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Original Article

Expression of the HNK-1 epitope is unaltered among early chick epiblast cells despite behavioral transformation by inducing factors *in vitro*

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ABSTRACT **Dissociated epiblast cells from pre-streak chick blastoderms have been exposed, in short-term culture on a fibronectin (FN) substratum, to recombinant mammalian activin and to mammalian basic fibroblast growth factor (bFGF). Such cultures have also been made on this substratum pre-conditioned by culture of a transfected cell line expressing the mammalian *Wnt-1* gene. The former two factors induce changes of FN adhesiveness and other behavior, such that the cultures after 6 h resemble cultures newly set up from the young primitive streak or substreak hypoblast region of similarly aged blastoderms that have developed onward across the intervening period (see Cooke and Wong, *Development 111:* 197-212, 1991 for related results). Over 95% of cells are potentially responsive, and with relatively high concentration of activin there is also production of nodular structures due to strong cell-cell adhesion, as in cultures from Hensen's node or the anterior streak. Pre-conditioning of the substratum by transfected *Wnt*-1-expressing cells does not appear specifically to alter behavior in such epiblast cultures, though these experimental cells, and not control-transfected ones, produce striking alterations of chick development in other experiments (Cooke *et al.*, in preparation). Up to 20% of cells in control cultures from central or 'marginal zone' pre-streak epiblast express the HNK-1 epitope, while up to 60% of cultured early streak cells cleaned of hypoblast do so. These figures tally with estimates from this laboratory for the *in vivo* incidence of such expressing cells in late pre-streak epiblast, and the maximum incidence within the early streak structure itself (cp. Canning and Stern, *Development 104:* 643-655, 1988). Despite the change in morphology caused by activin and bFGF, that closely mimics changes occurring during normal specification of early axial structures, the proportion of HNK-1-expressing cells is unaltered within any of these culture types. This suggests that expression of this epitope by cells is not a necessary concomitant of early phases of their induction into streak developmental pathways, either *in vivo* or experimentally in culture. The results are discussed in relation to published information on the experimental effect of *Wnt* on axis initiation in *Xenopus*, and to the results of grafting factor-producing transfected cells to whole blastoderms.**

KEY WORDS: *bird embryo, axis formation, gastrulation, activin, bFGF, wnt-1*

Introduction

There is now considerable circumstantial evidence of roles for activin and an FGF-like molecule in induction of mesoderm and initiation of axial pattern in amniote, specifically bird development (Mitrani *et al.*, 1990; Cooke and Wong 1991; Ziv *et al.*, 1992). This parallels the evidence for involvement of these molecules in the first inductive events of amphibian development (Slack *et al.*, 1987; Smith *et al.*, 1989; Cooke, 1991a,b; New *et al.*, 1991). At the same time, at a cellular level of description, the nature of primary induction is less clear in the bird blastoderm than it appears to be in the amphibian blastula. In the latter the interaction is principally an instructive one; an unknown number of signals, acting in parallel or in a cascade initiated from the yolky vegetal region, diversify the states of specification among cells of the animal hemisphere in a largely position-specific way. In birds, there is considerable evidence that the posterior 'marginal zone' epiblast and an adjoining region of the hypoblast play a comparable role in emitting signals that localize the site of axial pattern development (Eyal-Giladi, 1984), but other evidence that questions this can be found in Stern (1990). There is also a suggestion that selective aggregation of initially widespread but pre-specified cells, rather than localized

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instructive respecification, may underlie primitive streak formation. Stern and Canning (1990) propose that the initially scattered subpopulation of epiblast cells, which is distinguished by its progressive expression of cell surface-directed components bearing the HNK-1 epitope (Abo and Balch, 1981), gathers selectively so as to form the majority of the earliest streak structure. These cells then become distributed within most parts of the anterior axial system before the epitope is progressively lost (see also Canning and Stern, 1988).

The functional significance of the epitope expression is unclear, and evidence that the increased incidence of positivity at the streak site is due entirely to selective cell movement rather than position-specific initiation of expression in some new cells (i.e. induction) is not compelling (Canning and Stern, 1988). It is nevertheless reported that these HNK-1-positive cells are irreplaceable by any regulative process if immuno-ablated before streak formation, and that such blastoderms do not re-initiate axial development (Stern and Canning, 1990). A final point of interest is that as gastrulation (i.e. streak elongation and node regression) proceeds, this cell population appears to mingle in a largely 'pepper and salt' manner with non-expressing cells that are recruited into the streak structure (and hence into mesoderm). If expression of the epitope indeed defines a population with a specific competence in pattern formation, this intermingling with others to participate in single anatomical structures is a most unusual phenomenon within animal development, worth intensive further study.

The altered behavior of disaggregated cells from early epiblast in response to members of the TGFß and FGF families of peptide growth factors provides part of the evidence that streak induction does involve instructive respecification of cells, and that some of the same signals are effective in bird as in amphibian development. Such treated epiblast cells come to resemble behaviorally those from newly disaggregated young streak or substreak hypoblasts. Since both these latter structures show a high incidence of HNK-1 positivity *in vivo* (-100% in the case of hypoblast), whereas incidence in epiblast is low and scattered, it becomes relevant to ask whether treatment with the effective factors *in vitro* leads to increased incidence of HNK-1 expression.

In the present work I use a modification of a previous microculture system (Cooke and Wong, 1991). I find that when epiblast cells are disaggregated and exposed directly to activin, bFGF or both factors at stage XII (Eyal-Giladi and Kochav, 1976), the latest pre-streak stage at which they will make a full response in the assay, the massive increase in spreading on FN and (with activin) tight mutual adhesiveness begins after 4 h and is complete within 6-8 h. This is in good agreement with the time to initiation and elongation up to stage 3 (Hamburger and Hamilton, 1951) of the primitive streak, observed when such blastoderms are incubated whole in New culture (New, 1955). But no increased incidence of HNK-1 positivity occurs in these cultures, and no correlation exists between the transformation of adhesive behavior and HNK-1 positivity within individual cells.

Wnt-1 (a.k.a. the murine proto-oncogene *Int-1*) and other *Wnt* family members (including their homologue the *Drosophila* gene wingless) are able to initiate new axial development in the amphibian embryo among cells adjacent to those that have been experimentally injected with the messenger RNAs; that is, they act as a secreted signal. The *Wnt* signal may not by itself be an effective inducer of the axial state, acting instead to modulate or 'axialise' induced states initiated by other signals (Smith and Harland, 1991). Special interest attaches to proteins of this family as potential components of a system generating spatial pattern, because they seem to be secreted into particularly intimate association with extracellular matrix rather than being freely diffusible (Jue *et al.*, 1992). *Wnt-1*-secreting cell grafts share with activin-secreting ones the capacity to re-situate streak formation in whole blastoderms (Cooke *et al.*, in preparation). Thus, whether or not this signal family turns out to be utilized in the *in vivo* mechanism, it appears also to activate relevant intracellular response pathways in avian blastoderm cells.

The interest of testing exposure to *Wnt* protein for induction of HNK-1 positivity derives from the observation that in amphibians, axial development experimentally triggered by *Wnt* is distinguished from that due to activin signalling alone by its regular inclusion of the anteriormost levels of body pattern (Smith and Harland, 1991; Sokol *et al.*, 1991; Chakrabarti *et al.*, 1992; G. Guex and J. Cooke, unpublished observations). In chick early development, HNK-1-bearing streak cells would appear to be distinctively concentrated to anteriorly fated parts of the axial system. I report here that *Wnt* -transfected cells, when cultured to pre-condition the fibronectin substratum for microcultures, exert no significant inductive effect on epiblast cell behavior in themselves. Nor do they potentiate such behavioral effects caused by subsequent incubation with the soluble inducing proteins. Whether used alone or in combination with the subsequent addition of activin and bFGF, such substratum pre-conditioning is also without effect on incidence of HNK-1 expression in microcultures, despite the fact that these same *Wnt*-transfected cells are indeed active as 'axialisers' when localized grafts are made into whole blastoderms (Cooke *et al.*, in preparation). The significance of these observations is discussed in relation to the possible roles *in vivo* of 'activin' and *'Wnt'* signalling pathways, and of HNK-1-positive blastoderm cells.

Results

Twelve experiments were performed in all, each using separate suspensions of central and of lateral 'marginal zone' epiblast, pooled from 8-10 stage-matched blastoderms that were at pre-streak stages (Eyal-Giladi and Kochav, 1976) between XI and XIII+ in different experiments. Lateral marginal epiblast, adjacent to that lying immediately behind (i.e. peripheral to) Koller's sickle (see Eyal-Giladi *et al.*, 1992 for recent anatomical terminology), was studied separately in view of proposals by several workers that peripheral epiblast may play a special role in streak formation, or even that large parts of the early streak may fate-map to this location. It was reasoned that since new streaks can be induced by appropriate marginal grafting operations on blastoderms of these stages (Khaner and Eyal-Giladi, 1989; Cooke *et al.*, in preparation), a wide sector of this annulus of epiblast might already be in a primed or sensitized point along any pathway of induction into the axial (streak) state, both as regards behavioral transformation and possible initiation of HNK-1 expression. In fact, such epiblast proved indistinguishable from the general central region in its range of incidences of HNK-1 positivity and of spontaneous spreading on FN in culture, as well as in responsiveness to factors, and so will not further be considered separately.

Use of a range of precise blastoderm stages confirmed the finding using a different culture procedure (Cooke and Wong, 1991), that full response to either activin or bFGF (>90% cells transformed) was only obtained when exposure began by stage XII, with percent-

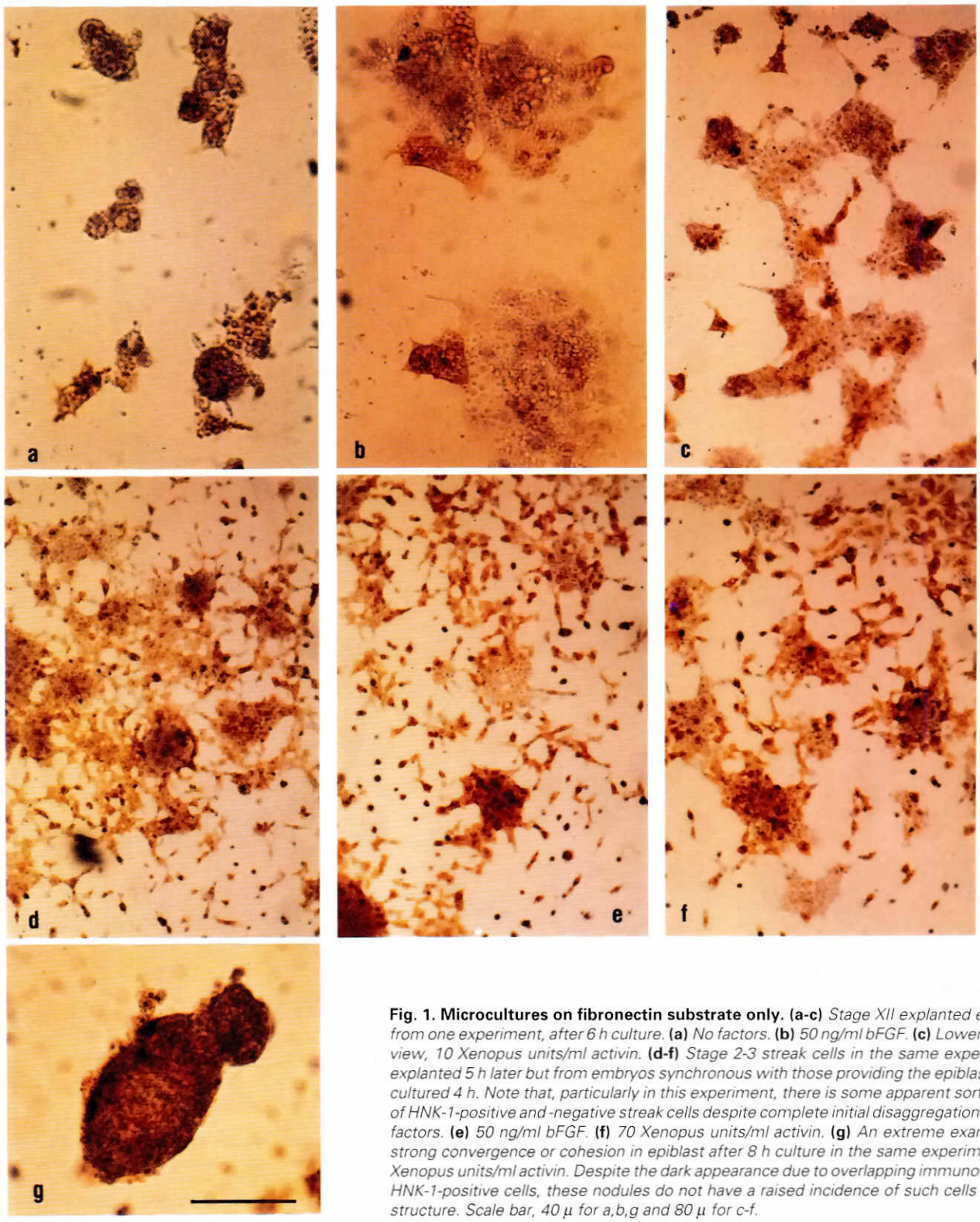

Fig. 1. Microcultures on fibronectin substrate only. (a-c) *Stage XII explanted epiblast from one experiment, after 6 h culture.* **(a)** *No factors.* **(b)** *50 ng/ml bFGF.* **(c)** *Lower power view, 10 Xenopus units/ml activin.* **(d-f)** *Stage 2-3 streak cells in the same experiment, explanted 5 h later but from embryos synchronous with those providing the epiblast, then cultured 4 h. Note that, particularly in this experiment, there is some apparent sorting out of HNK-1-positive and -negative streak cells despite complete initial disaggregation.* **(d)** *No factors.* **(e)** *50 ng/ml bFGF.* **(f)** *70 Xenopus units/ml activin.* **(g)** *An extreme example of strong convergence or cohesion in epiblast after 8 h culture in the same experiment, 70 Xenopus units/ml activin. Despite the dark appearance due to overlapping immunostained HNK-1-positive cells, these nodules do not have a raised incidence of such cells in their structure. Scale bar, 40 μ for a,b,g and 80 μ for c-f.*

age of cells responsive to bFGF falling rapidly, and that to activin more slowly, during XIII. Results of three typical experiments are given in Table 1. In one experiment only, epiblast from just pre-streak blastoderms (XIII-XIV) made a significant (40%) spreading response to activin but no appreciable one to bFGF (data not shown).

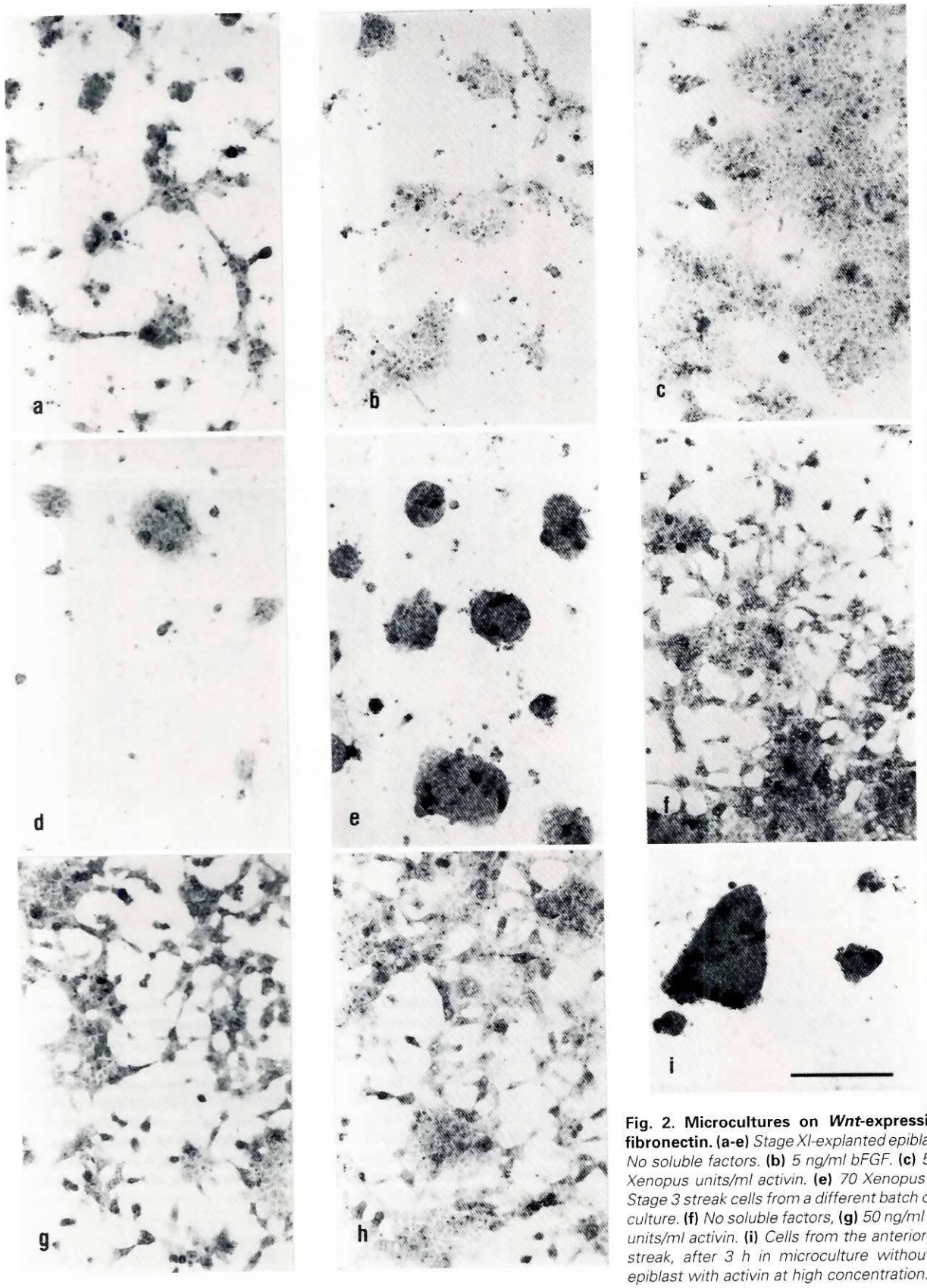

Fig. 2. Microcultures on *Wnt*-expressing cell-conditioned fibronectin. (a-e) *Stage XI-explanted epiblast after 8 h culture.* **(a)** *No soluble factors.* **(b)** *5 ng/ml bFGF.* **(c)** *50 ng/ml bFGF.* **(d)** *10 Xenopus units/ml activin.* **(e)** *70 Xenopus units/ml activin.* **(f-h)** *Stage 3 streak cells from a different batch of embryos, 4 h total in culture.* **(f)** *No soluble factors,* **(g)** *50 ng/ml bFGF.* **(h)** *70 Xenopus units/ml activin.* **(i)** *Cells from the anterior region of the stage 4 streak, after 3 h in microculture without factors. cp Fig. 1g, epiblast with activin at high concentration. Scale bar, 40 μ for a, and e-i, 80 μ for b-d.*

TABLE 1

TIMECOURSE AND FINAL % INCIDENCE OF THE SPREADING RESPONSE ON FN, FOR EPIBLAST MICRO-CULTURED WITH ACTIVIN AND BFGF

	2 h	3 h	4 h	6 h	8 h
Expt.1					
Stage XI					
Control	<5%	<5%	<5%	8%	6%
Activin					
10 X.u./ml	<5%	7%	73%	97%	74%
Expt. 2					
Stage XII					
Control	<5%	<5%	7%	10%	8%
Activin					
10 X.u./ml	<5%	17%	65%	93%	97%
bFGF					
50 mg/ml	<5%	11%	53%	95%	98%
Expt. 3					
Stage XIII					
Control	<5%	<5%	<5%	<5%	<5%
Activin					
10 X.u./ml	<5%	6%	24%	82%	70%
bFGF					
50 mg/ml	<5%	<5%	10%	36%	32%

*Time in culture (% spread cells scored in two replicate coverslip cultures - 3-400 cells - per time point).

Figs. 1a-c,g and 2a-e show typical appearances in epiblast microcultures from two experiments, photographed 6 or 8 h after setting up on the FN substratum in control medium and in the presence of activin or of bFGF. Cells in control cultures remained loosely mutually adherent in groups, and mostly only loosely adherent to substratum if contacting it, producing at most a slight 'skirt' of filopodia or lamellipodia. bFGF was used at 5 and 50 ng/ml and produced cell spreading. Only the proportion of responding cells, rather than the morphology produced, varied with concentration. Activin (bovine recombinant purified from culture supernatant) was used at concentrations of 10 and 70 *Xenopus* units (Cooke *et al.*, 1987), corresponding to ca. 1-7 ng, per ml. Individual cell spreading only was seen at the lower concentration. The spreading morphology produced by both factors strongly resembled that seen in freshly cultured early mesenchymal streak from blastoderms a few hours older. At the higher activin concentrations, a variable proportion of cells adopted an extreme mutual adhesiveness as well as a propensity to flatten on FN substratum if contacting it. This resulted in production of compact, smooth-surfaced balls of tissue, often attached to substratum *via* a fringe of flattened cells and lamellipodia. This appearance is extremely reminiscent of that spontaneously adopted by older (stage IV) streak cells from the node region when placed in these microcultures (see Fig. 2i). Paradoxically, however, it is adopted by epiblast cells under influence of activin at younger ages (say, pre-streak st. XI+8 h) than that from which anterior streak cells from cultured whole blastoderms will spontaneously display it. Stage IV is reached only ca. 16 h after stage XI in such blastoderms. When the factors are used together, the cells adopt the behavior seen in response to the activin alone in any one experiment. Inspection of cultures shows that significant numbers of cells have begun altered behavior by 3.5-4 h after introduction of factors but not before, and that spread adhesion is maximal by 6-8 h (Table 1). 'Stage IV node' morphology was not seen from epiblast before 6 h in culture and was strongest at 8 h.

The spontaneous incidence of HNK-1 positivity in epiblast cultures varied between 12 and 22% in different experiments, and was stable across the entire culture period as monitored by fixing and processing replicate control cultures at different times during three of the experiments. This incidence, and the intensity of HRP immunostaining was comparable to that seen within epiblasts of whole blastoderms processed from the egg or after brief culture with the 'ring' technique of New (1955). Positivity was 'all or none' in individual cells even though antigen was sometimes on the entire surface and sometimes largely within a juxtanuclear structure (presumably the Golgi apparatus). Only in some of the experiments with earliest-dissected central epiblast, where clean elimination of the population of still emerging 'polyinvaginating' hypoblast had been impossible, was the baseline elevated by presence of a few percent of these notably large and yolky appearing, very darkly immunostaining cells. Their appearance was sufficiently distinct from that of epiblast cells, both HNK-positive and -negative, for unambiguous scoring of HNK-1 incidence within the latter cell type. This was done by counting in six random, x25 microscope fields from each of three replicate culture coverslips for each treatment, in the six experiments where a maximal behavioral response was obtained using st. XI or XII epiblast. Three of these experiments included, as a variable, the use of simple FN coating and 'blocking' as a substratum in comparison with additional preconditioning by overnight culture of '*Wnt*-transfected' cells or their control cell line (see Materials and Methods).

Table 2 gives the results from an experiment of each of these types, involving the scoring of 800-1000 cells per treatment. In neither these nor the other experiments was any significant alteration in incidence of HNK-1 positivity associated with any experimental treatment. In other experiments where an appreciable but incomplete response to factors was seen, scoring revealed no

TABLE 2

% HNK-1 EXPRESSION IN EPIBLAST CULTURES

	4 h culture	8 h culture
Expt. 1		
Control	22.0%	21.5%
bFGF	24.5%	23.0%
Activin	21.5%	19.5%
Expt. 2		
Control (FN only)	14.5%	12.5%
+ pre-conditioning with control J558 cells	16.5%	13.0%
+ pre-conditioning with *'wnt*-transfected' J558 cells	15.0%	13.5%
FN with soluble factors activin + bFGF only	14.0%	15.5%
FN with soluble factors + *'wnt'* pre-conditioning	12.0%	15.0%

No within-experiment difference between a treatment and its control reached significance at the p= 0.05 level.

TABLE 3

% HNK-1 EXPRESSION IN CULTURES OF EARLY MESENCHYMAL STREAK CELLS

	1 h culture	3 h culture
Expt. 1		
Control	74.0%	61.5%
+ bFGF	76.5%	61.0%
+ Activin	78.0%	62.5%
Expt. 2		
Control	49.5%	41.5%
+ bFGF	50.5%	42.0%
+ Activin	48.0%	40.0%

No within-experiment differences of a treatment from controls were seen at either time point, but all differences between the 1 h and 3 h cultures were significant at the p= 0.01 level.

different incidences of HNK-positivity among behaviorally transformed *versus* unresponsive cells (data not shown).

During three of the experiments, the already mesenchymal cells from several pooled stage 2-3 streaks were disaggregated by brief EDTA treatment which allowed separation from the still epithelial epiblast. These were used to set up control microcultures and ones with either activin or bFGF at the high concentrations. These cultures, when examined an hour later — i.e. at the final scoring of the epiblast ones that had previously been set up from the same batch of incubated eggs, already showed essentially 100% cell spreading. Replicates from each treatment were then fixed either immediately, or after incubation for a further 3 h, before processing for HNK-1 immunoreactivity. Figs. 1d-f and 2f-h show regions from such cultures and Table 3, the positivity scores in the two experiments involving the highest and the lowest incidence of streak HNK-1. Although enhanced relative to that in epiblast generally, incidence of HNK-1 expression is by no means 100% among such mesenchymal streak cells, and varies widely between egg batches (as it does for the intact epiblast and early streak *in vivo*, J. Cooke, unpublished observations). There appears to be a slight decline over the culture period (overall mean; 65% positive 1 h after explantation, 52% positive 3 h later); a progression in line with what is reported during streak development *in vivo* (Canning and Stern, 1988). It can be seen that there is some evidence of selective aggregation or grouping of the flattened cells according to HNK-1 status, even though initial disaggregation had been complete. But once again, no appreciable effect of factor treatment upon incidence of positivity is seen, and there is no correlation between HNK-1 status and adhesive behavior among individual cells.

Discussion

The appreciably earlier loss of responsiveness by epiblast cells to the behavioral transforming effects of bFGF, in relation to those of activin, is in line with findings for these two classes of inducer using various *in vitro* assays for the mesodermal cell state in the amphibian system. Such loss of 'competence' to respond, some time before the earliest stage at which cells can first manifest the behavior change, and the subsequent time-course of that change that parallels the normal course of streak initiation in synchronous whole blastoderms, are additional evidence that the cellular response pathways being triggered are indeed those that bring about normal development (Cooke and Smith, 1990). This does imply that the streak is founded by genuinely instructive, localized inductive signals (Azar and Eyal-Giladi, 1979, 1981; Khaner and Eyal-Giladi, 1989), rather than only by selective relocation of cells pre-specified by some other mechanism (Stern and Canning, 1990). It remains uncertain, however, whether any of the precise *in vivo* inductive signals have been identified. The current state of the large body of amphibian evidence (Cooke, 1991a,b; New *et al.*, 1991; Hemmati-Brivanlou and Melton, 1992; Thompson and Slack, 1992) and the small but striking body of chick evidence (Mitrani and Shimoni, 1990; Mitrani *et al.*, 1990; Cooke and Wong, 1991; Ziv *et al.*, 1992), makes it increasingly likely that activin itself is involved in induction and in axial patterning of the embryo body, but perhaps less likely that an FGF (rather than some related factor) is part of the normal mechanism. It is of interest that strong convergent or cohesive behavior, indistinguishable from that of the anteriormost streak region explanted at stage IV, is attainable earlier by means of activin alone in culture than it is attained in the normal course of development. The effective concentration of the positive inducing signal *in vivo* is of course unknown, and in addition, modulatory or down-regulating signal components that must be part of the mechanism leading to stable patterning *in vivo* may affect timing of responses (Khaner and Eyal-Giladi, 1989; Cooke, 1991a).

While the functional significance of the HNK-1 epitope remains obscure, it is clear that at least the anteriormost part of the early streak is highly enriched with cells that express it. Canning and Stern (1988) suggest that most or all cells pre-designated for anterior axial meso-endodermal fates go through an HNK-1-positive phase that lasts while they are being selectively attracted to the streak site from an initially wide, near-random distribution in epiblast (see also Stern and Canning, 1990). Arrival in the mesenchymal part of the early streak by cells of epiblastic origin must involve their locomotory/adhesive transformation. This could occur either in individual cells from over a wide area followed by their selective aggregation, or more locally on a massive scale, as the visible ingression process that forms the later streak. It is hard to imagine, by contrast, selective migration of a scattered subpopulation (i.e. of HNK-1-positive cells) *within* the epithelial structure of the epiblast itself. Yet the above authors describe a progressively enhanced density, and individual intensity, of HNK-1 expression among cells in epiblast overlying the initial streak site as development advances. It seems likely that HNK-1 expression must be switched on inductively in epiblast-derived cells, by exposure to at least one of the *in vivo* inductive sequences that compose the normal axial pattern. However as noted in this study, and confirmed in related work on whole blastoderms (Cooke *et al.*, in preparation), other axial induced states, found in the early and later streak, do *not* involve a phase of HNK-1 expression. It is likely that those cells that have not been through an HNK-1 positive phase compose progressively more of the axis as more posterior levels of body pattern are laid down by the streak (Canning and Stern, 1988).

None of the *in vitro* regimes used in the present work induces from epiblast the *de novo* HNK-1-expressing state seen in anteriormost streak *in vivo*, or even acts to maintain or enhance HNK-1 incidence among explanted early streak cells. These regimes do, however, cause morphological or behavioral transformation identical to that undergone by many early streak cells during their recruitment from epiblast. A conclusion must be that other components of the total signalling situation, encountered in the

anteriormost streak region *in vivo*, are not replicated in any of these fibronectin substratum microcultures. Pre-conditioning of substrate by cells bearing a *Wnt-1*-expressing construct was included in this study because in contrast to those secreting activin alone, these cells do bring about induction of epiblast-derived HNK-1 positivity when grafted into whole blastoderms (Cooke *et al.*, in preparation). This indicates that these cells, which express *Wnt-1* RNA (A. McMahon, personal communication), do in fact synthesize the protein, and that its presence at an early stage triggers an equivalent step in both bird and *Xenopus* development. That step in *Xenopus* enables the formation of the anterior extreme of the axial sequence, which is seldom specified by activin alone (Smith and Harland, 1991; Sokol *et al.*, 1991). In bird embryogenesis, it is distinctively associated with *de novo* HNK-1 expression. The fact that this inductive step does not occur in the present cultures may again be due to requirements for yet other signalling components that are not present in them, or to inadequate secretion or presentation of the *Wnt* protein in the simple culture conditions.

These results must call into question the significance of the scattered endogenous HNK-1-positive epiblast cells from early stages, as opposed to those that seem to be induced subsequently in the streak-forming region (Cooke *et al.*, in preparation). These provide the background incidence of positivity in the present epiblast cultures, and show no differential competence to induction of behavioral change on treatment with factors. In no case has a clear functional correlation of the epitope expression with differential aspects of cell behavior been established.

Materials and Methods

Preparation of FN substratum and conditioning with wnt-transfected cell culture

13 mm coverslips were covered with 0.15 ml each of Hank's BSS (0.1 mM Ca^{++}), pH 7.3, containing 25 μg/ml of bovine fibronectin (FN-), and left in a humidified chamber in the cold overnight or at room temperature for 3 h. After brief rinsing in PBS they were 'blocked' by covering with Hank's BSS+10 mg/ml bovine serum albumen (BSA fraction V, Miles) for 2 h at room temperature. Following this treatment adhesion to FN is the only available mechanism of strong cell attachment and spreading. For tests of the soluble peptides activin and bFGF, cells were cultured directly on such coverslips, but possible effects of the *Wnt* protein, which is believed to be secreted into immediate association with extracellular matrix components, were tested by preconditioning coverslips with cultured transfected cells. A mixture of 1:4 RAT-1 cells: J558 myeloma cells transfected with a *Wnt-1*-expressing construct (courtesy of A. McMahon and S. Takada, Roche Institute, New Jersey, USA), or a similar mix where the J558 cells were transfected with a control construct, were allowed to settle and cultured overnight in 0.2 ml of Liebovitz medium with added glutamine +10% fetal calf serum. Density was adjusted to give a semiconfluent layer of the fibroblastic RAT-1 cells, interspersed and overlain with the more numerous, smaller non-adherent cell type. Such coverslips were washed for 3 min in glass-distilled water to lyse all cells, followed by Hank's BSS, followed by 0.2 ml of the same culture medium with immediate culture of the the chick blastoderm cells.

Micro-culture of chick blastoderm cells

Central epiblast was cut from pre-streak stage blastoderms that had been carefully cleaned of hypoblast in Hank's BSS (0.1 mM Ca^{++}), pH 7.2, containing 5 mg/ml bovine serum albumen (BSA Fraction V, Miles). Epiblast was pooled from sets of 8-12 blastoderms of closely similar stages and randomized by cutting into small scraps, followed by incubation for 15 min at 37.5°C in the same solution after addition of 2 mM EDTA. This tissue was then transferred in a 10 μl Finnpipette tip to 100 μl of Liebovitz medium, and the even suspension of single cells and small clumps produced by repeated passage through this tip immediately distributed to replicate coverslip cultures (0.2 ml of Liebovitz medium with added glutamine +10% fetal calf serum, in humidified air at 37.5°C). Recombinant human ßA activin and bovine bFGF were added as 50x concentrates in medium, and mixed in before cells settled. Incubation was for 6-8 h, by which time early streaks had formed in blastoderms whose re-incubation had started synchronously with the start of epiblast microculture. Primitive streak cultures were made in the above way but set up only 1 h before termination of the experimental epiblast cultures. Thus all cultures compared on each experimental occasion were of the same developmental age.

Immunocytochemistry

Cultures were washed for 10 seconds in warm PBS (1 mM Ca^{++}) to remove free protein, then fixed overnight in the cold in Bouin's fluid, followed by storage in 70% ethanol for 24 h in the cold (until colorless). Rehydration to PBS was via steps of 10 min and 20% concentration, followed by blocking for 1 h at room temperature in 5% normal goat serum in PBT (PBS+0.1% BSA and 0.05% Tween 80). Coverslips were then incubated with gentle rocking at room temperature in humidified air in the following series (0.15 ml/coverslip): anti HNK-1 mouse monoclonal supernatant diluted 1:3 with PBT 2 h, 4x PBT 30 min each, goat anti-mouse IgM (Sigma) 1:250 in PBT 2 h, 4x PBT 30 min each, 0.5 mg/ml tris buffered Diaminobenzidine (DAB)+0.3% H_2O_2 10 min precisely, followed by washing in excess PBS to stop the color reaction. Coverslips were then washed briefly in distilled water, stained for 20 seconds in Carazzi Haematoxylin, washed again in distilled water followed by tap water, dehydrated in an ethanol series, cleared in histoclear and mounted in XAM.

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