Almost all of the studies to date in which growth factors have been assessed for their ability to induce mesoderm from ectoderm have been done in the frog, *Xenopus laevis*, using the 'animal cap assay' developed by Nieuwkoop (reviewed in Refs 1, 2). In this assay, a piece of ectoderm is cut out from a blastula-stage embryo and either combined with vegetal tissue, as Nieuwkoop did, or incubated with soluble growth factors. Nanogram per ml concentrations of activin, TGF-β_2 and the FGFs can all cause some degree of mesodermal differentiation from animal caps, as assessed by morphology, by use of antibodies recognizing different cell types, or by RNase protection using probes such as cardiac muscle actin or the *T* gene (*Brachyury*)[2,3]. FGFs can cause differentiation of endothelium and muscle; activin, in addition, can generate notochord. These factors act in a concentration-dependent manner, low concentrations giving more 'ventral' mesodermal cell types (endothelium, 'mesenchyme'), higher concentrations producing 'intermediate', muscle differentiation, and the highest concentrations of activin giving 'dorsal' or 'axial' mesodermal differentiation (notochord). Activin can also induce the expression of the *goosecoid* gene[4], which is a marker for cells in the 'organizer' region of the embryo[5]. A combination of concentration gradients and multiple response thresholds is thought to specify dorsoventral pattern and the distribution of mesoderm in the early embryo[2]. More recently, it was found that injection of *goosecoid* mRNA[5] or of proto-oncogenes of the *wnt* family[6,7] can generate an ectopic axis in *Xenopus*.

The hypoblast and induction in the chick

The idea that the mesoderm of the chick embryo is induced from the epiblast (ectoderm) comes from Waddington's experiments[8]: if the hypoblast (which only gives rise to extraembryonic tissues and may be equivalent in some ways to the vegetal pole of frogs; Fig. 1) is rotated by 180° about its anteroposterior axis, the primitive streak forms at a site diametrically opposite to its original presumptive site. This experiment, along with more recent ones[9–11], led to the suggestion that the hypoblast is the inducer of mesoderm in the chick. But although the primitive streak is the source of mesoderm, it is not clear from this experiment that formation of a primitive streak is the same as 'mesoderm induction'.

In the chick, it is not yet possible to demonstrate mesoderm induction by growth factors in isolated chick epiblast explants, either by antibodies or by RNase protection, because cell type-specific markers are not available[12]. In the presence of a hypoblast (or of activin-containing medium; see below), the centre of the chick epiblast develops into an embryoid containing notochord and somites[13,14]. Moreover, when the epiblast is cultured alone, some mesoderm develops[14,15], suggesting that some of the mesoderm must be specified before the stage at which the epiblast is isolated (XII–XIII[16]; Fig. 1), which is the same stage at which the hypoblast can be rotated to reverse the axis of the embryo.

The mesodermal cells that differentiate in the absence of a hypoblast appear to be 'ventral' (blood,

Mesoderm induction and development of the embryonic axis in amniotes

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The mechanisms controlling the formation of the embryonic axis, and specifically those that give rise to the mesoderm, have received renewed attention recently. In the frog, some of these mechanisms have begun to be elucidated, and several factors have been found to cause uncommitted ectoderm cells to differentiate into mesoderm. All of the factors identified to date are related either to fibroblast growth factor (FGF) or to transforming growth factor β (TGF-β). Do the mechanisms that generate the embryonic axis of amphibians also operate in chick and mouse embryos? Here I address how amphibian and amniote embryos might provide complementary pieces of a puzzle.

mesenchyme, muscle). Since the hypoblast allows notochord and somites to form, it has been suggested that the hypoblast induces axial ('dorsal') mesoderm[14]. However, addition of a hypoblast allows the formation of an embryonic axis, and the tissues of the embryoids formed are organized into recognizable axial structures (notochord and somites). Therefore, the possibility cannot be excluded that the role of the hypoblast is to allow the formation of a primitive streak, which is required to organize, but not necessarily to induce, the mesoderm of the embryo. To resolve this problem requires further knowledge about the organization of the epiblast.

How complex is the epiblast?

Studies with the antibody HNK-1, which recognizes the earliest mesoderm cells in the chick embryo[17,18], showed that before mesoderm appears (stages XII–XIII[16]), the epiblast contains a random, salt-and-pepper pattern of HNK-1-positive cells. If these cells are labelled using HNK-1 directly coupled to gold, all the gold-labelled cells appear in tissues derived from the primitive streak (mesoderm and gut endoderm); if these cells are ablated using HNK-1 and complement, no mesoderm forms[18]. However, not all the mesoderm is derived from these early HNK-1 precursors. After the start of primitive streak formation, more epiblast cells, which were never HNK-1-positive, are recruited into it[18,19] (Fig. 2).

Despite the salt-and-pepper distribution of early mesoderm precursors, maps can be constructed depicting the fates of cells in different regions of the embryo, because not all of the mesoderm is derived from HNK-1-positive cells and because HNK-1-positive cells contribute to many different mesodermal cell types[18,19]. The dorsoventral character of the mesoderm derived from HNK-1-positive cells therefore depends on the position at which these cells find themselves, and therefore on cell interactions occurring relatively
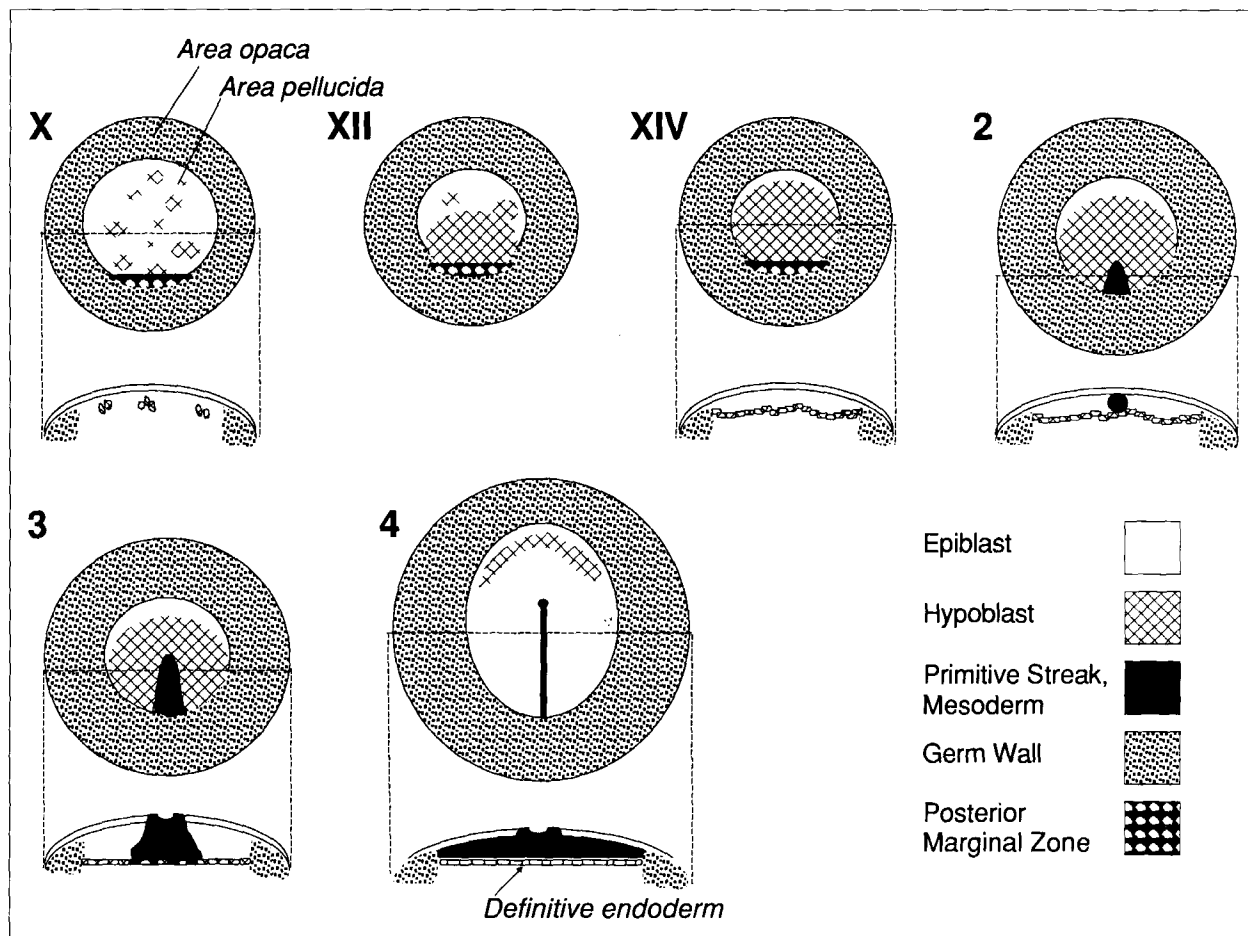
FIG 1

Diagrammatic views of chick embryos around the time of gastrulation. Two staging systems are used: Eyal-Giladi and Kochav's[16], in Roman numbers, for pre-primitive-streak stages, and Hamburger and Hamilton's[26], in Arabic numerals, starting at stage 2 with the appearance of the primitive streak. Each diagram shows the embryo viewed from its dorsal (epiblast) side, with the posterior end facing downwards. Some transverse sections through the embryos are also shown. At stages XIII–XIV, the embryo is a disc with two layers of cells: the epiblast (facing the egg white) and hypoblast (facing the yolk). The epiblast generates all the embryonic tissues and some extraembryonic ones (e.g. amnion), and the hypoblast gives rise only to extraembryonic structures (e.g. yolk sac stalk). This stage is equivalent to the amphibian blastula. Gastrulation (chick stages XIV–4) generates a third layer of cells, situated between the other two, which gives rise both to the mesoderm and to the definitive (gut) endoderm; both of these new tissues are derived from the epiblast. In stages 3 and 4, the mesoderm present beneath the epiblast is shown in the transverse section only.

late in development, after the diversification of HNK-1-positive and -negative cells. Thus, studies such as those of Lawson and collaborators[20], who showed that single cells in the mouse epiblast can contribute progeny to more than one germ layer, do not conflict with those obtained in the chick.

Fate maps and the timing of mesoderm induction in amniotes

Despite differences in overall shape and yolk content, amphibian embryos are not all that different from those of amniotes. Each region of one class of embryo can be traced to an equivalent region of the other class, although some tissues, notably some of the extraembryonic ones, are unique to amniotes. Figure 3 summarizes some homologies between amphibian, chick and mouse embryos.

In the frog late blastula, the region giving rise to dorsal structures is found near the dorsal marginal zone of the embryo, close to where the blastopore will appear shortly afterwards. Where is this region in

amniote embryos? Fate maps of the chick[21–25] and mouse[20] place it quite far from the posterior margin, where the primitive streak arises. But then it is difficult to visualize a gradient of an instructive, mesoderm-inducing factor that might generate such an arrangement at this stage. The concentration of such a factor should be highest at the point where the most axial (dorsal) mesodermal cells arise, and therefore near the middle of the chick blastoderm or apex of the mouse blastocyst (Fig. 3).

Examination of the fate maps of earlier embryos may help us to identify the time at which induction occurs and the source of the factor(s). Before the hypoblast is fully formed (stages X–XI[16]), the region of the chick embryo destined to form the notochord is located close to the posterior marginal zone. In the following few hours, massive cell movements take place, displacing the future notochord cells to the centre of the embryo (Fig. 3; Refs 22, 23; Y. Hatada and C.D. Stern, unpublished) even before the primitive streak appears. This suggests that induction of
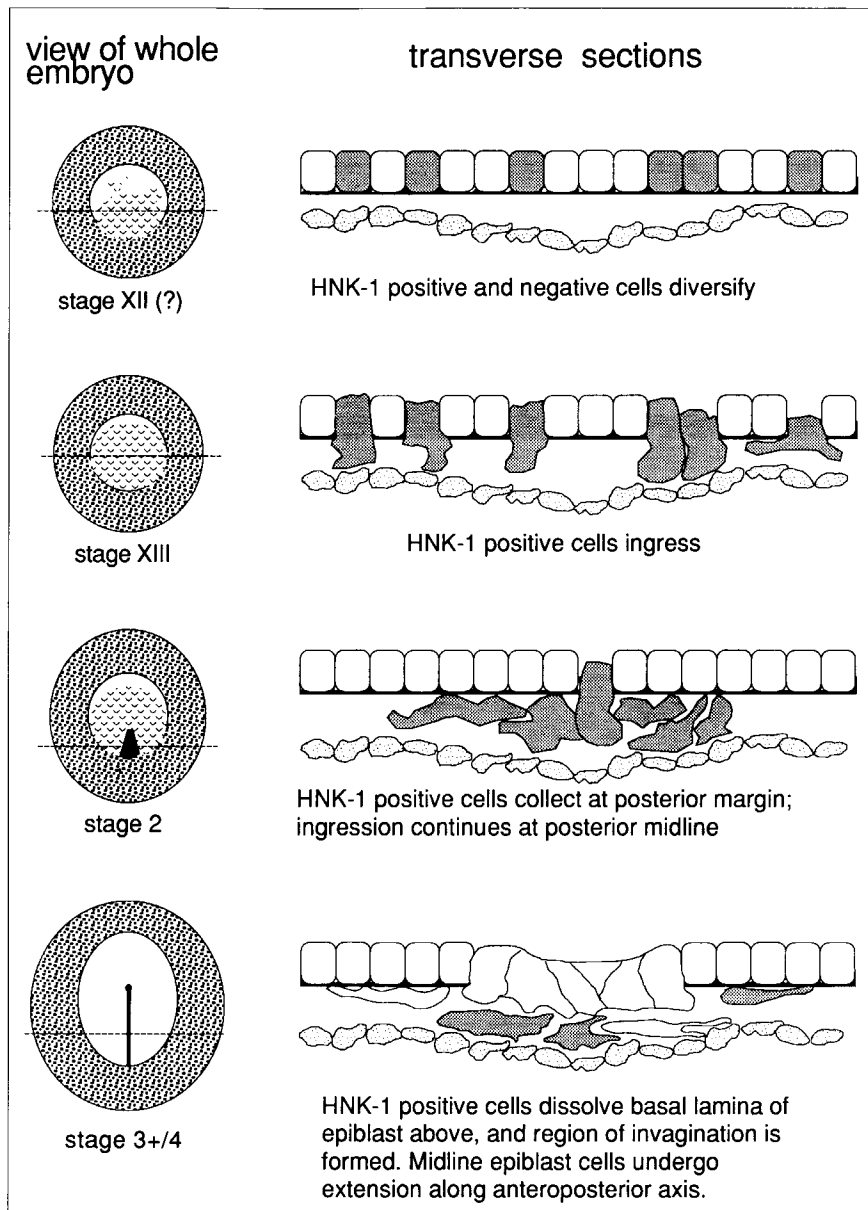
view of whole embryo

transverse sections

stage XII (?)

HNK-1 positive and negative cells diversify

stage XIII

HNK-1 positive cells ingress

stage 2

HNK-1 positive cells collect at posterior margin; ingression continues at posterior midline

stage 3+/4

HNK-1 positive cells dissolve basal lamina of epiblast above, and region of invagination is formed. Midline epiblast cells undergo extension along anteroposterior axis.

FIG **2**

A view of some of the events that might be involved in the early stages of primitive streak formation in the chick embryo. The overall appearance of the embryo at each of four developmental stages is shown on the left, and a transverse section through each stage is shown on the right. The primitive streak appears at stage 2 (Ref. 26), as mesodermal cells accumulate at the posterior end of the embryo.

FGF declines rapidly at the beginning of gastrulation. It also argues, once again, that the hypoblast cannot be the sole source of inducing factors in the chick, because by the late primitive streak stage it has moved out of the embryonic region (Fig. 1).

Induction of organizer cells

There is an alternative interpretation for the above results. In amphibians, the highest concentrations of activin cause treated animal cap cells to acquire inducing ability themselves[27]. According to the three-signal model[28] (Fig. 4) such 'organizer cells' should be able to pattern the mesoderm to generate a range of dorsoventral cell types. It is therefore possible that, in the chick, the induction of cells with 'organizer' properties takes place early in development, when these cells are close to the posterior marginal zone, which could be the source of the appropriate inducing factor. During gastrulation, the organizer cells could induce neighbouring epiblast cells to become mesoderm as the latter ingress into the embryo. These organizer cells are the same cells that are found in Hensen's node[19,25]. This idea also explains why Hensen's node can induce a second axis when grafted.

In conclusion, the specification of some cells as axial/dorsal (perhaps organizer cells) may begin before the hypoblast is fully formed, at about stage X–XI[16]. Other cells continue to become specified as axial/dorsal mesoderm at least as late as the beginning of neurulation [stage 5 (Ref. 26)], perhaps under the influence of the organizer cells. This late-forming mesoderm cannot be induced directly by the hypoblast.

Effects and expression of mesoderm-inducing factors

The discovery that soluble growth factors can cause mesodermal differentiation from uncommitted frog ectoderm has fuelled the expectation that such mechanisms should be universal among the vertebrates. Recent studies in the chick seem to justify this: growth factors shown to have mesoderm-inducing activity in *Xenopus* have some comparable effects in the chick, and are expressed in early amniote embryos.

axial/dorsal mesoderm begins before the hypoblast is a complete sheet of tissue, a conclusion that seems to conflict with the idea that the hypoblast is the source of axial/dorsal mesoderm-inducing factors at a later stage (XIII[16]), based on the results of its rotation.

However, epiblast cells continue to become specified as axial/dorsal mesoderm at much later stages in development. Even at the end of the primitive streak stage [stage 4 (Ref. 26)], single marked cells give rise to progeny that include both notochord and ectoderm[25], suggesting that at ·least some epiblast cells acquire axial/dorsal mesodermal characteristics very late during gastrulation. This result appears rather different from those obtained in amphibians, where the competence of the ectoderm to respond to activin and

FIG 3

Comparison of fate maps of the ectodermal layers of mouse (left), chick (centre) and urodele amphibian (right) embryos. The mouse embryo is shown flattened out and viewed from the inside of the cylinder (epiblast side). The chick is seen from the epiblast side, posterior end below. The amphibian is shown from its dorsal vegetal side (blastopore). In all three cases, the lower set of diagrams correspond to the early- to mid-gastrula stage. The middle row shows the fate maps of the ectoderm for the late blastula stage for the mouse (about 6.5 days, pre-streak) and chick (stage XIII[16]). A fate map for the chick 'early blastula' (stage X) is shown above. In the chick fate maps, note the anterior movement of the region containing presumptive notochord cells between the early and late blastula stages. Compiled from the findings of many investigators; lower set of diagrams for all three species mainly after Ref. 20 (with kind permission of Dr K.A. Lawson). Mouse late blastula compiled from Fig. 10 in Ref. 20. Chick fate maps based on Refs 20, 23–25 and Y. Hatada and C.D. Stern, unpublished.
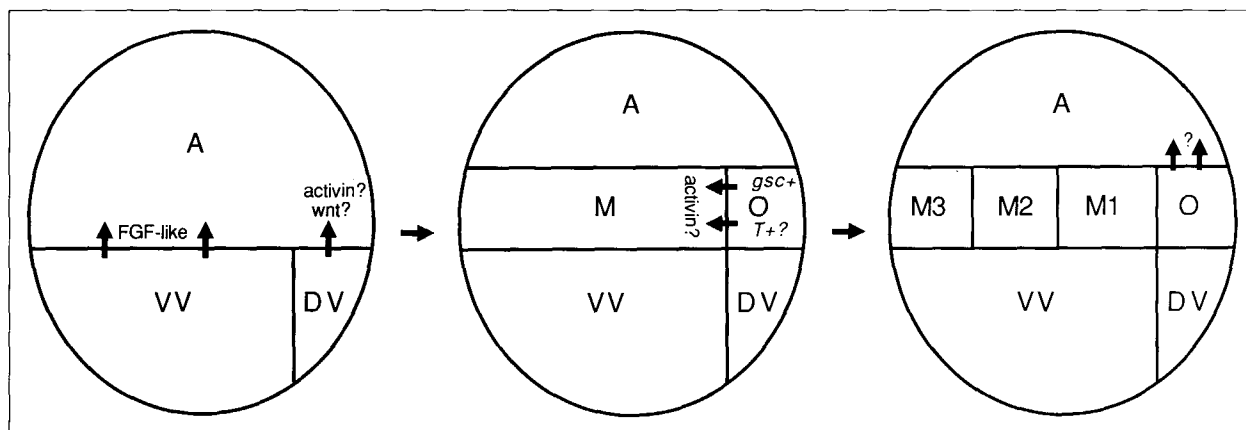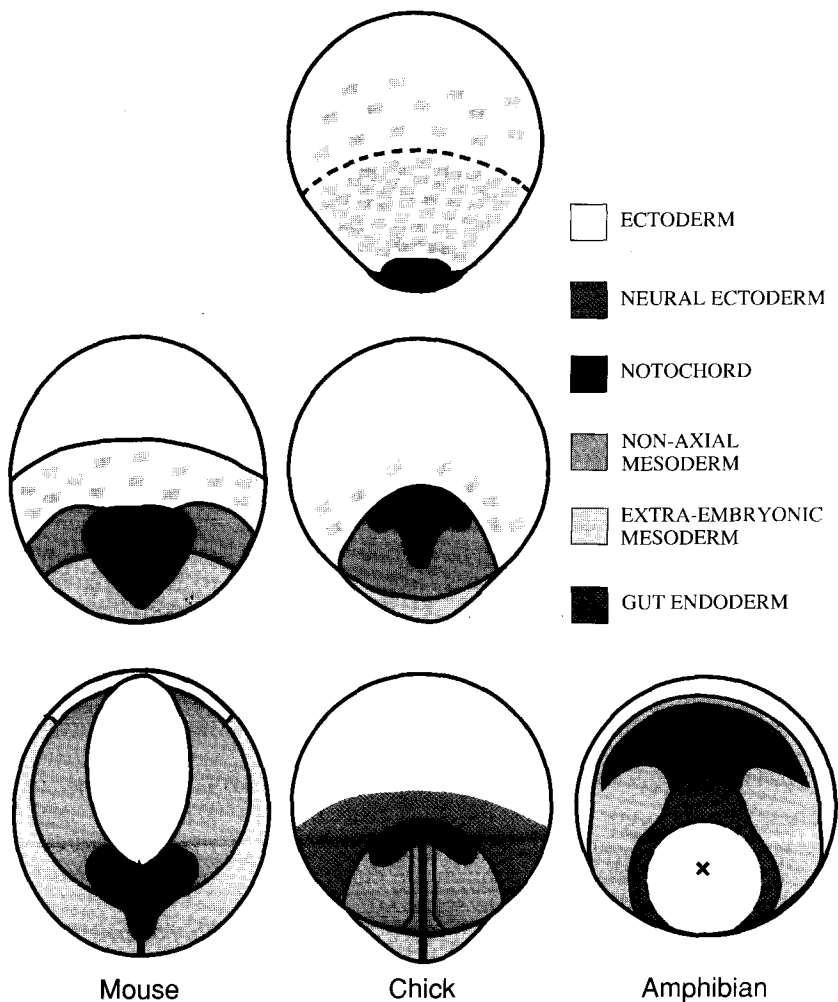
ECTODERM

NEURAL ECTODERM

NOTOCHORD

NON-AXIAL MESODERM

EXTRA-EMBRYONIC MESODERM

GUT ENDODERM

Mouse   Chick   Amphibian

FIG 4

A version of the three-signal model for frog mesoderm induction[28]. Early in development (perhaps as early as the 64-cell stage), two signals emanate from the vegetal side of the embryo: a ventral (VV) and a dorsal (DV) vegetal signal. The VV signal induces the overlying marginal zone to become mesoderm (M), while the DV signal induces organizer (O) activity in the overlying marginal zone. A dorsalizing signal from the organizer then regionalizes the belt of mesoderm into different dorsoventral mesodermal cell types (e.g. M1, notochord; M2, muscle; M3, mesenchyme, blood). Perhaps later in development, the organizer emits a (different?) signal responsible for neural induction in the overlying animal cap (A) ectoderm. Candidate molecules have been assigned to each of these signals. The VV signal might be FGF-related; the DV signal could be a *wnt*-related factor[6,7]. The DV signal could turn on the expression of the organizer-specific gene *goosecoid* (*gsc*)[4,5], and perhaps the *Brachyury* gene (*T*)[3]. However, expression of both *gsc* and *T* is a rapid response to activin; therefore, an activin-like molecule could correspond to the DV signal[5]. As the response to activin is dose dependent, with higher doses giving more dorsal types of mesoderm, an activin-like molecule could instead correspond to the O signal.

Effects of mesoderm-inducing factors on the chick

Heparin and suramin, which inhibit FGF action, interfere with axis formation in early chick embryos[29]. Dissociated chick embryonic cells, when treated with activin, undergo changes in behaviour comparable with some of those experienced by treated amphibian animal cap cells[30]. More dramatically, if the central part of the chick epiblast is cultured in medium conditioned by activin-secreting cells[13,14], an embryonic axis develops; thus, activin-containing medium can replace the hypoblast in such experiments. Activin can also induce the expression of *goosecoid* mRNA in mouse embryos (M. Blum *et al.*, unpublished).

Expression of mesoderm-inducing factors in amniote embryos

In the chick, transcripts related to basic FGF (which induces ventral mesoderm in the frog animal cap assay) were found in stage XIII[16] chick embryos by northern analysis[29]. mRNAs encoding various FGFs are also expressed in mouse embryos from 5.3 days (FGF-5; Refs 31, 32) and as early as 4.5 days p.c. for k-FGF (also known as FGF-4; Ref. 33). TGF-β_1 is also expressed in the chick embryo at late primitive streak stages[34]. Activin-A-related transcripts could not be detected at any stage in northern blots in the chick, but transcripts related to activin B were detected only at stage XIII and at early primitive streak stages[14].

These results argue against the idea that any of these growth factors can be solely responsible for the induction of axial/dorsal mesoderm in the chick. Basic FGF, which may be present before stage XIII[29], cannot be secreted by cells because it lacks a signal sequence[35] and cannot induce dorsal mesoderm in *Xenopus*. Activin, currently thought to be the most likely endogenous inducing factor for axial/dorsal mesoderm, is not expressed either early enough or late enough in the chick to account for the formation of all the mesoderm and for specifying its dorsoventral character. In frogs there is a similar problem: transcription of activin A mRNA does not begin until the late gastrula stage, and activin B is expressed from the late blastula stage[36], by which time the response of animal caps to added activin is beginning to decline. However, recent studies have reported the presence of several activin-related activities in the egg and early frog embryo (Ref. 37 and G-D. Guex and J.C. Smith, unpublished). It is therefore possible that activin-related substances that remain to be fully characterized induce axial/dorsal mesoderm in both amphibians and amniotes, but it seems increasingly unlikely that activins A and B themselves can be directly involved in this process in either group.

By analogy with the three-signal model, we might expect that factors inducing nonaxial ('ventral') mesoderm, perhaps related to FGF, might be produced by chick hypoblast around stages XI–XII, and affect mainly the posterior part of the embryo. Responding cells in the epiblast may be HNK-1-positive. The posterior marginal zone, on the other· hand, would be responsible for inducing organizer cells in the neighbouring epiblast at stages X–XI, and may therefore produce an activin-related and/or *wnt*-like activity. One might therefore predict that some cells in this region of the epiblast will express the organizer-specific genes *goosecoid* and/or *T*(*Brachyury*).

Relationship between mesoderm induction and axis formation

Frog animal caps, when treated with appropriate growth factors, undergo complex changes in addition to the differentiation of cells into various mesodermal cell types. Initially, the explants elongate and, eventually, generate miniature embryonic axes[2,38,39]. The same is true in the chick: isolated chick epiblast, in the presence of either activin or a hypoblast, develops an axis; when cultured alone, some disorganized mesoderm develops (mesenchyme, blood and muscle) but no axis forms. It is possible, therefore, that activin or a hypoblast are required at the late blastula stage for primitive streak formation, but not for mesoderm induction. In turn, the primitive streak is required for the subsequent organization of the mesoderm into a coherent axis, which allows the axial/dorsal mesoderm to be recognized in the absence of cell type-specific markers.

One possibility is that activin is required for the elongation that accompanies axis formation: the rapid elongation that frog animal caps undergo after treatment with activin could be equivalent to the massive cell movements that displace the future notochord region to the middle of the chick embryo. Thus, an activin-like activity, produced by the posterior marginal zone and/or by the hypoblast, may be required for such extension movements.

Prepatterns

If the hypoblast is dissociated and then combined with an intact epiblast, the embryo formed follows the orientation of the epiblast[40]. More dramatically, in the converse experiment, the orientation of embryos formed from dissociated epiblasts follows the polarity of the intact hypoblast[41]. These results suggest that both the hypoblast and the epiblast have polarity, as suggested in amphibians[38,42].

The finding that the epiblast is polarized confirms the speculation that by the time at which the hypoblast can be rotated to reverse the axis (stage XIII), some induction has already taken place in the epiblast. That the hypoblast has polarity suggests that two properties are associated with this tissue: one, which does not require organization and is mimicked by activin, is required for primitive streak formation from its original site; the other, which does require organization, induces a new primitive streak when the hypoblast is rotated. The two properties have been reconciled by the suggestion that the hypoblast contains a gradient of an activin-related activity, decreasing in the posterior-to-anterior direction[14].

Conclusions

The above discussion suggests that mesoderm induction and formation of an embryonic axis are different and separable events, perhaps involving different inducing factors. But despite the spectacular results obtained with the frog animal cap assay and the similarities between amphibians and amniotes, there is still much to be learnt about induction of mesoderm and

formation of the embryonic axis in both classes, especially at the cellular level. Single treated animal cap cells do not appear to differentiate into mesoderm, and even in intact animal caps not all cells respond. Nor do all differentiating cells become the same mesodermal cell type as one another: animal caps treated with enough factor to produce muscle also contain blood and endothelium as well as epidermis. These results have been attributed to 'developmental noise', to patchy distribution of the factors, to a prepattern of determination or to cooperation between responding cells[43]. Whatever the reasons, mesoderm induction and axis formation appear to be more complex than the notion of gradients of single factors specifying multiple cell types suggests. Data now becoming available from amniote embryos will no doubt help us to understand, in the near future, how the axis of vertebrate embryos is laid out.

Acknowledgements

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