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Original Article

Uncommitted *Xenopus* blastula cells can be directed to uniform muscle gene expression by gradient interpretation and a community effect

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ABSTRACT The animal cap cells of *Xenopus* blastulae behave as multi-potent stem cells in so far as they can differentiate along many unrelated pathways according to the kind and amount of signal factor that they experience. At first, animal cap cells activate early zygotic genes across a broad range of TGFβ concentrations; soon after this, they activate later genes more intensely and over a narrow concentration range. Here we show that uncommitted blastula cells can be directed, by the sequential influence of a particular concentration of a TGFβ morphogen and an FGF-mediated community effect, to form a homogeneous single cell type. As a result of these two signalling processes, an entire population of animal cap cells can be converted, in the absence of other signals, to a uniform population of one tissue type. Mesoderm cells that experience a particular concentration of activin increase their *XMyoD* expression by 10-fold and become distinct from neighbouring cells that received lower or higher concentrations of activin. The signalling processes that we employ here may be important in normal development and useful in guiding stem cell differentiation.

KEY WORDS: *activin, community effect, FGF, morphogen, TGFβ, Xenopus*

Introduction

A common characteristic of gene activity in early vertebrate development is a low level of expression across a broad region of the embryo, encompassing cells that are lineage ancestors to several different later cell-types. A few hours later, the same or other genes become much more strongly expressed in a subset of those cells with an initial weak expression. This second stage forecasts the differentiation of a final cell-type, with very strong expression of genes that usually carry out a structural as opposed to regulatory function.

This sequence of events is exemplified by *Xenopus* genes that are expressed in the muscle lineage and that form the basis of the results described here. The early zygotically-expressed *Xenopus* genes *Xbra* (Smith *et al.*, 1991), *Eomesodermin* (Ryan *et al.*, 1996) and *Antipodean* (zygotic VegT; Stennard *et al.*, 1996; Stennard *et al.*, 1999) are expressed across the entire equatorial region of mid-blastulae, and therefore in cells of all different mesodermal fates. These primary, cycloheximide-insensitive responses to mesoderm inducers are followed a few hours later by the secondary mesodermal response genes *XMyoD* (Hopwood *et al.*, 1989) and *XMyf5* (Hopwood *et al.*, 1991). These myogenic genes are at first expressed at a low level throughout the lateral and ventral mesoderm, but are not expressed in the dorsal notochordal region of the mesoderm; their later expression is confined to the cells of the future somitic muscle (Hopwood *et al.*, 1989; Harvey, 1990; Frank and Harland, 1991; Harvey, 1991; Hopwood *et al.*, 1991; Rupp and Weintraub, 1991; Harvey, 1992; Hopwood *et al.*, 1992). The third level of mesodermal gene expression includes structural genes such as muscle-specific *cardiac actin* (Mohun *et al.*, 1984) and the myotome marker 12/101 (Kintner and Brockes, 1984), which are strongly and exclusively expressed in muscle, which by this stage is totally demarcated from adjacent mesoderm-derived cells of other types, including the notochord and lateral plate mesoderm. There is therefore a progression from a gradient of early gene expression across the broad mesodermal region to a highly localised, demarcated and uniform expression of later genes in one cell type (Fig. 1).

Abbreviations used in this paper: BSA, bovine serum albumin; (e)FGF, (embryonic) Fibroblast Growth Factor; FGF-R, Fibroblast Growth Factor Receptor; TGFβ, transforming growth factor beta; pM or pMol, picomolar concentration.

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We ask here whether this sequence of events, and a uniformity of cell differentiation within a cell group, can be induced in blastula cells by an appropriate exposure to defined signal factors. The uniform differentiation of mouse embryonic stem cells has been very difficult to achieve; aggregates of such cells in embryoid bodies usually contain a mixture of different cell-types, and this diversity of cells may contribute to the differentiation of cells within the aggregate. An extensive series of experiments has been conducted by Asashima and colleagues (Uchiyama and Asashima, 1992, Ninomiya *et al.*, 1999, Ariizumi and Asashima, 2001) who have cultured amphibian whole animal caps of blastulae in activin and other molecules; their work has emphasized the diversity of organs that can be obtained, rather than the homogeneity of cells within a cell population.

Results

A Narrowing and Intensification of Activin *Gene Response takes place in Animal Cap Cells*

Using transiently dissociated *Xenopus* blastula cells to obtain uniform exposure to a defined concentration of activin, we have previously found that the primary response genes *Antipodean*, *Eomesodermin* and *Xbra* are expressed across a broad concentration range, as they are in the mesoderm of normal early gastrula embryos (Dyson and Gurdon, 1998; Heasman, 1997). We now find that the strength and concentration sensitivity of expression of the secondary response genes *XMyoD* and *XMyf5* change substantially in reaggregated cells between gastrula and neurula stages. At stage 11 (mid-gastrula) these genes are expressed at a low level across a broad activin concentration range from 10 pM to 90 pM (Fig. 2A). By stage 16 (mid-neurula) their expression profile has narrowed, strong expression being seen only after a 10 pM dose of activin, and not at all at the higher doses (Fig. 2B), a result seen in all four independent experimental series carried out. The same response to activin is not seen in other unrelated pathways of differentiation. High concentrations of activin induce endodermal genes (Hudson *et al.*, 1997 and references therein), and the endodermal marker gene *XSox17β* was indeed expressed at stage 16 in cells treated with 30 and 90 pM activin, while the neural marker *N-tubulin* (Richter *et al.*, 1988) was not strongly expressed at any dose (Fig. 2C).

At the same time as the narrowing of its concentration response, we also observe a nearly ten-fold amplification of *XMyoD* expression in cells that received a 10 pM dose of activin (compare histograms in Fig. 2 A,B). Therefore, the lack of *XMyoD* expression at neurula stages after treatment with non-myogenic concentrations of activin (≤ 3 and ≥ 30 pM) is accompanied by a great enhancement of expression after muscle-inducing doses.

We have used *in situ* hybridisation of activin-treated cell reaggregates to ask whether the concentration-dependent gene responses seen above apply to all the cells in a population. In Fig. 3 we see uniform expression of *XMyoD*, *cardiac actin* and the endoderm marker *endodermin* (Sasai *et al.*, 1996) at appropriate activin concentrations in all non-pigmented cells, that is, in those derived from the inner animal cap cells of a blastula; the outer pigmented cells do not respond to activin and they become epidermis. The tertiary response gene *cardiac actin*, normally expressed in axial muscle at this stage, is expressed strongly after 10, but not after 3.4 or 30 pM activin, just as is observed for the myogenic genes.

We draw four conclusions from our results so far. First, the progression from a primary early mesodermal response to activin concentration to a tertiary muscle-specific response can take place in uncommitted blastula animal cap cells, without any signalling or other contribution from non-mesodermal cells of the embryo, in agreement with the results of others (Green *et al.*, 1994; Wilson and Melton, 1994). Second, the transition from a broad to a narrow response to activin concentration takes place at the level of myogenic gene expression during gastrulation. This narrowing of gene expression has been described previously for *Xbra, goosecoid, noggin,* and *Xwnt8,* but not for *cardiac actin,* which is not expressed at stage 10.5, nor for the myogenic genes, which were not tested (Green *et al.*, 1994; Wilson and Melton, 1994). Third, a regulatory gene, namely *XMyoD*, undergoes an approximately 10-fold amplification of expression in response to the same signal molecule. The narrowing of concentration response, coupled with a large amplification, provides a mechanism by which a clear demarcation is achieved between muscle and non-muscle derivatives of the mesoderm. Fourth, the uniformity of gene expression seen by *in situ* hybridisation shows that, apart from a few non-responding epidermal cells, non-mesodermal cell-types are not present in the reaggregates, and indeed that, at a particular activin dose, all the responding cells differentiate in the same way.

The Narrowing and Amplification of Gene Response Requires an FGF-mediated Community Effect

Our results so far have excluded a need for signalling from outside the activin-treated blastula cell group, but have not tested

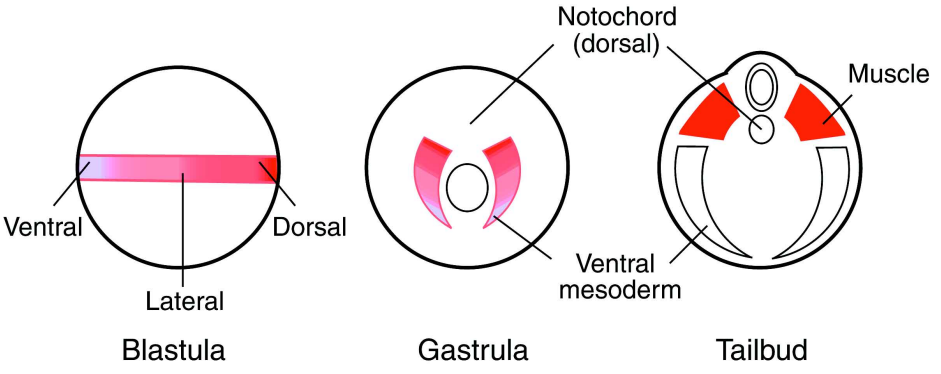

Fig. 1. Mesoderm, myogenic and muscle gene expression in the *Xenopus* embryo. *Schematic diagrams of blastula (side view), mid-gastrula (vegetal view) and tailbud (transverse section) stages. Early mesoderm genes such as* Apod, Eomes *and* Xbra *are expressed throughout the blastula mesoderm (pink gradient). During gastrulation, myogenic genes are expressed throughout the lateral and ventral muscle-generating mesoderm (pink), but are not expressed in the dorsal presumptive notochord. In the tailbud, the somites are distinct from surrounding tissues, and all the cells within the boundaries of the myotome express genes such as* cardiac actin *and the 12/101 antigen (red).*

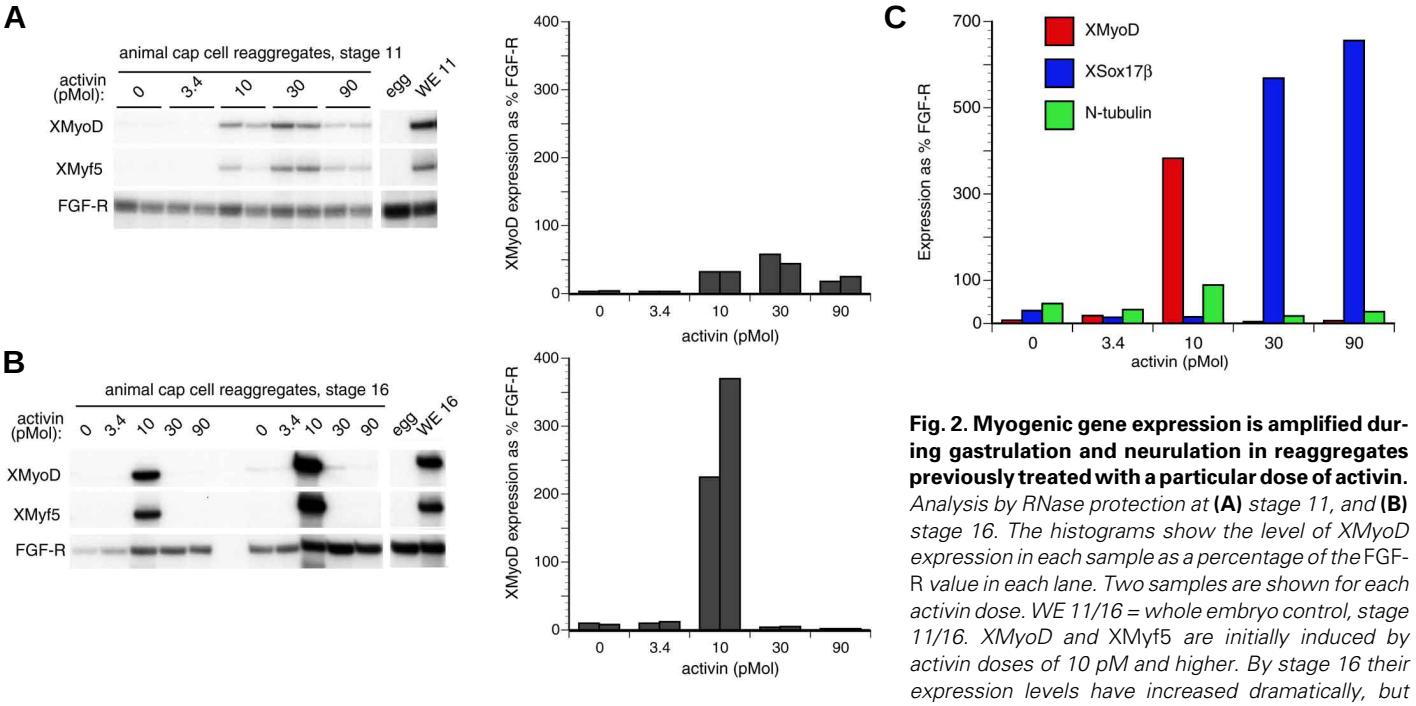

Fig. 2. Myogenic gene expression is amplified during gastrulation and neurulation in reaggregates previously treated with a particular dose of activin. *Analysis by RNase protection at* **(A)** *stage 11, and* **(B)** *stage 16. The histograms show the level of XMyoD expression in each sample as a percentage of the* FGF-R *value in each lane. Two samples are shown for each activin dose. WE 11/16 = whole embryo control, stage 11/16. XMyoD and* XMyf5 *are initially induced by activin doses of 10 pM and higher. By stage 16 their expression levels have increased dramatically, but only in samples treated with 10 pM activin.* **(C)** *At stage 16,* XSox17β *is expressed in response to higher activin doses than XMyoD, while* N-tubulin *is not strongly expressed at any dose. In this and the remaining figures pMol indicates a picomolar concentration.*

whether communication within a reaggregate is required. We have therefore cultured activin-treated cells as a widely dispersed monolayer of cells, to compare their gene expression profiles with those of the reaggregates in Fig. 2. Cells kept in a dispersed configuration from the time of activin treatment showed the same broad response to activin concentration at stage 18 (Fig. 4) as reaggregates do at stage 11 (Fig. 2A). Dispersed cells therefore underwent neither the narrowing of dose response nor the amplification of myogenic gene expression that is characteristic of reaggregated cells at the effective dose. The culture of cells in a dispersed state has been found before to eliminate or greatly reduce expression of *Xlhbox6, Xbra, Xwnt8,* and *cardiac actin,* and to eliminate the narrowing of concentration-dependent expression of *goosecoid* (Green *et al.*, 1994; Wilson and Melton, 1994).

Our previous work has shown that an eFGF-mediated community effect is required for normal gastrula mesoderm cells to progress to muscle gene expression (Standley *et al.*, 2001). We now ask whether this effect is required for activin-treated blastula cells to undergo the transition from a broad to a narrow and amplified gene response. We have used the tyrosine kinase inhibitor SU5402 (Mohammadi *et al.*, 1997; Standley *et al.*, 2001) to block activity of the FGF receptor in reaggregates that have already made their primary response to activin. Three sets of activin-treated animal cap cell reaggregates were prepared as before and cultured to stage 11, when one set was frozen. SU5402 was added to one of the two remaining sets of reaggregates, both of which were frozen at stage 18 and analysed by RNase protection. Reaggregates treated with 1-4 ng/ml activin (but with no SU5402) strongly amplified *XMyoD* and *XMyf5* expression between stages 11 and 18 (Fig. 5 A,B). The weak expression of *XMyoD* seen at stage 11 in reaggregates that were exposed to 8 ng/ml activin was not amplified. The amplification of tissue-specific gene expression within a certain dose range of activin, and the resulting sharpening of the expression profile thus showed the same pattern as seen in

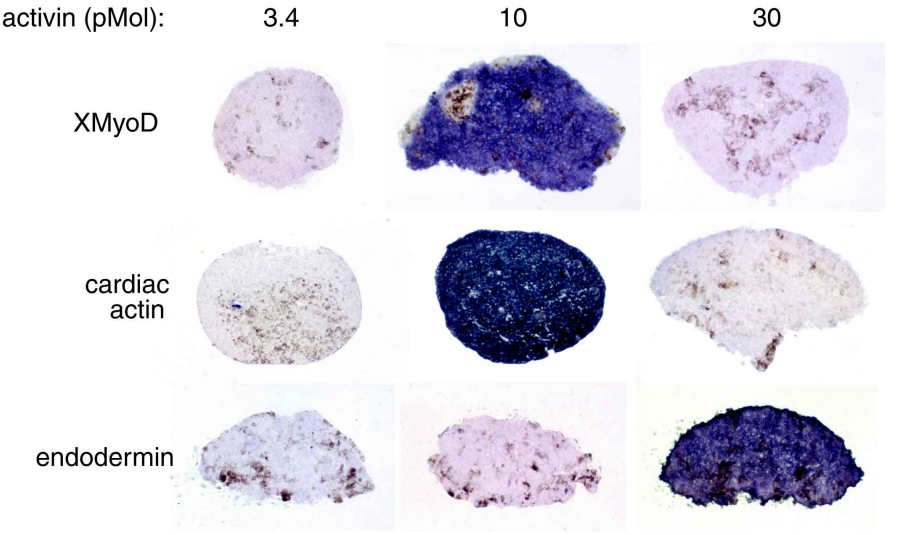

Fig. 3. Sectioned reaggregates assayed by *in situ* hybridisation for *XMyoD* **(stage 16), *cardiac actin* (stage 16) and *endodermin* (stage 26).** *The expression profiles follow the same dose-response as seen in Fig. 2 and gene expression is uniform throughout each reaggregate, except for small patches of non-responsive ectoderm.*

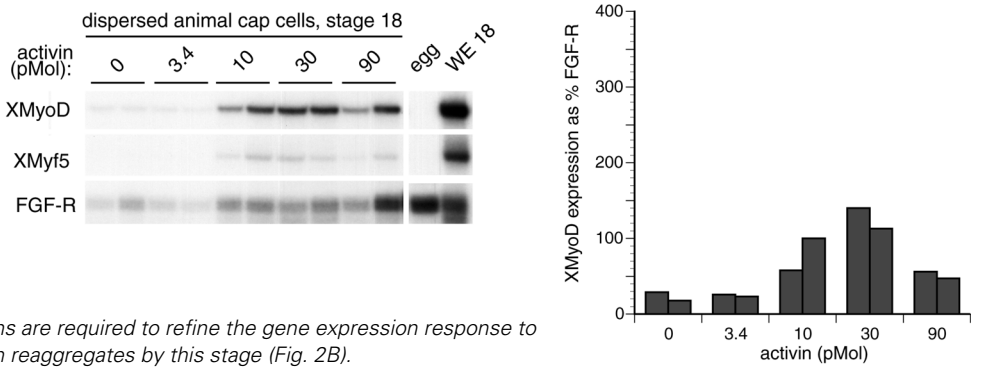

Fig. 4. Myogenic genes are expressed in activin-treated dispersed animal cap cells, but they are not significantly amplified at the 10 pM activin dose. *Analysis by RNase protection. The histogram shows the level of XMyoD expression in each sample as a percentage of the* FGF-R *value in each lane. Two samples are shown for each activin dose. This profile suggests that cell interactions are required to refine the gene expression response to activin, resulting in the profile normally seen in reaggregates by this stage (Fig. 2B).*

Fig. 2. Treatment of cultures with SU5402 prevented the amplification of *XMyoD* and *XMyf5* expression that normally occurs during gastrula and neurula stages (Fig. 5C). As a result, the dose response profile for the SU5402-treated samples analysed at stage 11 (Fig. 2A). We conclude that FGF-mediated signalling is required for the up-regulation of muscle-specific gene expression in animal cap cell reaggregates previously exposed to the appropriate concentration of activin, just as it is at the same stage in normal development.

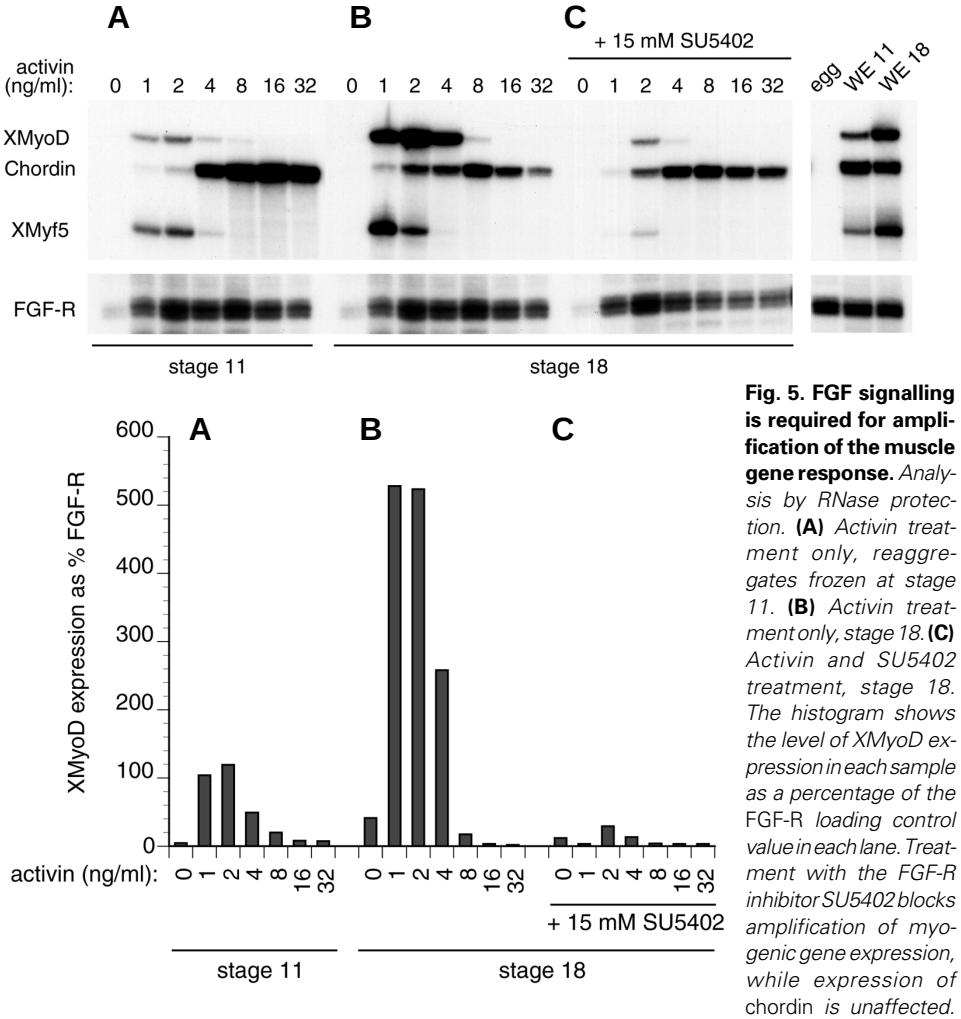

Fig. 5. FGF signalling is required for amplification of the muscle gene response. *Analysis by RNase protection.* **(A)** *Activin treatment only, reaggregates frozen at stage 11.* **(B)** *Activin treatment only, stage 18.* **(C)** *Activin and SU5402 treatment, stage 18. The histogram shows the level of XMyoD expression in each sample as a percentage of the* FGF-R *loading control value in each lane. Treatment with the FGF-R inhibitor SU5402 blocks amplification of myogenic gene expression, while expression of* chordin *is unaffected. As noted under Materials and Methods, the activin used for the results in this figure was from a different source compared to that used in Figs. 2-4. The absolute concentration (expressed here as ng/ml) is not the same as in Figs. 2-4 (expressed as pMolar), although the effects of changes in concentration are indeed the same. The different source of activin may also account for the less sharp demarcation seen in Fig. 5B compared to Fig. 2B.*

We note that the dorsally expressed gene *chordin* (Sasai *et al.*, 1994) also shows a dose-dependent response, reaching maximal levels after higher doses of activin than those at which *XMyoD* and *XMyf5* are induced (Fig. 5A). While induction of *chordin* requires FGF signalling (Mitchell and Sheets, 2001), we have previously observed that cell contact is not required for maintenance of *chordin* expression during gastrulation (Standley *et al.*, 2001). In confirmation of this, and in contrast to the myogenic genes, *chordin* expression levels here are unaffected by treatment with SU5402 (Fig. 5C); thus this inhibitor is not having a non-specific or toxic effect.

Discussion

Our results concern three aspects of cell differentiation, exemplified in the present work by muscle. These are (i) the progression from a broad expression of early zygotic genes across the whole mesoderm to expression of cell type-specific genes in only the muscle pathway, thereby sharpening the border between muscle and non-muscle; (ii) an increase in the intensity of muscle gene expression in the muscle lineage; and (iii) a uniformity of gene expression among all cells belonging to the muscle lineage. We show that uncommitted blastula cells can be made to undergo all these three steps by the operation of two signalling events. These two events are the interpretation of position in a signal factor concentration gradient, and a community effect within a group of adjacent cells that

have responded to a particular concentration of the first signal process.

Our results also contribute to an understanding of how a gradient of gene expression across a broad region of an embryo can be converted into adjacent demarcated areas of strong as opposed to ones of weak or nil expressions. The 10-fold amplification of *XMyoD* in cells experiencing a middle concentration of activin requires a community effect that is also responsible for the remarkably homogeneous *XMyoD* expression in strongly responding cells (Fig. 3). This process that is thought to sharpen the distinction between muscle and non-muscle derivatives of the mesoderm takes place during gastrula and neurula stages.

Previous work has described the concentration-dependent activation of mesodermal genes normally expressed in the equatorial cells of the blastula, and the progression of muscle progenitor cells in a gastrula to muscle differentiation through a community effect (Green *et al.*, 1992; Gurdon *et al.*, 1993; Green *et al.*, 1994; Gurdon *et al.*, 1994; Wilson and Melton, 1994; Standley *et al.*, 2001). The results described here extend previous work by showing that blastula cells of prospective epidermal or neural fate can be switched to a uniform muscle fate by the operation of two signalling processes, each involving a defined signal factor. These processes are likely to operate in the normal mesoderm-muscle pathway.

Much recent interest centres on the extent to which mouse embryonic stem (ES) cells can be induced to form uniform populations of one cell type (Donovan and Gearhart, 2001). ES cells differentiate best in the configuration of embryoid bodies; these contain a mixture of cell types and continue through unknown signals to generate cell diversity. ES cells in a monolayer or dispersed configuration do not differentiate well. Our work with amphibian blastula cells contributes to an understanding of processes that can be used to direct the differentiation of embryonic cells in culture.

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). Animal caps were dissected at stage 8.5 in 1X MBS. To disaggregate the cells, the caps 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, and the excess medium discarded, leaving a known volume (100 or 150 µl). Activin protein was then added at the desired concentration, and the tubes agitated gently over a period of 10 minutes, to ensure that the activin was evenly distributed. The cells were washed 3 times to remove unbound activin, the final wash being in normal (calcium-containing) 1X MBS, 0.1% BSA, 1 µg/ml gentamicin, to allow the cells to reaggregate for culture. Alternatively, after the third wash, the cells were dispersed into agarose lined dishes containing 1X MBS, 0.1% BSA, 1 µg/ml gentamicin, for culture as single cells. The activin protein used for Figs. 2, 3 and 4 had previously been prepared as described (Wilson and Melton, 1994), giving a stock concentration of 7 nMolar (Dyson and Gurdon, 1998). The activin protein used for Fig. 5 was purchased from R&D Systems (Cat. no. 338-AC). The tyrosine kinase inhibitor SU5402 was purchased from Calbiochem (Cat. no. 572630) and dissolved in DMSO. When reaggregates reached stage 11, the culture solution was replaced with fresh medium supplemented with 15 µM SU5402 for the remainder of the culture period. At the end of the culture period, the caps were frozen on dry ice for analysis by RNase protection, or fixed for 2 hours in MEMFA (Hemmati-Brivanlou *et al.*, 1990), followed by storage in methanol at -20°C, for subsequent analysis by *in situ* hybridisation.

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: *chordin* (Ryan *et al.*, 1996; EcoRI, T3 polymerase), *FGF-R* (Lemaire and Gurdon, 1994; BamHI, T7 polymerase), *N-tubulin* (K. Ryan, unpublished; EcoRI, T3 polymerase), *XMyf5* (Standley *et al.*, 2001; PmeI, T7 polymerase), *XMyoD* (Standley *et al.*, 2001; EcoRI, T7 polymerase), *XSox17β* (Standley *et al.*, 2001; BamHI, T7 polymerase). The *XMyoD* probe detects *XMyoDb* transcripts only, and therefore no signal is seen in maternal mRNA samples. Quantitation was carried out using a Fujifilm Phosphor Imager and MacBAS 2.5 software.

In Situ *Hybridisation*

MEMFA-fixed animal cap cell reaggregates and whole embryo controls were embedded in 98% Histoplast (Shandon): 2% beeswax and sectioned. The samples were de-waxed for 20 minutes in xylene and rehydrated through an ethanol series prior to analysis as described (Butler *et al.*, 2001). Plasmid templates were linearised, and digoxygenin-substituted antisense RNA probes were transcribed using a MEGA Script kit (Ambion) as follows: *XMyoD* (Hopwood *et al.*, 1989; BamHI, SP6 polymerase); *cardiac actin* (Mohun *et al.*, 1984; EcoRI, SP6 polymerase); *endodermin* (Sasai *et al.*, 1996; EcoRI, T7 polymerase).

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