control whole embryos had completed gastrulation (stage 13-14), and were therefore beyond the stage when the community effect takes place. The cells were then analysed by RNase protection or immunohistochemistry for expression of the muscle-specific genes XMyoD and XMyo5 (Hopwood et al., 1991; Fig. 1A). In the dispersed culture, the cells were not in contact with their normal neighbours, and so could not participate in any intercellular signalling. The endogenous secreted community factor would have been diluted to a concentration below its threshold for activity in the large volume of medium. Therefore, if these cells expressed muscle-specific gene products, this would mean that the injected factor had compensated for the lack of cell contact, implicating that factor in the community interaction in normal development.
In each experiment, intact dorsolateral mesoderm tissue pieces provided controls for myogenic gene expression and detection. Reaggregates (the cells disaggregated and immediately reaggregated) demonstrated that it was not the mechanical procedure of cell disaggregation that blocked muscle development in the dispersed cells, but rather the loss of some crucial community factor(s). These samples serve as positive controls because the cells are in contact with each other during gastrulation and are thus able to undergo the normal community interaction.

The Xwnt8 signalling pathway alone cannot mediate a community effect

Each plasmid DNA was tested in the community assay at least twice, and one set of results is shown in Fig. 1B. Intact tissue pieces strongly expressed XMyoD and XMyf5, while dispersed cells failed to maintain expression of these genes. At none of the doses of Xwnt8, dishevelled and β-catenin tested was there significantly more XMyoD in the dispersed injected samples than in the dispersed uninjected samples. Dispersed cells taken from injected embryos and stained with an antibody against XMyoD protein (Hopwood et al., 1992) confirmed these results at the single cell level, with no stain for XMyoD visible either in isolated single cells or in those which had settled in small clusters (data not shown). Tailbud stage embryos reared from embryos injected dorsally at the four-cell stage with any of the three plasmids lacked eyes and cement glands, as previously published (Christian and Moon, 1993; Sokol, 1996; Yost et al., 1996), showing that the plasmids were biologically active at the doses tested (data not shown). It was concluded that stimulating the Xwnt8 pathway in dispersed dorsolateral mesoderm cells does not allow them to express muscle-specific genes, and therefore the Xwnt8 pathway alone is not sufficient to account for the community effect.

These experiments also confirmed that the community factor acts at the level of XMyoD and XMyf5 mRNAs, both of which are sensitive to cell dispersal, by stimulating transcription, or possibly by increasing mRNA stability. Previous community effect studies assayed at the level of muscle-specific protein expression (Carnac and Gurdon, 1997; Gurdon et al., 1993b), leaving open the question of the level at which the community factor acted. Here, we exclude other possibilities, such as a role for the community factor in facilitating translation, or in promoting export of mRNA from the nucleus.

Candidate community factors can be tested for community activity by supplying them in protein form to dispersed cells

Potential community factors available in protein form were tested by adding them to the culture medium of disaggregated muscle precursor cells, with the aim of replacing the endogenous community factor (Fig. 2A). eFGF (Isaacs et al., 1992), bFGF (Kimelman et al., 1988; Slack and Isaacs, 1989), BMP4 (Dale et al., 1992; Jones et al., 1992) and TGFβ2 (Rosa et al., 1988) were tested in this way. These factors have previously been implicated in mesoderm development. For example, expression of a dominant-negative FGFR after MBT is known to result in a loss of later mesoderm gene expression (Kroll and Amaya, 1996), and an autoregulatory loop has been identified between eFGF and the transcription factor Xbra (Isaacs et al., 1994; Schulte-Merker and Smith, 1995). BMP4 has been shown to behave as a morphogen in mesoderm patterning, with a moderate level of BMP4 being required for expression of XMyf5 (Dosch et al., 1997). TGFβ2 is a convenient generic TGFβ factor, representative of a class of molecules that includes Xnr1 and Xnr2, which are implicated in promoting dorsal mesoderm patterning (Jones et al., 1995). These factors were all tested in the community assay at concentrations that we found reliably induced mesoderm genes in intact blastula stage animal caps (data not shown).

eFGF can behave as a myogenic community factor

RNase protection results for all the proteins tested in the community assay are shown in Fig. 2B. eFGF supported expression of XMyoD and XMyf5 in dispersed muscle precursor cells, a result observed in seven separate experiments, with a stimulation in the level of XMyoD mRNA of 10- to 20-fold, compared with the level in dispersed untreated cells. In contrast, bFGF, BMP4 and TGFβ2 did not result in any increase in the level of XMyoD mRNA. The level of chordin mRNA, which is less sensitive to cell dispersal, remained relatively constant in most samples, but is reduced by BMP4 treatment, presumably owing to ventralisation of the cells.

Having shown an increase in the level of XMyoD mRNA in the cell population on eFGF treatment, it was important to use immunohistochemistry to determine whether XMyoD protein was expressed from the nucleus.

**Fig. 2.** (A) The community assay for testing candidate factors – protein treatment method. Muscle precursor cells were dissected from the dorsolateral mesoderm (shaded) of embryos at stage 10.25. The cells were disaggregated and cultured in the presence of the candidate factor, which was added in protein form to the medium. At stage 13 the cells were analysed by RNase protection or immunohistochemistry. (B) eFGF (100 ng/ml), bFGF (100 ng/ml), TGFβ2 (100 ng/ml) and BMP4 (1.0 μg/ml) were tested in the community assay. Analysis by RNase protection. The expression level of XMyoD is shown as a percentage of the cognate FGFR loading control value beneath each lane. eFGF stimulates expression of XMyoD and XMyf5.
was being produced in single isolated cells. This was to eliminate the possibility that the XMyoD mRNA seen by RNase protection might be entirely due to small groups of cells present in the sample, owing to insufficient dissociation or local reaggregation during culture. After culture in the presence or absence of eFGF, the cells were transferred to slides and fixed. Intact dorsolateral mesoderm pieces disaggregated and fixed immediately at the end of the culture period 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. A small patch of somite cells taken from neurula embryos and added to every slide acted as a positive control for the antibody staining procedure. After antibody treatment and detection, the cells were stained with the DNA dye Hoechst to label the nuclei. At least 500 cells were counted on every slide and scored as being positive or negative for XMyoD protein. Only cells in groups of ten cells or fewer were counted in this way.

As shown in Fig. 3A, single cells treated with eFGF protein expressed XMyoD protein. Typically, the percentage of XMyoD-positive cells in the eFGF-treated dispersed cell samples approached or even exceeded the level in the late dispersal control sample (Fig. 3B). In one experiment, the cells were cultured to stage 23 and assayed with a marker for later muscle differentiation, the 12/101 antibody (Kintner and Brockes, 1984). Stained cells were observed in the eFGF-treated dispersed cell sample, indicating that the expression of XMyoD at stage 13 represented cells firmly established on the pathway to terminal muscle differentiation (Fig. 3A).

Untreated cells and those treated with bFGF or BMP4 did not express XMyoD (Fig. 3B).

Animal cap cells dissected at stage 10.25 and dispersed did not express XMyoD when treated with eFGF protein (data not shown). This suggests that eFGF was not inducing cells of other types to become muscle, and hence that it was truly the muscle precursor cells that responded in the community assay. eFGF can therefore behave as a myogenic community factor.

**eFGF is expressed in the muscle precursor cells during the period of the community effect and is unaffected by cell dispersal during this time**

Three criteria must always be satisfied in order to demonstrate that a factor is responsible for a given process. These criteria are: normal expression, in that the factor must be present at the right time and in the right place to be responsible; sufficiency,
in that it must be biologically active in an appropriate test system; and necessity, in that specific inhibition of its action must prevent the process occurring in vivo. Having demonstrated that eFGF is sufficient to allow expression of myogenic genes, the remaining criteria were addressed.

A series of in situ hybridisations were carried out on embryos of stage 11.5-12 (Fig. 4A). At this stage, XMyoD is expressed in the dorsolateral mesoderm. As previously published (Isaacs et al., 1995), eFGF is expressed most strongly in the dorsal mesoderm, but also extends in a complete ring around the blastopore, so that its expression domain does overlap with that of XMyoD. eFGF mRNA is never very abundant, but is found in dissected dorsolateral mesoderm pieces analysed by RNase protection (Fig. 4B). The level of eFGF mRNA relative to FGFR mRNA is maintained in dispersed cells, and is unaffected by addition of eFGF protein to the culture medium. Therefore, eFGF is expressed in the right place and at the right time to be involved in the community effect in muscle development. The presence of the FGFR receptor in the muscle precursor cells, another important requirement, was confirmed as it was used as a loading control in all RNase protection assays described here.

The muscle precursor cells are sensitive to a wide range of doses of eFGF and show a dose-dependent response

To further characterise the response of the muscle precursor cells to eFGF protein, dispersed cells were treated with a range of doses of between 0.3 and 300 ng/ml. A percentage of the cells responded by expressing XMyoD at all these doses, showing that they are sensitive to eFGF over a very wide range of concentrations (Fig. 5). At the low end of the scale, the percentage of cells responding increased slightly with ascending eFGF concentration, indicating a dose-responsive effect. It then appeared to plateau at doses of 10 ng/ml and higher, around the level of XMyoD expression seen in the late dispersal control samples, rather than continuing to increase. A dose of 100 ng/ml eFGF was used in all subsequent experiments, as it consistently gave a strong stimulation of XMyoD.

The period of sensitivity to eFGF coincides with the endogenous community interaction

We wanted to determine whether the time period during which the cells are sensitive to eFGF is consistent with the endogenous community effect. Dispersed muscle precursor cells were incubated in the presence of eFGF, which was then washed away after various intervals (Fig. 6A), or alternatively eFGF was added to dispersed cell culture after various periods (Fig. 6B). The cells were then analysed by immunohistochemistry for XMyoD protein. Washing away the eFGF before the end of the culture period led to a dramatic reduction in the percentage of cells expressing XMyoD, even if the cells had been exposed to the eFGF signal for 2 hours. The cells that were washed and returned to fresh culture medium containing eFGF did express XMyoD at levels comparable with the cells that were incubated continuously in eFGF-containing medium, showing that the washing procedure itself was not responsible for the loss of XMyoD (Fig. 6A). Cell samples that had eFGF added to their culture medium within 3 hours of dissociation (around stage 12 or earlier) did express XMyoD, but adding eFGF later than this resulted in little or no stimulation. In general, the earlier the addition of eFGF, the higher the percentage of dispersed cells that responded by expressing XMyoD. The percentage of positive cells in the late dispersal controls was relatively constant at the different stages of fixing (stages 12.5-16) (Fig. 6B).

To additionally determine whether the endogenous community factor acts on the same timescale as eFGF in the community assay, dispersed cell samples were reaggregated after different time periods by collecting the cells and gently centrifuging them, aiming to restore their receipt of the endogenous community factor from their neighbours. After 3 hours of culture as a reaggregate, each sample was frozen for analysis by RNase protection, together with an intact control sample. The results from one such experiment (carried out four times) are shown in Fig. 6C. The cells reaggregated immediately after dispersal have a level of XMyoD mRNA equivalent to that in intact pieces, while samples reaggregated after one or more hours of being dispersed have much less. Chordin was included in the analysis because it is less sensitive to cell dispersal, and so its mRNA could be detected in all samples. This experiment showed that the cells need to be in contact with each other for most of the gastrulation period if the community signal is to be operative. When it is too late to rescue muscle differentiation in dispersed cells by adding eFGF to their medium, it is also too late to rescue them by reaggregating them.

These results, shown schematically in Fig. 6D, show that the period of sensitivity to added eFGF is during gastrulation, and is roughly the same as the period of sensitivity to the endogenous community factor when the cells are reaggregated. This coincides with the community effect as previously determined by single cell transplantation experiments (Kato and Gurdon, 1993). These transplantation experiments identified the stage when a muscle cell becomes independent of its neighbours for further muscle differentiation; the experiments presented here extend this by defining the time windows of both the community signal and the competence to respond to that signal.
Inhibition of FGF signalling prevents expression of XMyoD and XMyf5 in muscle precursor cell reaggregates

The final criterion necessary to show that eFGF is responsible for the community effect is that of necessity; that is, blocking the FGF signal should prevent the community interaction and hence muscle cell differentiation. To directly target the community effect, rather than earlier mesoderm induction or patterning events, two inhibitors were used that could be applied directly to the muscle precursor cells at the desired time and that interfered with the FGF signalling pathway at different levels.

SU5402 is an FGF receptor-specific tyrosine kinase inhibitor that binds to the catalytic domain of the cytoplasmic region of the FGF receptor and prevents its enzymatic activity (Mohammadi et al., 1997). It thus blocks FGF signalling from within the cell. Muscle precursor cells dissected at stage 10.25 were disaggregated and then immediately reaggregated for culture in the presence of 15-40 μM SU5402 (Calbiochem). At stage 14, they were analysed by RNase protection; one example from two experiments is shown in Fig. 7A. Intact and untreated ‘mock’ reaggregates expressed XMyoD and XMyf5, while reaggregates treated with all doses of SU5402 did not, showing that FGF signalling is necessary for myogenic gene expression. SU5402 did not affect the level of expression of XSox17β (Hudson et al., 1997), an endodermal gene that requires TGFβ signalling for maintenance (Yasuo and Lemaire, 1999). DMSO alone, the solvent for SU5402, had no effect on gene expression. Treatment with SU4984 (Calbiochem), a second tyrosine kinase inhibitor with a broader target range than SU5402, generated similar results (three experiments, data not shown).

A second method to block FGF signalling made use of a soluble version of the FGF receptor. The FGFR1 alpha (IIIc)/Fc chimera (R&D Systems) consists of the extracellular domain of human FGFR1 fused to the Fc region of human IgG, and can therefore be produced as a secreted protein in a mouse myeloma cell line. It binds the FGF ligand outside the cell and stops much of it from accessing its endogenous functional receptor, and has previously been used to look at necessity for FGF signalling in development (Jung et al., 1999). Muscle precursor cells dissected at stage 10.25 were disaggregated and then immediately reaggregated for culture in the presence of 5.0-10.0 μg/ml FGFR1 alpha (IIIc)/Fc. At stage 13 they were analysed by RNase protection; one example from three experiments is shown in Fig. 7B. The pattern is very similar to that in Fig. 7A, with 1.0 μg/ml soluble FGFR eliminating XMyoD and XMyf5 but having little effect on XSox17β.

Blocking FGF signalling both inside (SU5402) and outside (FGFR1 alpha (IIIc)/Fc) the cell resulted in a failure of the muscle precursor cells to upregulate expression of myogenic...
genes at the normal time, despite being in contact with each other. It was concluded that FGF signalling is an essential component of the endogenous community signal.

**DISCUSSION**

We have demonstrated here that eFGF is likely to be involved in mediating the community effect in *Xenopus* myogenesis for the following reasons: (1) it can support stable expression of myogenic genes and later muscle differentiation markers in single muscle precursor cells; (2) it is expressed in the muscle precursor cells during gastrulation; (3) the time window of sensitivity to eFGF coincides with the period of the endogenous community effect; and (4) specific inhibition of FGF signalling prevents community signalling. These results are consistent with a model in which each cell in the dorsolateral mesoderm secretes eFGF, and its neighbours respond by maintaining stable expression of myogenic transcription factors prior to terminal differentiation.

Two potential causes for concern arise from our experiments. First, the percentage of cells that express XMyoD protein, both in dispersed cell samples treated with eFGF and in late dispersal control samples, is not high (10-20%). The reason for this is that the majority of cells in the dissected dorsolateral mesoderm pieces are not in fact muscle precursor cells, but other mesoderm cells and also ectoderm and endoderm cells. This is because it is not possible to reliably dissect starting material with a higher proportion of presumptive muscle cells at the early gastrula stage. However, although most of the cells in our samples are not muscle precursor cells, they do not affect our results or our conclusions.

Second, XMyf5 expression in reaggregates is variable. Unlike XMyoD, which is generally expressed at the same level in reaggregates as in intact pieces, XMyf5 is sometimes poorly expressed in reaggregates (compare intact and reaggregated samples). This variation seems to be independent of any other experimental treatment. It appears that XMyf5 is more sensitive than XMyoD to cell dispersal, and so it does not always recover to control levels on reaggregation. The reason for this is unclear, but the overall trends are consistent between experiments, as both XMyoD and XMyf5 clearly require cell contact for maintained expression, and both respond to eFGF treatment of single cells.

**FGFs as candidate community factors**

In addition to bFGF and eFGF, three other FGFs have so far been identified in early *Xenopus* embryos; FGF3 (Tannahill et al., 1992), FGF8 (Christen and Slack, 1997) and FGF9 (Song and Slack, 1996). eFGF is unique to *Xenopus*; it is about equally related to mammalian FGF4 and FGF6, while the other *Xenopus* FGFs are much more closely related to their mammalian counterparts (Isaacs, 1997; Slack et al., 1996). During gastrulation eFGF, FGF3, FGF8 and FGF9 are all expressed in complete rings around the blastopore, with eFGF being most strongly expressed dorsally (Isaacs et al., 1995), FGF3 is concentrated laterally and ventrally (Lombardo et al., 1998), and FGF8 and FGF9 are initially expressed at even intensity throughout (Christen and Slack, 1997; Song and Slack, 1996). However, as gastrulation proceeds, FGF3 and FGF8 become restricted to posterior domains, while eFGF and FGF9 are still expressed in the dorsal and dorsolateral
mesoderm, and are thus most consistently associated with the muscle precursor cells. Zygotic transcription of bFGF does not start until the mid-neurula stages, too late to be involved in the community effect. eFGF, FGF3 and FGF8 are all secreted proteins, and would seem to be the most probable candidates for mediating an intercellular signalling phenomenon like the community effect, rather than bFGF and FGF9, which lack recognisable secretory peptides and are not thought to be efficiently secreted from *Xenopus* cells. All five *Xenopus* FGFs are active as mesoderm inducers when their mRNAs are injected into the early embryo. eFGF has a specific activity approximately ten times greater than FGF2, FGF3, FGF8 and FGF9 in this assay (Christen and Slack, 1997; Isaacs et al., 1992; Lombardo et al., 1998; Song and Slack, 1996).

Four high-affinity FGF receptor genes are known, and alternative splicing generates a greater diversity of receptors. While different FGFs do bind preferentially to particular FGF isoforms, there is a great degree of crossreactivity, and ligand-receptor specificity is not well characterised. All four FGF receptors are expressed during gastrulation, each with a different dynamic profile (Hongo et al., 1999).

In this study we have focussed on eFGF, because it is active in our community assay and fulfils all the criteria expected of the community factor. However, the large degree of spatial and temporal overlap of the FGF expression domains, and the crossreactivity between the FGF ligands and their receptors, makes it difficult to absolutely determine which FGF is responsible for the community effect. Although eFGF appears to be the most likely candidate for the natural community factor, it is possible that FGF3 or FGF8, or a combination of the two, may contribute to the community signal in normal development. Indeed, in a preliminary experiment, human FGF5, FGF6, and FGF8 had a similar level of myogenic community activity to *Xenopus* eFGF, based on the percentage of single cells expressing *XMyoD*. Human FGF4, FGF9, FGF17 and FGF18 showed a lower level of muscle-promoting activity, while FGF1, FGF10 and FGF16 were inactive (H. J. S., unpublished). However, it is not yet clear whether this activity of the human FGF proteins is applicable to *Xenopus* development.

Our experiments do not exclude the possibility that other factors such as Xwnt8, BMP4 or Xnrs are also involved in the community effect, though necessarily acting in combination with eFGF. While the other factors tested were unable to mediate a community effect alone (Figs 1, 2), other evidence still points to the likelihood of them having a role in muscle development at some level (Dosch et al., 1997; Hoppler et al., 1996; Jones et al., 1995). It may be that they are involved in the earlier mesoderm patterning events and initial activation of XMyoD and XM Myf5 in the mesoderm, but are no longer required for regulating muscle gene expression by the time the community effect comes into operation during gastrulation.

**The mode of action of eFGF in the community effect**

The muscle precursor cells need to be supplied with eFGF over an extended period of several hours if they are to respond by expressing muscle-specific genes (Fig. 6). This is in marked contrast to the rapid induction of mesoderm genes in animal caps, which can be achieved by incubating disaggregated cap cells in the presence of activin for only ten minutes (Dyson and Gurdon, 1998). This may be because activin has a very high affinity for its receptor, and the ligand-receptor interaction is extremely stable. FGFs are thought to require interactions with cell surface heparan sulphate proteoglycans in order to bind their receptors (Rapraeger et al., 1991; Yayon et al., 1991). It is possible that cell dispersal may disrupt the extracellular matrix, and this might make eFGF less effective at binding, so eFGF would be required in the medium during the whole of the signalling period to enable an effective amount to access the FGF receptor.

We favour an alternative explanation for the differences in timing between the activities of activin and eFGF. We propose that the community effect is a fundamentally different process from that of initial mesoderm induction. As we have shown, in community interactions, each cell must receive the signal over an extended period to both consolidate its differentiation pathway and to enable all the cells in the community to differentiate co-ordinately. The results presented here highlight the difference between initial induction, which activates gene expression, and a longer-term community signal, which maintains gene expression and tissue identity.

**The community effect in the muscle and the notochord**

Here, we have shown that eFGF can behave as a community factor in the developing muscle. The expression pattern of eFGF suggests that it could also be the community factor in the notochord (Weston et al., 1994). Direct confirmation of this would involve testing whether notochord differentiation markers, such as the MZ15 antigen (Smith and Watt, 1985), can be expressed in single dorsal mesoderm cells treated with eFGF. eFGF and the transcription factor *Xbra* (Smith et al., 1991) are involved in an autocatalytic loop, with Xbra inducing expression of the gene for eFGF, which the cells then require for their continued expression of *Xbra* (Casey et al., 1998; Isaacs et al., 1994; Schulte-Merker and Smith, 1995). This feedback loop would provide a mechanism for the community effect in the notochord, stabilising expression of *Xbra*, which is then required for terminal notochord differentiation.

This would lead to the question of how one factor could mediate the community effect in two cell populations of different fates. It may be that the community factor is non-instructive, and that its role is purely one of maintenance. It could tell the cells to continue on the appropriate differentiation path (notochord or muscle), and co-ordinate differentiation within the group, without providing any information about which pathway a particular cell should take. Information regarding cell fate could come entirely from other patterning molecules. In this scenario eFGF would have a general, rather than tissue-specific role, and so could be the community factor in both tissues. In our assay, the dorsolateral mesoderm cells have already received the instructions to become muscle, and just require eFGF to carry out these instructions to the point of stable differentiation. However, from our experiments we cannot rule out the possibility that there is an instructive component to the community signal. It is possible that different doses or periods of eFGF exposure may provide this information to the mesoderm.

It is possible that *Xbra* is involved in the community effect in the muscle as well as in the notochord, as in early gastrulae it is expressed throughout the mesoderm. An alternative possibility, that we plan to investigate further, is that
Antipodean/VegT/Xombi/Brat (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996) may have an equivalent role in the muscle to Xbra in the notochord. Antipodean/VegT encodes a similar type of protein (T-box transcription factor), and it is expressed in the paraxial mesoderm, in a complementary pattern to Xbra, at late gastrula stages.

The community effect in other systems and in cancer

A community effect has been identified in mammalian somitogenesis (Cossu et al., 1995). Community interactions are also implicated in Drosophila neurogenesis (Stuttem and Campos-Ortega, 1991), in the enveloping layer of the zebrafish mesoderm, in a complementary pattern to T-box transcription factor, and it is expressed in the paraxial patterning in mouse (Aubin-Houzelstein et al., 1998) and Wood, 1992; Kuwabara et al., 1992) and in coat pigment also implicated in somitogenesis (Cossu et al., 1995). Community interactions are an important goal, both to contribute to a molecular description of development, and as a first step in designing metastatic capacity, because they promote cell differentiation rather than proliferation. In cancer, deficiency of a community factor may cause cells to lose their identity and become migratory and invasive. Characterisation of community factors is an important goal, both to contribute to a molecular description of development, and as a first step in designing specific approaches in the prevention and treatment of metastasis.

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