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Hypoblastic tissue and fibroblast growth factor induce blood tissue (haemoglobin) in the early chick embryo

C. Gordon-Thomson[1] and B. C. Fabian[2]

[1]Biology Department, University of Natal, King George V Avenue, Durban 4001, South Africa
[2]B. I. Balinsky Laboratory, Zoology Department, University of the Witwatersrand, P O Wits, Johannesburg 2050, South Africa

SUMMARY

We have investigated the temporal and the causal basis of blood tissue specification in the chick embryo. Earlier workers have shown that the prospective blood-forming area is specified in a horseshoe-shaped area at the posterior side of the embryo. We found that cultured explants from the posterior marginal zone at stages XI to XIII (consisting of the posterior marginal zone and part of Koller's sickle) have a high propensity to form haemoglobin (Hb), which could be inhibited at stage XI by adding antibody against basic fibroblast growth factor (bFGF) to the neutral culture medium; this treatment had no effect from stage XII onwards. The same result was found when whole embryos were cultured with an antiserum raised against bFGF, or with heparin.

In another series of experiments, we found that cultured pieces from the inner-core of stage XIII epiblasts (with or without hypoblast tissue) were able to form Hb, whereas inner-core pieces from the pre-hypoblast stages, namely stages X and XI, did not form Hb. The capacity to form Hb, however, could be conferred upon the inner-core pieces from stage X epiblasts if bFGF at a concentration of 75-150 ng/ml was added to the culture medium. Furthermore, and most pertinently, the capacity to form Hb could be conferred on stage X inner-core pieces when they were co-cultured with hypoblast from a stage XIII embryo in a sandwich explant. Thus the inductive role of the hypoblast appears to be mediated via bFGF. This conclusion was confirmed by our finding that the antibody to bFGF inhibited the formation of Hb in these hypoblast sandwich explants.

We propose that the erythropoietic cell lineage, i.e. blood tissue, is determined in the chick embryo before stage XII by a bFGF-type of inductive signal from the emerging hypoblast adjacent to Koller's sickle, in the area encompassing the posterior marginal zone. The morphogenetic changes that take place in the epiblast and hypoblast between stages X and XIII are considered in the discussion, especially with respect to how the posterior horseshoe of blood-forming tissue arises in response to the inductive signal.

Key words: mesoderm determination, haemoglobin induction, hypoblast, bFGF, chick blastoderm

INTRODUCTION

The determinative events that generate the spectrum of mesodermal tissues in *Xenopus* embryos, ranging from ventral blood to dorsal organizer, have been incorporated into a unitary model that is driven by growth factor signalling (see reviews by Slack, 1990; Kimelman et al., 1992; Jessell and Melton, 1992; Beddington and Smith, 1993). Some equivalent components of this model have been proposed for the chick embryo, such as the induction of the dorsal 'axial' system by activin (Mitrani et al., 1990b; Ziv et al., 1992). This is not the case for the other end of the mesodermal spectrum where the factors that control the specification of the erythropoietic lineage in the chick blastoderm await clarification. This investigation addresses the latter question and will show that both hypoblastic tissue and bFGF are able to induce uncommitted cells of stage X blastoderms to proceed along a haemoglobin differentiative pathway.

Wilt (1974) demonstrated that a commitment towards haemoglobin (Hb) differentiation in the chick embryo occurs prior to gastrulation in those cells of the blastoderm that occupy a horseshoe-shaped area that overlaps the marginal zone, i.e. the posterior and lateral parts; but not in the enclosed area, which comprises the inner central disc of the epiblast. He demonstrated this by culturing small pieces of blastoderm when the embryo was "a simple disc of cells ..... in the process of forming two embryonic tissues (epiblast and hypoblast)"; i.e. within the XI-XII window of the normal tables of Eyal-Giladi and Kochav (1976). By stage XIII of EG-K, the isolated central disc, stripped of the marginal zone and hypoblast layer, has been shown by Azar and Eyal-Giladi (1979) to have the capacity to form blood islands (in terms of a histological evaluation) when cultured in a neutral medium.

The commitment of the posterior horseshoe-shaped area of cells to form blood at stage XI or XII needs to be interpreted in the context of the key morphogenetic events that take place at this stage, namely, the emergence of Koller's sickle and the formation of the hypoblast at the posterior, ventral side of the

blastoderm. The crescentic ridge of cells constituting the sickle forms at stage X and demarcates the inner border of the posterior marginal zone. The coherent hypoblast layer is first seen anterior to Koller's sickle and emerges from this posterior region (Stern, 1990; Eyal-Giladi et al., 1992). By stage XII, the horns of the sickle have extended laterally bordering the emerging hypoblast at the posterior end of the area pellucida. The emerging hypoblast integrates with the islands of polyingressed epiblast cells as it migrates anteriorly to form the fully formed hypoblast layer by stage XIII (see Eyal-Giladi, 1991; Stern, 1991; and Khaner, 1993, for reviews). We propose that the emerging hypoblastic tissue at the posterior end of the blastoderm induces cells in the adjacent epiblast to enter the erythropoietic lineage in the chick, possibly at the posterior and posterolateral points of contact between the two layers. The latter interpretation is consistent with the subsequent location of the blood-forming (Hb) horseshoe-shaped area that overlaps the marginal zone and Koller's sickle in the posterior and posterolateral area pellucida. Our basic proposal rests on the capacity of uncommitted cells in the epiblast to respond to an inductive signal and form blood. It is thus a hypothesis that can be tested.

An 'inductive' or 'salutary' stimulation of mesoderm along a Hb differentiative pathway has been observed at a later stage of development (Wilt, 1965, 1967; Kessel and Fabian, 1986, 1987). At this later stage, stage 4 of the tables of Hamburger and Hamilton (1951), the prospective mesoderm has translocated from the epiblast into the space between the epiblast and hypoblast and spread centrifugally through the area pellucida (AP), thereby establishing the area opaca vasculosa (AOV) (Wilt, 1967; Kessel and Fabian, 1985). At this stage, Wilt (1965) found that the endoderm of the AOV was a potent stimulus for the amount of Hb synthesized by the AOV mesoderm. As the mesoderm synthesized some Hb even in the absence of endoderm, it was appropriate to describe the effect as 'salutary' rather than 'inductive', although the salutary response may represent the end point of an instructive induction that was initiated at an earlier stage, requiring a continuing supply of inducing factor to complete its differentiative pathway. The existence of an earlier inductive event is supported by a population of mesodermal cells within the AP at stage 4 that does not normally form blood, but can be induced to do so by AOV endoderm (Kessel and Fabian, 1986, 1987).

As to the nature of the inducing signal that initially determines the blood lineage at the earliest hypoblast stage, according to our proposal, we previously reported that Hb differentiation can be blocked by heparin treatment of whole embryos at stage X and XI (Gordon-Thomson et al., 1988), and that bFGF was able to neutralize this inhibitory effect (Gordon-Thomson et al., 1991), suggesting that bFGF played a role in the induction of blood tissue in the chick at these stages. Mitrani et al. (1990a) have shown that bFGF is expressed in all parts of the chick blastoderm from stage XI onwards. These workers, however, were only able to detect the bFGF transcripts in the marginal zone at the full hypoblast stage (stage XIII) and not at earlier stages, possibly due to the low level of transcript.

In this study, we have found that explants including the posterior marginal zone (PMZ) had a high propensity to form Hb at stages XI to XIII, while the inner core of the epiblast (ICD) before stage XII did not, in accordance with Wilt's findings. We show further that anti-FGF is able to inhibit Hb formation in explants from the PMZ at stage XI, but not in explants of PMZ at stage XII and onwards. Similarly, treatment of whole embryos at stages X and XI with an antibody to bFGF inhibited erythropoiesis, as well as the formation of other posterior structures, namely somites and heart. We also show that the addition of a hypoblast or the addition of bFGF to a stage X ICD leads to the formation of Hb, and that the antibody to bFGF is able to inhibit this response.

Based on these findings we propose that the source of the inductive signal for the determination of blood mesoderm is in the hypoblast, having its origin in those cells that emerge from the posterior and posterolateral marginal zone, and that bFGF is the inducing agent that commits cells before stage XII into the Hb differentiative pathway in the chick.

MATERIALS AND METHODS

Culturing methods for blastoderm and blastodermal pieces

Freshly laid hens' eggs were collected and stored at 14°C to prevent further development of the embryo in the egg before treatment, and the embryo explants prepared within 30 hours. The stage XIII blastoderms were obtained from eggs incubated for 8 hours at 38°C. The explanted blastoderms were cleaned of yolk in Ringer's solution (DeHaan, 1967) under aseptic conditions. Special attention was given to identifying the stage of each embryo accurately, using the tables of Eyal-Giladi and Kochav (1976).

Whole embryos (stages X-XIII) were transferred to rafts prepared by stretching the vitelline membrane over a glass ring (according to New, 1955). Each embryo was arranged in the centre of the raft with its ventral surface facing up and the excess Ringer's solution was aspirated off by pipette. Each raft with its embryo was settled over a pool of the more fluid component of the egg albumen in a watch glass (refer to Fig. 1), placed inside a Petri dish containing cotton wool swabs soaked in sterile water and incubated for 48 hours at 38°C.

Pieces (approximately 0.75 mm^2 in size) of the blastoderm (as outlined in Fig. 1) were dissected by means of hair loops from whole blastoderms at stages X to XIII in Ringer's solution. The pieces of the blastoderm were cultured using one of the following three methods: (1) ventral surface up on a vitelline membrane raft over egg albumen, (2) ventral surface up on solid agarose plates made of 1.8% agarose in a 1:1:1 mixture of egg albumen, Ringer's solution and serum-free Dulbecco's modified Eagles medium (DMEM; purchased from Highveld Biological Supplies, RSA) in organ culture dishes or (3) in suspension in a 45 μl drop of serum-free DMEM diluted 1:1 with Ringer's solution. (Only serum-free medium was used, as preliminary tests showed that serum-enriched medium 'stimulated' erythropoiesis.) The blastodermal pieces suspended in the drop of medium were cultured in a chamber made from two silicone-coated coverslips (prepared by immersing in 2% dichlorodimethylsilane in carbon tetrachloride then baked for 3 hours at 105°C). The coverslips were supported by an aluminium slide and sealed to form an air-tight chamber with a molten wax, vaseline and wool fat mixture (refer to Fig. 1). All cultures of blastodermal pieces were incubated for 3 days at 38°C in a humidified atmosphere. (It should be noted that treatment of the blastodermal pieces using any one of these three culture methods produced equivalent results. The suspension culture method allowed for (1) economic use of growth factor and antisera additives and (2) easy manipulation of the pieces of blastoderm in the drop of medium to form the tissue recombinations in the sandwich explants.)

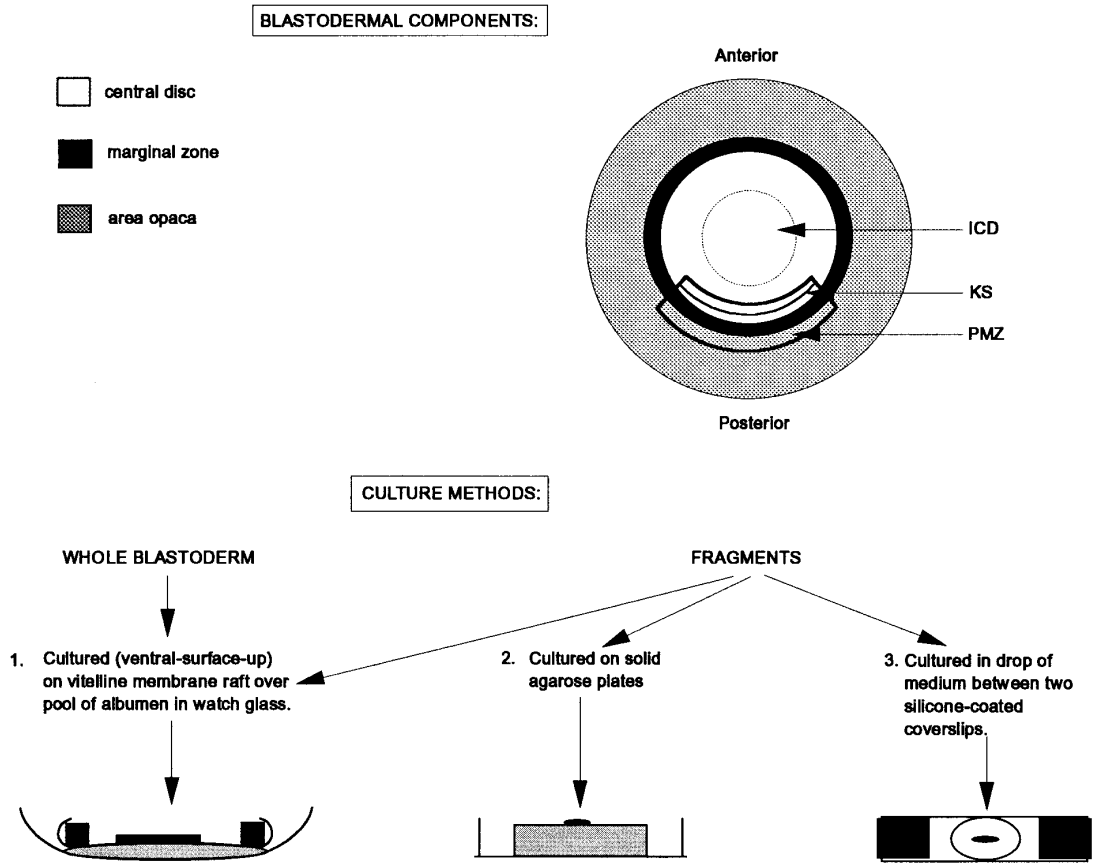

Fig. 1. Diagram outlining the pieces (described as components or fragments in the figure) dissected from the blastoderm and the culturing procedures used. (Abbreviations: ICD = inner core of the central disc of the area pellucida. The ICD from the stage X embryo consisted of the epiblast and islands of polyingressed epiblast cells; the ICD from the stage XI embryo consisted of the epiblast and polyingressed epiblast cells and excluded Koller's sickle and posterior emerging hypoblast; the ICD component from embryos from stages XII and XIII consisted of the epiblast and hypoblast layers but excluded Koller's sickle region. PMZ = posterior region of the epiblast including Koller's sickle, posterior marginal zone and adjoining part of the area opaca). Whole blastoderms and some pieces of blastoderm were cultured on vitelline membrane rafts. Other blastodermal pieces were cultured either on solid agarose plates, or in a suspended drop of medium.

Treatment of whole blastoderms with antiserum to bFGF and heparin

A rabbit polyclonal antibody raised against bovine brain bFGF (anti-FGF) was purchased from R and D Systems, USA. (The blastodermal pieces were treated with anti-FGF purchased from Sigma Immunochemicals, USA; see below). Whole blastoderms were treated with anti-FGF at concentrations of 250 to 1000 μg/ml anti-FGF in phosphate-buffered saline.

Embryo explants of stages X and XI were treated with anti-FGF by incubating each blastoderm ventral surface down on the surface of a drop of the antibody solution in a silicone-coated Petri dish for 1 hour. During this treatment the embryos were incubated at 38°C in a humidified atmosphere to ensure uptake of the antibody. Each embryo was then transferred to a vitelline membrane raft and incubated over a pool of albumen for 2 days, as described above. An additional 5 μl of the antibody solution was dispensed at the edge of each embryo by means of a micropipette, at a time ranging between 1 and 6.5 hours after the initial dose. Control embryos were treated similarly with Ringer's solution, or with two other irrelevant rabbit polyclonal antisera diluted 1:1500 (anti-human albumen and *Otomys irroratus* liver IgGs were used).

Treatment with heparin has been described previously (Gordon-Thomson et al., 1991). Briefly, embryo explants (stages X-XIII) were cultured on vitelline membrane rafts over a pool of 3 mg/ml heparin (Sigma, USA) in the more fluid component of the egg albumen. (It should be noted that the concentration of heparin in the pool of albumen beneath the vitelline membrane does not necessarily represent the concentration in the embryo; it represents the minimum concentration that had a developmental effect in our experiments.) Control embryos were cultured on vitelline membrane rafts over egg albumen alone, or with the addition of another glycosaminoglycans, namely chondroitin sulphate (Sigma), at a concentration of 3 mg/ml egg albumen.

Treatment of the posterior marginal zone components with antiserum to bFGF

PMZ components dissected from embryos at stages XI-XIII were each cultured in a 45 μl drop of medium containing a rabbit polyclonal antibody raised against bovine brain bFGF (supplied by Sigma Immunochemicals, USA) at concentrations of 180 to 400 μg/ml anti-FGF in serum-free DMEM: Ringer's solution (1:1). Two other irrelevant rabbit IgGs (as described above) were substituted for the anti-FGF in the controls. A separate immunoneutralization test was made by incubating the anti-FGF diluted 1:2000 (final concentration = 200 μg/ml IgG) with recombinant, bovine bFGF at concentrations ranging from 2 to 100 ng/ml bFGF before treatment of the stage XI PMZ pieces.

bFGF treatment of the stage X central disc

The inner core of the central disc (ICD) dissected from stage X-XI embryos were cultured either on vitelline membrane rafts or in suspension. The ICD components cultured on the vitelline membrane rafts were dosed at the beginning of the culture period with two 5 μl drops of recombinant, bovine bFGF (supplied by Boehringer Mannheim, Germany) at concentrations ranging from 50 to 150 ng/ml in serum-free DMEM, administered at the edge of the explant with a micropipette. The ICD components cultured in suspension were incubated in a drop of serum-free DMEM:Ringer's solution (1:1), containing 50 to 150 ng/ml bFGF. Some controls were incubated in medium containing 100 ng/ml bFGF together with 400 μg/ml anti-FGF.

Tissue recombination sandwich explants

The fully formed hypoblast (HYP) was dissected with hair loops from the central disc of the stage XIII blastoderm under Ringer's solution and transferred by pipette to a drop of medium on a silicone-coated coverslip. An inner core of the central disc (ICD) of a stage X blastoderm was transferred to each drop of medium containing a HYP, and the two components combined to form a sandwich explant suspended in the drop of medium. Hair loops were used to juxtapose the ventral surface of the ICD component against the inner (i.e. the dorsal) surface of the HYP. The drop of medium was replaced with fresh medium, by aspirating approximately two-thirds of the volume of the drop without disturbing the sandwich explant, and replacing it with fresh culture medium, repeated twice. The suspension cultures were supported on aluminium slides as described above. Four different controls were cultured in suspension or on solid agarose plates and consisted of the following components: the HYP alone, the stage XIII ICD without a hypoblast, the stage X ICD alone and the stage X ICD combined with the endoderm of the AP of a stage 4 blastoderm (explanted from eggs incubated for 10-12 hours and staged using the tables of Hamburger and Hamilton, 1951),

In another series of experiments sandwich explants of the stage X ICD combined with a stage XIII HYP were cultured in a 45 μl drop of medium containing a rabbit polyclonal antibody raised against bovine brain bFGF (supplied by Sigma Immunochemicals, USA) at a concentration of 200 μg/ml anti-FGF in serum-free DMEM:Ringer's solution (1:1). Two other irrelevant rabbit IgGs (as described above) were substituted for the anti-FGF in the controls.

O-dianisidine test for haemoglobin

Determination of erythropoiesis in whole blastoderms and blastodermal components was by the o-dianisidine test of O'Brien (1960). The small pieces of the blastoderm were attached to slides precoated with poly-L-lysine (Sigma Diagnostics, USA) before staining. The stain was prepared from four parts o-dianisidine stock solution (100 mg o-dianisidine in 70 ml absolute ethanol), one part 0.1 M sodium acetate (pH 4.6), 0.2 parts 30% hydrogen peroxide and 1.5 parts distilled water. Explants were counterstained with 1% aqueous methyl green, dehydrated in p-dioxane, cleared in xylol and mounted with DePeX mountant (BDH Chemicals, UK). The specificity of the test for the assay of blood at these stages of chick development is well documented (O'Brien, 1960; Wilt, 1967, 1974; Kessel and Fabian, 1986, 1987). Microscopic observations and photography were performed with bright-field optics.

RESULTS

Erythropoietic potential of blastoderm and blastodermal pieces cultured in a neutral medium

After 2 days in culture, blood tissue (Hb) developed in the AOV of approximately 80% of the control whole embryo explants. Hb was identified cytochemically with a sensitive modification of the benzidine stain which uses o-dianisidine to detect the pseudo-peroxidase activity of Hb, according to O'Brien (1960). This test has been used by others for the identification of Hb in the early stages of chick development and the cytochemistry has been correlated with the histology by these earlier workers (see Wilt, 1967, 1974; Kessel and Fabian, 1986, 1987). In the present investigation, the differentiating red blood cells in blood islands and the erythrocytes within vascular channels of the control whole-mount preparations stained brown, while all other tissues stained green with the counterstain (Fig. 2A).

The blastodermal pieces after 3 days in culture had formed either a compact cluster of cells, or a swollen vesicular structure. The incidence of erythropoiesis in the pieces of the blastoderm cultured in a neutral medium depended on the area of the blastoderm from which the component was dissected, as well as on the stage of the embryo at explantation. The posterior marginal zone (PMZ) component dissected from stage XI-XIII embryos showed a tendency to form vesicular (cyst-like) structures, and approximately 80% of these components contained groups of cells that gave a positive o-dianisidine reaction (refer to Fig. 2B, and Table 1). In some cases, these vesicles contained tissue that pulsated rhythmically in the culture medium, indicating cardiac muscle differentiation.

The inner core of the central disc (ICD) dissected from stage X-XI embryos developed into small compact clusters of cells, which did not form haemoglobin (Fig. 2C). However, aggregates of cells in approximately 80% of the ICD components dissected from the older stage XIII embryos did stain positively with o-dianisidine (refer to Fig. 2D). Erythropoiesis in stage XIII ICD components was at a lower percentage (58%) after removal of the hypoblast, whereas the isolated hypoblasts

Table 1. The potentiality of various pieces of the blastoderm dissected from stage X to XIII embryos to form erythropoietic tissue (o-dianisidine-positive tissue) when cultured alone, or recombined with another component, in a neutral medium or with various additives

Blastodermal component	No. of tests	Percentage erythropoiesis
Stages X and XI		
ICD	34	9
ICD + serum	14	50
ICD + 50 ng/ml bFGF	9	11
ICD + 75 ,,	9	67
ICD + 100 ,,	12	75
ICD + 150 ,,	16	69
ICD *X* + HYP	12	75
ICD *X* + HYP + anti-FGF	6	0
ICD *X* + endoderm (stage 4)	2	0
Stage XI		
PMZ	32	84
PMZ + rabbit IgG	14	79
PMZ + 180 μg/ml anti-FGF	6	33
PMZ + 200 ,,	18	5
Stages XII and XIII		
PMZ	31	74
PMZ + anti-FGF	10	80
ICD *XIII* with HYP intact	12	83
ICD *XIII* without HYP	12	58
HYP alone	6	0

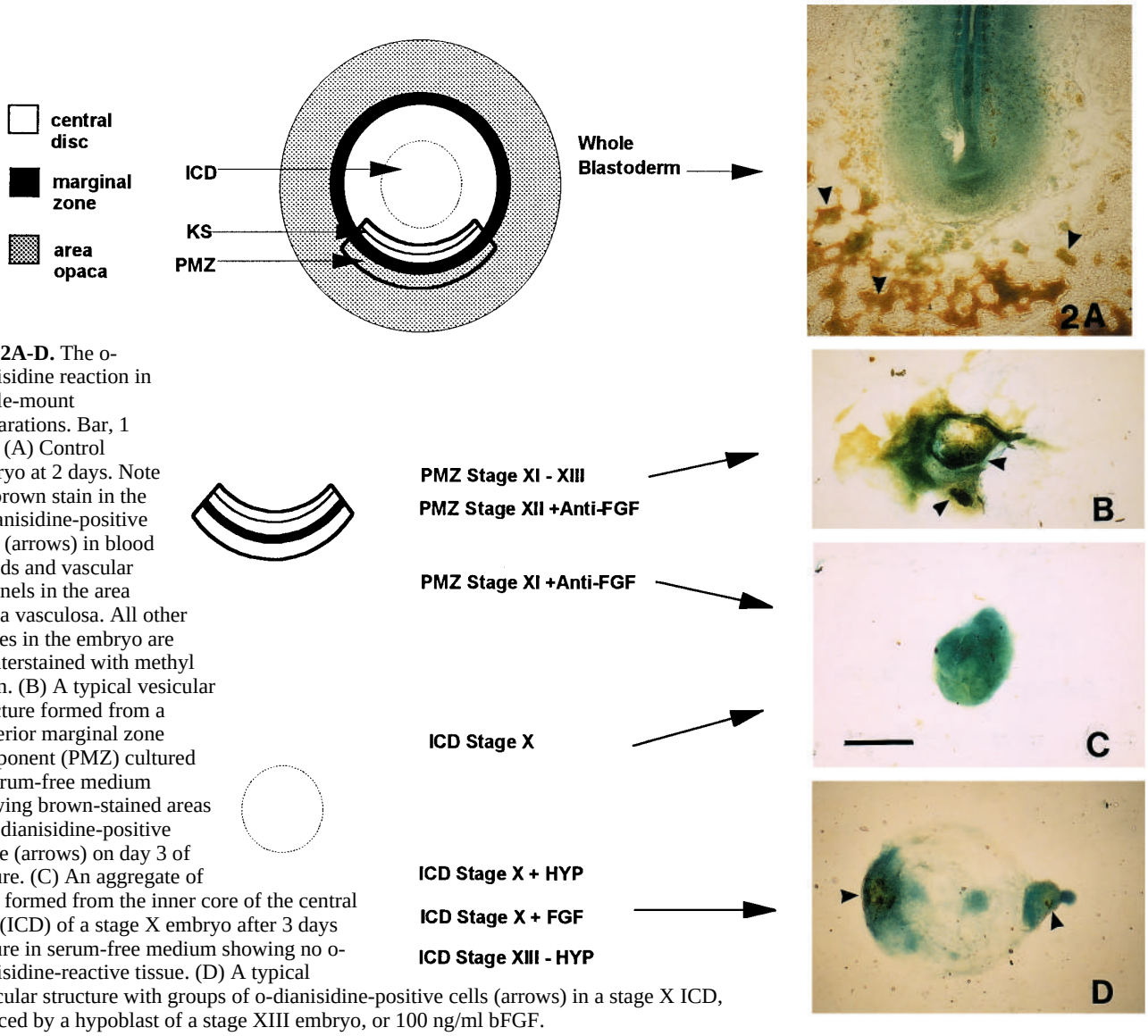

Fig. 2A-D. The o-dianisidine reaction in whole-mount preparations. Bar, 1 mm. (A) Control embryo at 2 days. Note the brown stain in the o-dianisidine-positive cells (arrows) in blood islands and vascular channels in the area opaca vasculosa. All other tissues in the embryo are counterstained with methyl green. (B) A typical vesicular structure formed from a posterior marginal zone component (PMZ) cultured in serum-free medium showing brown-stained areas of o-dianisidine-positive tissue (arrows) on day 3 of culture. (C) An aggregate of cells formed from the inner core of the central disc (ICD) of a stage X embryo after 3 days culture in serum-free medium showing no o-dianisidine-reactive tissue. (D) A typical vesicular structure with groups of o-dianisidine-positive cells (arrows) in a stage X ICD, induced by a hypoblast of a stage XIII embryo, or 100 ng/ml bFGF.

formed loose sheets of cells, which dissociated easily and showed no positive o-dianisidine staining (refer to Table 1).

Inhibition of erythropoiesis by early treatment with anti-FGF or heparin

Treatment of whole embryo explants at stages X and XI with anti-FGF or heparin inhibited the development of erythropoietic tissue, while erythropoietic tissue developed in approximately 80% of the controls (Refer to Fig. 3, and Table 2.) Other abnormalities were observed in the treated embryos. Heparin treatment inhibited primitive streak formation and axis development (as reported previously by Gordon-Thomson et al., 1988, 1991). In approximately 70% of the embryos treated with

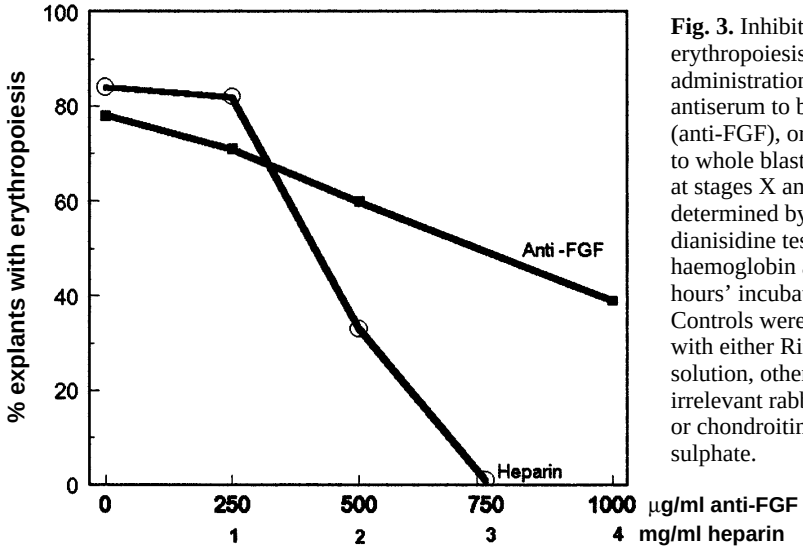

Fig. 3. Inhibition of erythropoiesis by the administration of an antiserum to bFGF (anti-FGF), or heparin, to whole blastoderms at stages X and XI, as determined by the o-dianisidine test for haemoglobin after 48 hours' incubation. Controls were treated with either Ringer's solution, other irrelevant rabbit IgGs, or chondroitin sulphate.

Table 2. The effect on erythropoiesis by various treatments to whole blastoderms at the earlier stages X and XI, compared with the later stages XII and XIII; as determined by the o-dianisidine test for haemoglobin

Treatment	No. of tests	Percentage erythropoiesis
Stages X and XI		
Controls	48	79
250 μg/ml anti-FGF	7	71
500 ,,	10	60
1000 ,,	18	39
3 mg/ml heparin	10	0
Stages XII and XIII		
Controls	15	80
3 mg/ml heparin	14	64

Controls were treated with Ringer's solution, chondroitin sulphate, or other irrelevant rabbit IgGs.

anti-FGF, development was seen to be abnormal. The treated embryos had well-developed head structures, while the posterior trunk region appeared defective as heart, somites and blood tissue were absent.

The PMZ components dissected from stage XI embryos normally form o-dianisidine-positive tissue in culture. The addition of anti-FGF to these cultures inhibited erythropoiesis. The dose required for this inhibitory response was at a lower concentration of anti-FGF than that used with the whole blastoderm. Anti-FGF at concentrations of 200 μg/ml and higher, inhibited erythropoiesis in the stage XI PMZ (refer to Fig. 4, and Table 1). The PMZs cultured with anti-FGF did not develop into vesicular structures, but instead formed loosely packed aggregates or sheets of cells. Immunoneutralization tests showed that 2 ng/ml recombinant bFGF could block the inhibitory effect of 200 μg/ml anti-FGF on erythropoiesis in the PMZ components.

Erythropoiesis is not inhibited by anti-FGF or heparin at later stages

In our earlier investigations, we noted that heparin treatment of whole embryos at the later stage of XII and XIII did not inhibit erythropoiesis (refer to Table 2), although these whole blastoderms developed abnormal axial structures. In a parallel study using the smaller PMZ components dissected from stage XII and XIII embryos, instead of whole blastoderms, it was found that anti-FGF treatment did not inhibit erythropoiesis. The PMZs treated with 200 μg/ml anti-FGF developed into vesicular structures and erythropoietic tissue differentiated in 80% of the tests, similar to the controls (see Fig. 5, and Table 1).

Induction of erythropoiesis in stage X-XI inner core of the central disc by bFGF

The blastodermal pieces dissected from the inner core of the epiblast (ICD) of stage X and XI embryos did not form any o-dianisidine-positive tissue after 3 days of culture in a neutral medium. The addition of bFGF at concentrations ranging from 75-150 ng/ml to these explants, induced the formation of o-dianisidine-positive tissue (refer to Figs 2D, 6). This response was abolished when

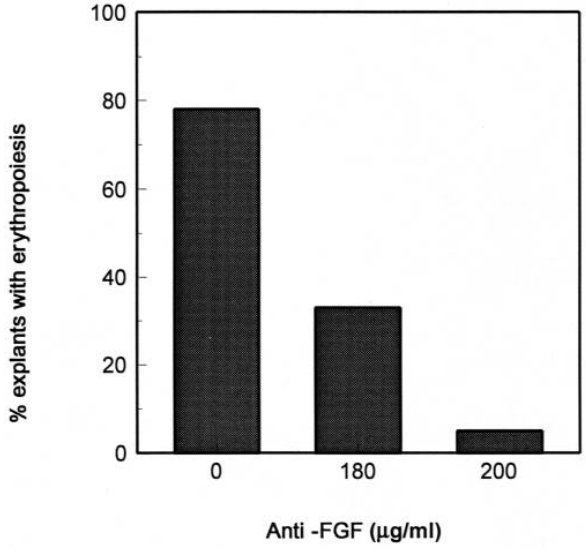

Fig. 4. The percentage of stage XI posterior marginal zone components (PMZ) in which erythropoiesis was inhibited by the addition of different concentrations of anti-FGF to the culture medium. Controls were treated with other irrelevant rabbit IgGs.

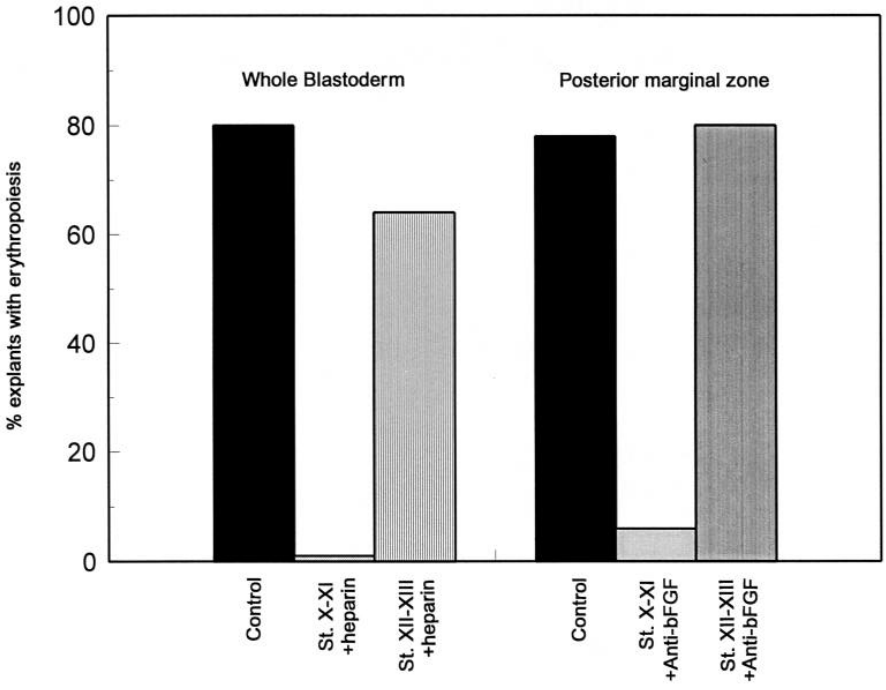

Fig. 5. The percentage of whole blastoderms or posterior marginal zone (PMZ) components that developed erythropoietic tissue after early (stages X and XI) or later (stages XII and XIII) treatment with antisera to bFGF or heparin. Controls were treated with chondroitin sulphate in the heparin series or with other irrelevant rabbit IgGs in the anti-FGF series.

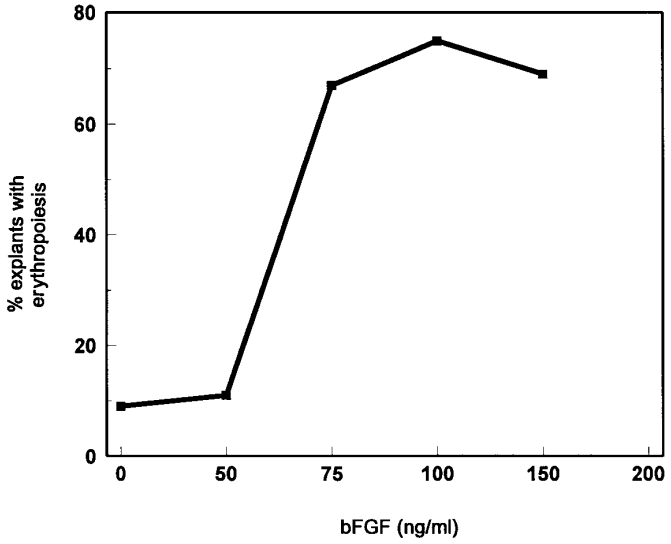

Fig. 6. Induction of erythropoiesis by bFGF in ICD components of stage X and XI embryos. Controls were cultured in serum-free medium without bFGF.

ICDs were incubated with bFGF and anti-bFGF simultaneously.

The bFGF concentration required to induce erythropoiesis in the chick explants was three-fold higher than that used to induce ventral mesoderm in the animal caps of *Xenopus* (refer to Slack et al., 1987; Green et al., 1990); however, the 'blood-like' cells reported to be seen in the frog animal cap bioassays have never been conclusively identified as fully differentiated erythrocytes (see Green et al., 1990). In the present study of the chick, histological examination of sectioned material revealed recognizable red blood cells within large spaces lined by 'endothelial-like' cells in the ICDs treated with bFGF, as well as positive o-dianisidine staining for Hb in the whole mounts.

In support of our results, and also for bFGF's role as an inducer of blood mesoderm in the chick, Flamme and Risau (1992) have recently shown that a similar concentration of human recombinant bFGF is required to induce cultured cells of dissociated quail blastoderms to form endothelial and hemopoietic cells. It should be noted, however, that in our experiments the explants were cultured in a neutral medium, i.e. without serum, thereby eliminating the extraneous introduction of unknown growth factors.

Induction of erythropoiesis in hypoblast sandwich explants

The sandwich explants of the stage X ICD combined with a stage XIII HYP cultured in a neutral medium developed into vesicular structures. Groups of cells that stained positively for Hb with o-dianisidine were seen in 75% of these sandwich explants (refer to Fig. 2D, and Table 1). Functional cardiac muscle tissue was also observed in 3 out of 12 explants. No o-dianisidine-positive tissue developed in the stage X ICDs cultured alone or when recombined with AP endoderm, or in the hypoblasts cultured alone (refer to Table 1).

DISCUSSION

The early determination of the blood (Hb) differentiative pathway

The posterior marginal zone (PMZ) has the highest propensity to form blood (Hb) in cultured whole embryos as well as in explants. We have shown that the formation of blood (Hb) is inhibited at stage XI, but not at stage XII, when PMZ explants are cultured with an antibody raised against bFGF, suggesting that a commitment to the Hb differentiative pathway takes place prior to stage XII. This finding was further supported by treating whole embryos with anti-FGF, or heparin, at stages X to XIII. Once again, it was found that anti-bFGF, and also heparin, inhibited Hb formation in embryos treated at stage X and XI, but not at stages XII and XIII. We conclude that there are uncommitted cells in the PMZ of stage X-XI embryos that can be induced by bFGF into a Hb differentiative pathway.

In support of bFGF's role as an inducer of Hb in uncommitted blastoderm, we found that the treatment of an explant of uncommitted cells from a stage X blastoderm with bFGF (75-150 ng/ml) resulted in the formation of Hb. The explant of uncommitted cells was taken from the inner central disc (ICD) of stage X embryos, which we have shown does not form blood in culture. We presume that the uncommitted cells in the ICDs are part of, or equivalent to, the population of uncommitted cells that extends into the PMZ and which are competent to respond to bFGF by stage XI.

The pertinence of these results needs to be interpreted against the morphological changes taking place between stages X and XIII, which involves the emergence of cells from the Koller's sickle region at the inner border of the posterior marginal zone (PMZ) and their migration anteriorly into the central disc region. Between stages XI and XIII, the deeper cells from the Koller's sickle region spread anteriorly beneath the epiblast to form the hypoblast (Khaner et al., 1985; Stern, 1990; Eyal-Giladi, 1991; Eyal-Giladi et al., 1992). At the same time, the horns of the sickle have extended more laterally (stage XII) and form the posterior and lateral borders of the emerging hypoblast (Khaner, 1993).

We have shown using cultured sandwich experiments that the hypoblast can induce Hb in uncommitted ICD cells, and that no blood was formed in control experiments when ICD or hypoblasts were cultured alone. Furthermore, and most relevantly, the addition of anti-FGF to the sandwiches (hypoblast plus ICD tissue) inhibited the formation of Hb. We are currently using an immunocytochemical approach to localise the source of this FGF-type signal.

Finally, it should be noted that the hypoblasts' role in the induction of blood (Hb) is supported by a related experiment carried out at a later stage of development, which showed that supernumerary hypoblasts from stage XIII embryos grafted onto pregastrula blastoderms induced the formation of multiple erythropoietic sites (Zagris, 1982).

The posterior horseshoe of blood-forming tissue

We propose that the posterior horseshoe pattern of blood tissue specification becomes committed following the induction of cells in the epiblastic layer along the Koller's sickle area, by cells of the emerging hypoblast with which they are in intimate contact. This induction would not be effective at the most

central part of the epiblast, which becomes separated from the mature hypoblast with the formation of the blastocoelic space. The stage X central disc cells would not therefore normally receive a FGF-type signal. We have shown, however, that at least some of these cells are competent to respond as they can be induced to produce Hb if contact is made with the hypoblast in a sandwich culture or in response to bFGF treatment. The width of the horseshoe area of blood-forming tissue in the posterior blastoderm may be determined by the extent to which the FGF signal is transmitted inwardly along the epiblast in a planar direction; an event that may well explain the Hb commitment of some cells of the ICD after stage XII. The localization of Hb commitment to the posterior part of the marginal zone may be a function of a FGF concentration gradient with the highest point at the posterior marginal zone and decreasing in the lateral-anterior direction, in accordance with the specification map of Wilt (1974). We have carried out experiments that support a high posterior concentration of bFGF in the blastoderm and will be reporting on our findings.

A role for bFGF in the harmonious patterning of the mesodermal spectrum of tissues in the chick

The posterior-to-posterolateral 'horseshoe' of Hb commitment by stage XII can be equated with the ventral mesoderm of the amphibian blastula, with tissues from both species being induced by bFGF into a prospective blood lineage. It appears that the PMZ anchors a bFGF-like signal for the blood-forming end of the mesodermal spectrum in the chick. The other end of the mesodermal spectrum, i.e. the axial end, corresponds to embryonic tissue in the chick that can be induced by activin (Mitrani et al., 1990b), in agreement with the frog model (reviewed by Ruiz i Altaba and Melton, 1989; Jessell and Melton, 1992; Green et al., 1992). The fate map of the chick, prior to gastrulation, reflects the mesodermal spectrum, showing prospective blood mesoderm at the posterior end of the blastoderm grading through to lateral plate, somitic and head mesoderm, and through to notochord at the anterior end (Balinsky, 1975; Gilbert, 1991; Stern et al., 1992).

In support of the role of bFGF in the balanced patterning of mesodermal morphogenesis in chick development, we found that the development of stage X and XI embryos was morphologically abnormal after treatment with antiserum to bFGF. While the anti-FGF blocked the formation of Hb, it did not affect the development of head structures, but resulted in the absence of the somites and heart. It should be mentioned that we found a similar result, i.e. the inhibition of Hb and suppression of heart and somite formation, in a series of experiments using a rabbit polyclonal antibody raised against the bFGF receptor (data not shown). The lack of heart tissue in these experiments is consistent with the results of our sandwich explant experiments in which the stage X ICD combined with a hypoblast or bFGF led to the formation of Hb as well as beating bits of heart tissue, but not of recognisable axial tissue.

In conclusion, our cardinal finding is that the determination of the erythropoietic sequence in the chick is induced before stage XII by cells of the hypoblast and that a bFGF-like molecule is the causal trigger of this event.

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