THE ANALYSIS OF ERYTHROPOIESIS AND OTHER EARLY DEVELOPMENTAL EVENTS IN THE CHICK EMBRYO USING MESODERMAL-INDUCING FACTORS

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PREFACE

The investigative studies described in this thesis were undertaken in the Department of Biology, University of Natal, Durban. The experimental work was carried out on a part-time basis between January 1987 to January 1994. This study was supervised by the appointed co-supervisor, Professor Barry C. Fabian of the Zoology Department, University of the Witwatersrand, Johannesburg. Professor John C. Poynton of the Biology Department of the University of Natal acted as supervisor for administrative purposes from 1987 until his retirement in 1991; and was replaced by Professor Anne J. Alexander, Biology Department, University of the Natal.

These studies represent original work by the author and have not been submitted in any form to another university. Where use was made of the work of others it has been duly acknowledged in the text. In accordance with the guidelines for publishing in the British journal *Development*, italics have not been used for latin words such as "et al", "in vitro", "in utero" and "in situ". The use of italics has been reserved for highlighting the names of genes, generic names, and journal titles.

Chapters Two and Four are direct transcripts from manuscripts that have been submitted for publication[1] [2]. A detailed description of the methodology is included in the appendices, and all references are listed in a single reference section at the end of the thesis. Sections of this work have also been presented at conferences in South Africa and in the United States of America, and the titles of the abstracts are listed below[3].

Clare Gordon-Thomson

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[1]GORDON-THOMSON, C., SALM, N. AND FABIAN, B.C. (1991). Fibroblast growth factor can neutralize the inhibitory effect of heparin on erythropoiesis but not on primitive streak formation in chick embryos. *South African Journal of Science*, **87**, 227-233.

[2]GORDON-THOMSON, C. AND FABIAN, B.C. (1994). Hypoblastic tissue and fibroblast growth factor induce blood tissue (haemoglobin) in the early chick embryo. *Development* (manuscript has been reviewed and accepted for publication).

[3]Abstracts in Conference Proceedings are listed below:
GORDON-THOMSON, C., SALM, N. AND FABIAN, B.C. (1988). The inhibitory effect of heparin on early development in chick embryos. *Proceedings of the Electron Microscope Society of Southern Africa*, **18**, 57-58.

GORDON-THOMSON, C. AND FABIAN, B.C. (1993). The competence of the bFGF response in blood formation in chick development. Proceedings of the *52nd Annual meeting of the American Society of Developmental Biology.*

FABIAN, B.C. AND GORDON-THOMSON, C. (1994). The blood lineage in chick embryos is induced by hypoblast and FGF. Proceedings of the *53rd Annual meeting of the American Society of Developmental Biology.*

GORDON-THOMSON, C. AND FABIAN, B.C. (1994). Determination of blood tissue in the chick is induced by fibroblast growth factor and the hypoblast. Proceedings of the *Zoological Society of Southern Africa.*

I DEDICATE THIS TO MY FAMILY

WITH APPRECIATION AND LOVE.

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ABSTRACT

The causal and temporal aspects of blood tissue specification in the chick embryo were investigated in this study. The main focus was on the role of basic fibroblast growth factor (bFGF) in the determination of the erythropoietic tissue, particularly in context with its representation as a non-axial mesodermal derivative which arises in the posterior domain of the chick embryo.

The initial strategy employed in this study was the use of agents that are known to block the activity of bFGF, and to determine their effects on erythropoiesis. Treatment of unincubated chick embryo explants with heparin, which binds specifically to the FGF family, was found to inhibit primitive streak formation and erythropoiesis, and also inhibited the formation of other mesodermal tissues. These initial findings suggested that one or more growth factors had become bound to the heparin, and that their activity is important for the specification of primitive streak formation and mesodermal patterning. The development of the erythropoietic tissue was assayed by a cytochemical test for haemoglobin using o-dianisidine; and by histological examination for blood islands and red blood cells in serial sections of the embryos after 48 hours incubation. Microscopic examination of the embryos at the stages of gastrulation on the first day of incubation revealed that heparin caused holes to appear in the ventral layer; and although a primitive streak did not form, a middle layer of mesenchymal cells were seen to accumulate between the ectodermal and ventral "endodermal" layers. It was significant that heparin's inhibitory effect on erythropoiesis could be reversed after the addition of a recombinant bovine bFGF to the heparin-treated embryos. However, the exogenous bFGF did not neutralize the inhibitory effect of heparin on the primitive streak and other mesodermal derivatives (Chapter Two).

The inhibition of erythropoiesis by heparin was also reversed by the addition of a mesodermal-inducing factor extracted from a *Xenopus* embryonic cell line, namely XTC. The XTC mesodermal-inducing factor (MIF), which belongs to the transforming growth factor-β family and is a homologue of activin, could also reverse the inhibitory effect of heparin on primitive streak formation; but no recognizable axial mesodermal structures subsequently developed. Of consequence, was that both bFGF and XTC-MIF blocked heparin's effect on the ventral layer, preventing the gaps forming. Therefore, it is suggestive that the

development of an intact ventral layer is important for the determination of the erythropoietic sequence (Chapter Three).

By taking a more specific approach using antisera to bFGF (anti-FGF) and the bFGF receptor (anti-FGFR) on whole embryo explants, it was found that anti-FGF and anti-FGFR were able to inhibit erythropoiesis, but not primitive streak formation. However, these antisera caused defects in the posterior region of the embryonic axis. These embryos not only lacked posterior blood tissue, but heart and somites were missing; whereas the anterior head structures were well formed. These results therefore suggest that bFGF signalling is important for the development of the posterior body plan, which includes erythropoiesis (Chapter Four).

Further evidence for the role of bFGF in the determination of the blood mesodermal tissue line was reached in an in vitro bioassay. In this part of the investigation, specific pieces of the blastoderm, namely pieces dissected from the posterior marginal zone (PMZ) and inner core of the central disc (ICD) were able to form haemoglobin under particular conditions. The PMZ components were found to have the capacity to form haemoglobin when dissected from blastoderms of stages X to XIII when cultured in serum-free medium. This commitment to form haemoglobin could be blocked by treatment with anti-FGF at stages X and XI, but not at the later stages of XII and XIII. The ICD components were found to have a commitment to form haemoglobin only if this component was dissected from embryos at stage XII and XIII, but not before. These results suggest that a determinative event for the haemoglobin differentiative pathway occurs between stages XI and XII. It was also found that the stage X central disc component could be induced to form haemoglobin if a stage XIII hypoblast was added to it in tissue recombination sandwich cultures, or if bFGF (75 - 150 ng/ml) was added to the medium. These results lend further support that bFGF plays an important role in the determination of erythropoiesis; and furthermore, suggest that the hypoblastic tissue is the source of this induction (Chapter Four).

Finally, immunocytochemical labelling with a polyclonal antibody to bFGF has revealed that bFGF increases significantly from stage XI in cells within the developing hypoblast layer and in the middle mesodermal layer. These cells are located predominantly in the posterior domain of the embryo. This polarized distribution of bFGF with the high value of bFGF concentration in the posterior area, is presumably responsible for inducing the overlying epiblast to form the posterior horseshoe-shaped region from which blood tissue is seen to arise. An immunocytochemical analysis of the distribution of the FGF receptor was

assessed, as an indicator of the possible competence of the cells to respond to the bFGF signal. The bFGF receptor was found to be expressed at stage XII in cells that appeared to be in register with those immunoreactive to the bFGF ligand; therefore suggesting an autocrine function. It was interesting that at stage XIII an intense immunostaining with the anti-FGFR developed in the nuclei of cells within the epiblast layer (Chapter Five).

In conclusion, this study has demonstrated that the initial determination of the erythropoietic cell lineage in the chick is at the time when the hypoblast is in the process of forming beneath the epiblast, i.e. between stages XI and XII. Furthermore, it was found that an induction by an FGF-like signal from the hypoblast layer (or middle mesodermal cells that may be closely associated with the hypoblast) induces "competent" cells (i.e. FGFR-positive cells) in the epiblast to form blood tissue in the posterior domain of the chick embryo.

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INTRODUCTION

1.1 GENERAL AIM OF THIS STUDY

A core problem in development is how cells of the embryo become programmed to form specialised cell types in specific domains of an organism. This process known as regional specification is defined by Slack (1991a) as the mechanism by which cells in different positions acquire different developmental commitments. The general consensus at present (see Slack, 1991a; Gilbert, 1991; Davidson, 1993) is that regional specification in vertebrates is controlled by two processes. In the one process, a cell becomes committed to develop along a particular pathway by virtue of inherited cytoplasmic determinants which regulate the developmental decisions of that cell. The second process is conditional, in that one group of cells will follow a particular developmental pathway only after an induction from another closely associated tissue has instructed it to do so. These two ideas of cytoplasmic determinants and tissue interactions were first formulated almost a century ago by Boveri (who revised Weismann's theory of nuclear determinants) and Spemann respectively (reviewed in Spemann and Mangold, 1924; Horder et al., 1986).

From the many investigations made on different animal systems, it appears that the specification of the body plan occurs in an epigenetic manner. In the chick, a sequence of highly co-ordinated processes bring about axis determination. The first step occurs during oogenesis when cytoplasmic components and yolk accumulate in the egg in a polarized fashion establishing its animal-vegetal axis. The second pattern determining event occurs after fertilization, when the dorsal-ventral axis is set by the formation of a subgerminal cavity beneath the cleaving, radially symmetrical blastoderm. Following this, prior to the egg being laid, the anteroposterior axis is established during the formation of the area pellucida (reviewed by Spratt, 1966; Eyal-Giladi, 1991; Schoenwolf, 1991; Khaner, 1993.)

The third change in polarity in the chick determines the region of the embryo which will subsequently form the posterior marginal zone, a region that has been found by various workers to be highly regulative. An axis (primitive streak) can only develop in separated parts of the blastoderm cultured with this posterior marginal zone intact (Spratt and Haas,

1960b; Eyal-Giladi and Spratt, 1965); while, anteroposterior rotation of the posterior marginal zone results in a reversal of the cranio-caudal orientation of the axis (Azar and Eyal-Giladi, 1979; Khaner et al., 1985; Khaner and Eyal-Giladi, 1986, 1989; Eyal-Giladi and Khaner, 1989).

The first known inductive influence in the chick occurs shortly after the anteroposterior axis has been established. The hypoblast which emerges from the posterior marginal zone interacts with the epiblast and induces embryonic axis formation, i.e. the primitive streak. Without the presence of the hypoblast beneath the epiblast a primitive streak does not form (Eyal-Giladi and Wolk, 1970; Azar and Eyal-Giladi, 1979). This hypoblastic influence appears to be involved in determining the polarity of the primitive streak. This has been demonstrated by separating the hypoblast from the epiblast and reorientated it on the epiblast. The embryonic axis will always develop in accord with the original anteroposterior axis of the hypoblast (Waddington, 1933; Azar and Eyal-Giladi, 1979, 1981). Although mesodermal tissue is derived from the primitive streak, there is no clear evidence whether the induction of the embryonic axis also specifies the mesodermal derivatives (see Khaner, 1993, for a recent discussion). From the time that Spemann put forward the concept of embryonic induction much attention has been directed at identifying the molecular nature of this signal and other inducing signals. It is only recently, with the advent of the more powerful techniques of molecular biology, that significant advances have been made in the causal analysis of induction and the identification of the signal molecules, especially with respect to mesoderm induction in the amphibian system (reviewed in section 1.4 below). Two key papers published in 1987 reported that peptide growth factors could act as the putative signal molecules in the specification of mesodermal tissue in *Xenopus* (Smith, 1987; Slack et al., 1987). The members of the fibroblast growth factor family, particularly basic fibroblast growth factor (bFGF), can change the fate of a proportion of the cells in isolated ectoderm explants of the blastula to yield ventral-type mesoderm, such as muscle, mesothelium, and "blood-like" cells (Slack et al., 1987).

This evidence suggesting that a heparin-binding growth factor is the signal molecule that induces the development of "blood-like cells" in the frog, was the cue that initiated this study. In the chick, there is a wealth of information regarding the process of red blood cell differentiation (Wilt, 1967; Kessel and Fabian, 1985, 1986, 1987); but, the factors that specify this non-axial mesodermal tissue type are not known. It is thought that non-axial mesoderm

in the chick is determined before the induction of the primitive streak, as a commitment to form blood tissue has been observed in cultures of small pieces of the blastoderm isolated before primitive streak induction occurs (reviewed in Eyal-Giladi, 1991; Slack, 1991a). However, the causal mechanisms that determine the erythropoietic cell lineage have not been examined, and the stage at which this event takes place is not known.

The aim of this study therefore is to investigate whether bFGF has a causal role in the determination of the erythropoietic cell lineage in the chick, as has been found in the frog; and to elucidate the stage at which this determinative event takes place.

1.2 MORPHOLOGY OF CHICK DEVELOPMENT, WITH AN EXPLANATION OF A NEW STANDARD TERMINOLOGY

Usage of terms that referred to different parts of the chick embryo by the various investigators in the past, has led to many misunderstandings in the literature. Recently, a consensus has been reached regarding the terminology used by a group of investigators of early chick embryology (see Khaner, 1993). A description of normal development, using this adopted standard terminology with reference to the older terminology is reported below. Normal development from cleavage to the formation of blood tissue with emphasis on the morphogenetic processes associated with blood tissue formation is explained. The earlier stages of the embryo referred to in this study follow the tables of Eyal-Giladi and Kochav (1976), and are given in Roman numerals. The later stages follow the tables of Hamburger and Hamilton (1951), and are given in Arabic numerals.

1.2.1 Early developmental stages of the chick in utero

The fertilized egg undergoes a series of cleavage divisions in the cytoplasmic region of the animal pole resulting in the formation of a radially symmetrical disc-like blastoderm of 5 to 6 layers of cells. This meroblastic cleavage takes place in utero, and is followed by complex and important morphological changes. The first of these events occurs at the designated stage VII in the tables of Eyal-Giladi and Kochav (1976). The layers in the central area of the blastoderm thin-out by the shedding of the deeper cells to eventually form a single-layered area pellucida in the centre of the blastoderm. The cast off cells fall into the subgerminal cavity in the direction of the future anterior region of the blastoderm where they disintegrate (Eyal-Giladi and Kochav, 1976; Kochav et al., 1980; Eyal-Giladi, 1984, 1991).

1.2.2 The morphogenetic processes in the pregastrula stages after the egg is laid

At the completion of the formation of the area pellucida, the egg is laid. This stage is designated stage 1 in the tables of Hamburger and Hamilton (1951), or stage X in the high resolution tables of Eyal-Giladi and Kochav (1976). By this stage the embryo is said to consist of approximately 60,000 cells. Only 500 of these cells make up the single-layered area pellucida and will give rise to the adult structures, while the remaining cells in the peripheral ring of the area opaca, which is several layers thick, will form the extraembryonic membranes (Spratt and Haas, 1960b).

At stage X, the blastoderm has become bilaterally symmetrical. The central area pellucida is made up of a single layer of organised epithelium known as the epiblast, with many islands of 5 to 20 cells scattered on its ventral surface. These islands of cells originate by a polyinvagination process from most of the epithelium excepting from the posterior border between the area pellucida and area opaca. These cells are called polyingressed cells by Eyal-Giladi and her colleagues (Eyal-Giladi, 1984; 1991) or primary hypoblast by others (Stern and Canning, 1988; Stern, 1990; 1991; Schoenwolf, 1991). More recently these authors and others have agreed to call these cells "polyingressed epiblast cells" (see Khaner, 1993). This invagination of polyingressed epiblast cells is regarded as the first signs of hypoblast formation (Kochav et al., 1980). The posterior region demarcated by a transparent strip where no polyingressed cells can be seen is known as the marginal zone which borders the area opaca and area pellucida.

A condensation of cells begins to form on the ventral surface of the inner border of the marginal zone in the shape of a crescent called Koller's sickle. It is from this posterior region that complex morphogenetic processes occur from stage X onwards. One of these morphogenetic processes involves the anterolateral expansion of hypoblast cells from the posterior region (Spratt and Haas, 1960a; Vakaert, 1962). Cells emerge from this region by proliferative activity (Weinberger and Brick, 1982) forming a sheet of cells originally called the primary hypoblast by Eyal-Giladi (1984, 1991) or the secondary hypoblast by Stern (1990). This new layer of cells migrates over the ventral surface of the epiblast integrating with the polyingressed epiblast cells on its way to the anterior marginal zone. This process takes place between stages XI and XIII to form a ventral layer of cells now called simply,

the hypoblast. (These morphogenetic movements are shown diagrammatically in Fig. 1.1).

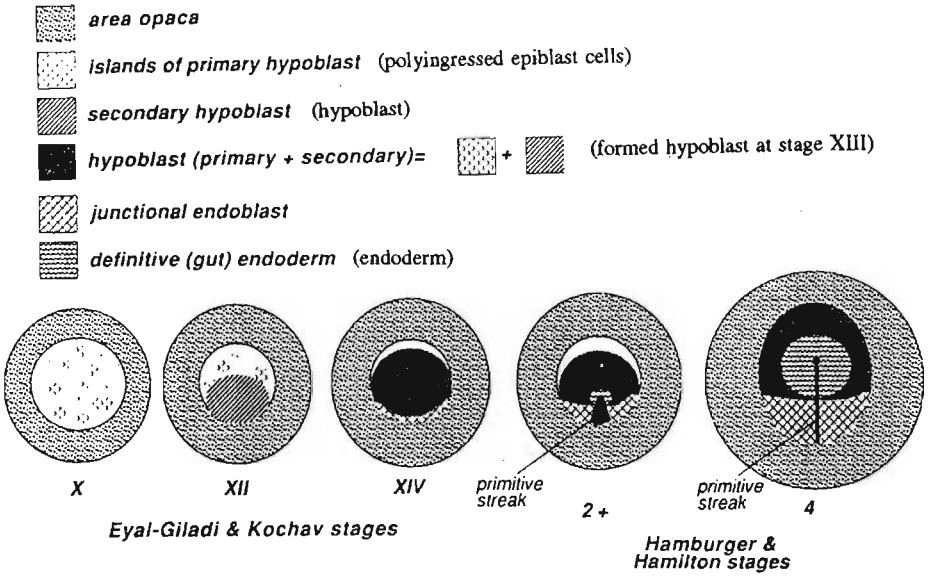

Fig. 1.1 Morphogenetic movements from stage X to primitive streak formation according to Stern (1990, 1991). The hypoblast emerges from the posterior marginal zone, migrates anteriorly, coalescing with the polyingressed epiblast cells between stages XI and XIII. The hypoblast layer becomes displaced to an anterior position after stage XIII, by the junctional endoblast (which forms from the posterior marginal zone in the wake of the hypoblast), and by the endodermal cells arising from the primitive streak.

There is some controversy as to which parts of the posterior region give rise to the hypoblast. Stern (1990, 1991) suggests that the deep endodermal cells of the germ wall which forms a ventral flap of cells situated beneath the posterior marginal zone are the progenitor cells of the hypoblast. His proposal is based on findings that these deep endodermal cells are immunoreactive with the HNK-1 monoclonal antibody, and that the fully formed hypoblast at stage XIII is also labelled with this marker. Ablation of these deep endodermal cells at stage XI and XII was found to inhibit the formation of the hypoblast in his experiments. Contrary to Stern's findings, Eyal-Giladi et al. (1992) have shown that by labelling the stage X posterior marginal zone component with lysine-rhodamine-dextran, the hypoblast emerges from the labelled posterior marginal zone and Koller's sickle.

A separate morphogenetic process occurs shortly after the hypoblast begins to spread anteriorly. At stage XII a subpopulation of cells from a more superficial region of either the posterior marginal zone (Stern, 1990), or a narrow strip of the epiblast anterior to Koller's

sickle (Eyal-Giladi et al. 1992) converge in a narrow region along the midline to the centre of the epiblast forming the anlage of the primitive streak by stage 2.

Despite these conflicting ideas of the exact source of the hypoblast and the central epiblast cells, it seems that the posterior region of the marginal zone, and its adjacent tissues, is the epicentre of developmental processes in the chick from which epiblast and hypoblast cells arise and spread anteriorly. By stage XIII, the area pellucida is composed of a bilaminar disc of upper epiblast and lower hypoblast with a blastocoel cavity separating the two layers. This stage is reached after approximately nine hours of incubation and is considered to be the blastula stage of the chick embryo.

1.2.3 **The formation of mesoderm and endoderm associated with blood tissue formation**

The process of gastrulation basically involves the migration of cells from the surface of the embryo into the interior resulting in the formation of the three primary germ layers of ectoderm, mesoderm and endoderm. In the chick embryo all these germ layers arise from the epiblast layer of the area pellucida. The classical view of cells invaginating through a primitive streak is now considered by several authors to be inaccurate. Various workers have observed a middle layer of cells between the epiblast and hypoblast forming long before the primitive streak arises, and before the anterior migration of the hypoblast is complete. It appears that cells in the posterior region of the epiblast ingress by a de-epithelialization (see Fig. 1.2) to form a middle layer of cells from stage XII onwards (Vakaert, 1962, 1984; Azar and Eyal-Giladi, 1979; Stern and Canning, 1988; Stern, 1991; Schoenwolf, 1991); or possibly even earlier from stage XI (Izpisua-Belmonte et al., 1993).

As mentioned above, cells within the epiblast converge to the median line of the blastoderm forming the primitive streak, a process known as primitive streak progression (Bellairs, 1986). This convergence stops once cells begin to ingress from the lower surface of the primitive streak. Various fate mapping studies have shown that the first cells to ingress are the presumptive endodermal cells. These cells leave the cranial half of the primitive streak at stage 2 (Vakaert, 1962; Rosenquist, 1972; Selleck and Stern, 1991), and dock into the hypoblast layer initially forming a mosaic of hypoblast and endodermal cells. As the cells of the hypoblast layer are loosely attached (Sanders et al., 1978) they allow for this integration, and eventually become displaced to a peripheral position. (Other terms used for this endodermal layer have been endoblast, tertiary hypoblast, definitive endoderm and gut

endoderm). According to some authors a further recruitment of cells from the posterior marginal zone migrates into this endodermal layer. This has been called the junctional endoblast and forms in the wake of the anteriorly migrating hypoblast layer after stage XII, eventually displacing the hypoblast to the anterior germinal crescent (refer to Fig. 1.1) by stage 4 (Vakaert, 1970; Stern and Ireland, 1981; Stern, 1991). An alternate view is that the hypoblast is displaced by the endoderm to the periphery of the area pellucida (refer to Fig. 1.3) by stage 7 (Rosenquist, 1972; Azar and Eyal-Giladi, 1983; Eyal-Giladi, 1991). Whichever route it takes, it is agreed that the hypoblast by stage 10 is situated at an anterior position in the germinal crescent (Spratt and Haas, 1960a; Azar and Eyal-Giladi, 1983; Eyal-Giladi, 1991; Stern, 1991) where it eventually gives rise to the endodermal lining of the yolk sac stalk (Rosenquist, 1972).

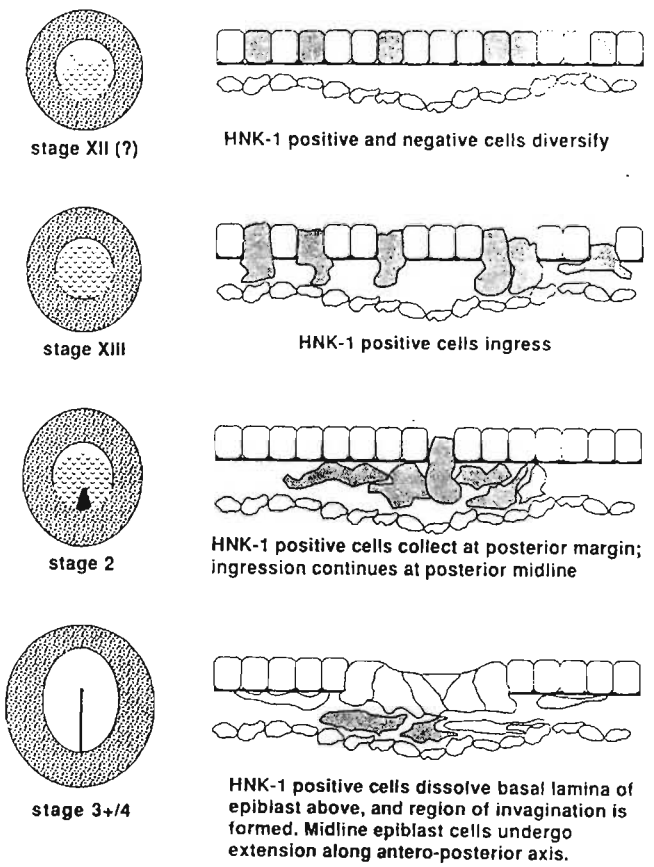

Fig. 1.2 A revised view of chick gastrulation, according to Stern (1991), showing HNK-1 positive cells in the epiblast layer migrating to form a middle mesodermal layer before primitive streak formation.

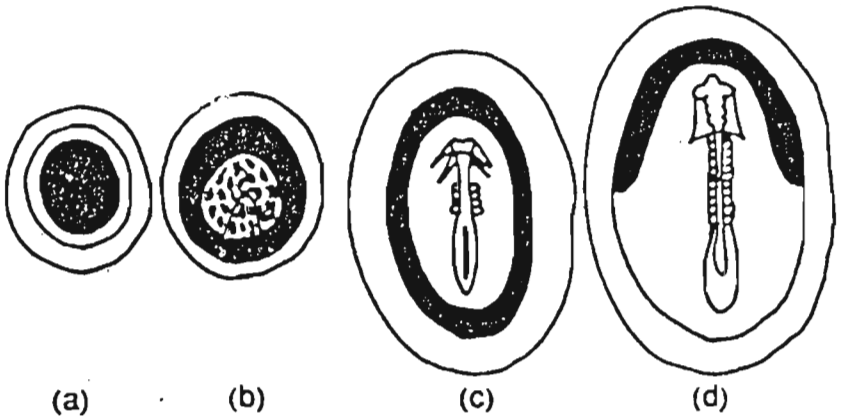

Fig. 1.3 The position of the hypoblastic cells (shaded area) in the blastoderm, according to Eyal-Giladi (1991), at (a) stage XIII; (b) stage 3; (c) stage 8; and (d) stage 10.

Following the migration of the prospective endoderm from the primitive streak is the ingression of the prospective mesoderm (or mesoblast) at stages 3+ to 10. The first mesodermal cells that migrate from the primitive streak are cells that originate from the posterior part of the epiblast. Earlier fate mapping studies have traced the prospective blood tissue back, and have shown that the prospective blood cells arise from a horseshoe-shaped area in the posterior region of the "late prestreak stage" epiblast (Settle, 1954; and reviewed by Wilt, 1967). Further studies using tritiated thymidine-labelled grafts showed that the progenitor blood cells were the first mesodermal cells to leave the caudal half of the primitive streak and migrate laterally inside the blastocoel between the epiblast and hypoblast (Rosenquist, 1966; Nicolet, 1970). Labelling and tracing studies by Eyal-Giladi et al. (1992) have shown that epiblast cells in the posterior region (anterior to Koller's sickle) converge anteriorly along the median line of the epiblast during hypoblast formation; and that these labelled cells later form the distal leading edge of the laterally migrating mesoblast inside the blastocoel (Eyal-Giladi et al., 1992). Whether these are the progenitor blood cells traced by the earlier workers has not been validated. The location of the progenitor blood cells in the epiblast, and their migration to the middle layer therefore is not clear.

There is no doubt however that the first wave of mesodermal cells that spread centrifugally between the epiblast and hypoblast of the area pellucida to the posterior area opaca will form the blood tissue. This mesoderm extends into a region described as a "horseshoe-shaped area" in the posterior and posterolateral parts of the area opaca by stage

4, establishing the area opaca vasculosa (AOV). It is in this AOV region of the embryo that the first vascular system and haemoglobin-containing cells develop (Fig. 1.4).

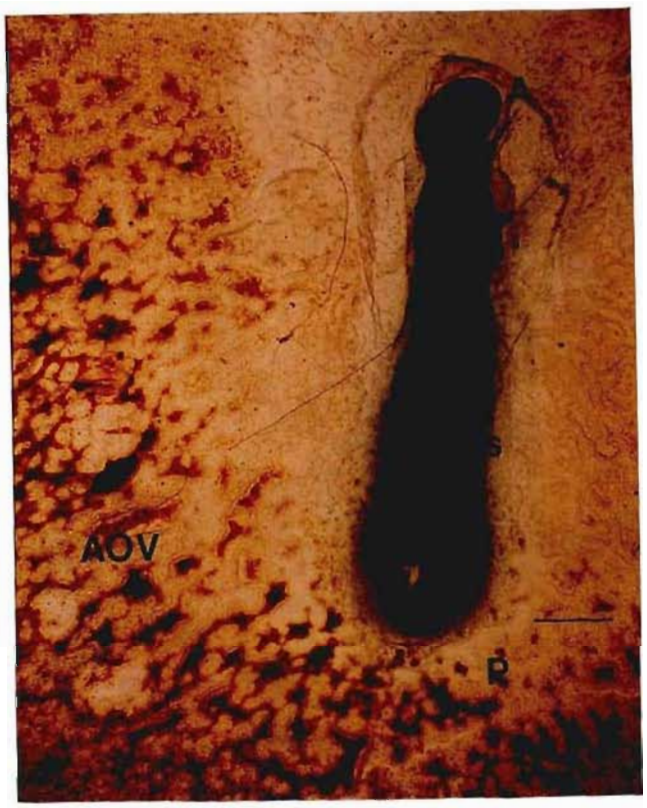

Fig. 1.4 A two day old chick embryo showing the distribution of the red blood cells (stained brown with the o-dianisidine test for haemoglobin), in blood vessels of the area opaca vasculosa (AOV). The AOV begins to form in a posterior horseshoe-shaped area. (Abbreviations: A = anterior head region of the embryonic axis; P = posterior region; S = somites.) Bar, 1 mm.

1.2.4 **The haemoglobin differentiative pathway in the AOV**

Once the distal edge of the migrating mesodermal cells reaches the posterior area opaca, groups of mesodermal cells start transforming into an organised system of blood vessels and red blood cells. This transformation begins at stage 5 when aggregates of mesodermal cells form tight clusters of cells called blood islands in close association with the underlying AOV endoderm (Fig. 1.5). It is interesting to note that the AOV endoderm at this stage is a thick stratified epithelium composed of large yolk-filled cells, derived either from the displaced hypoblast, or from the junctional endoblast (see section 1.2.3; and Figs 1.1 and 1.3 above). When the mesoderm splits, the blood islands remain in the splanchnic mesoderm adjacent to

the AOV endoderm, and subsequently differentiate into endothelial-lined vessels surrounding haemoglobin-filled erythrocytes (Wilt, 1967; Gonzalez-Crussi, 1971; Kessel and Fabian, 1985).

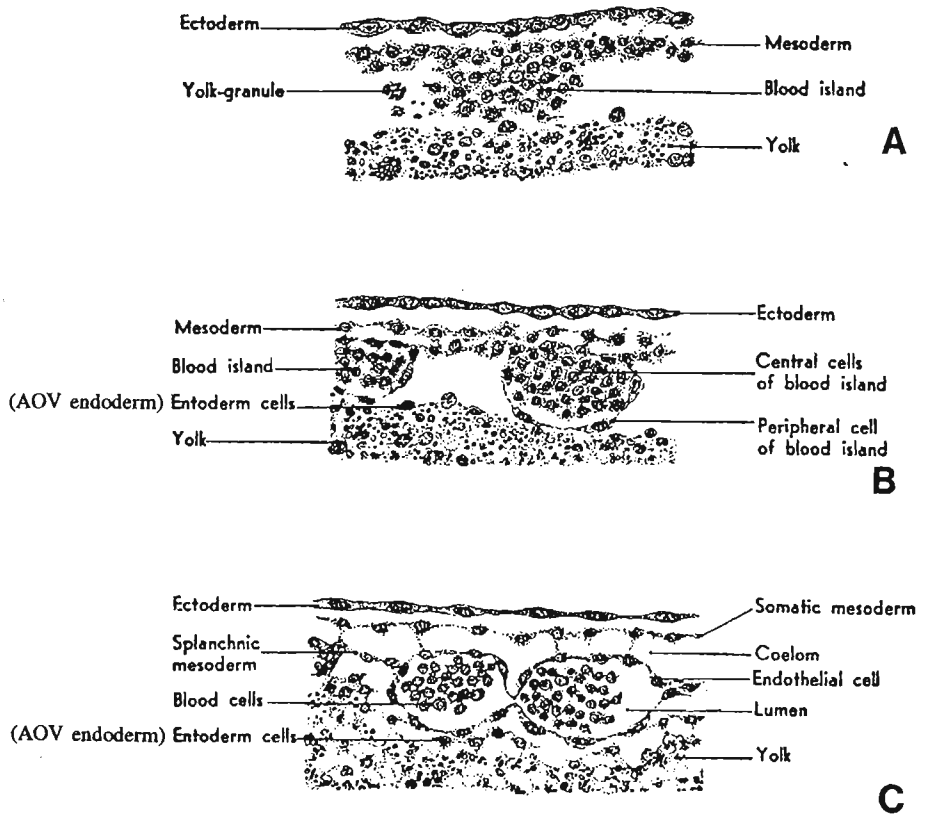

Fig. 1.5 Diagrammatic representation of the cellular organization of blood islands in the area opaca vasculosa; (A) at 18 hours; (B) 24 hours; and (C) 33 hours. (From Browder, 1980, after Patten, 1971.)

1.3 PRESENT VIEWS ON BLOOD TISSUE SPECIFICATION IN THE CHICK

Haemoglobin (Hb) formation in the blood islands of the AOV mesoderm requires the influence of the AOV endoderm at stage 4. This has been demonstrated by culturing pieces of the AOV mesoderm with and without AOV endoderm from stage 4 blastoderms (Wilt, 1965, 1967; Muira and Wilt, 1969; Kessel and Fabian, 1986, 1987). Wilt (1965) described this tissue interaction as "salutary" rather than an instructive induction because of (1) the low level of blood tissue (blood islands) developing in AOV mesoderm in the absence of the AOV endoderm, (2) the low levels of globin transcripts found in the mesodermal cells by stage 4 before blood island formation, and (3) the sensitivity of haemoglobin synthesis to actinomycin

and 5-bromodeoxyuridine (BUdR) treatment becoming refractory before stage 4 (Wilt, 1967). The AOV endoderm also stimulates erythropoiesis in isolated fragments of area pellucida mesoderm that is normally non-erythropoietic (Kessel and Fabian, 1987). Clearly, this evidence supports the idea that a decision is made for the specification of the erythropoietic cell lineage at an earlier stage in the chick embryo.

Wilt (1974) proposed that the Hb differentiative pathway is determined before the egg is laid, as small pieces of the unincubated blastoderm were found to be already committed towards Hb differentiation, as identified by the o-dianisidine test of O'Brien (1960). He found that those parts dissected from a horseshoe-shaped area comprising the posterior and posterolateral parts of the marginal zone and adjacent area pellucida (refer to Fig. 1.6) could form Hb when grown in culture. He describes the blastoderm from which he isolated the small pieces, as "a simple disc of cells"....."in the process of forming the embryonic tissues (epiblast and hypoblast)" (Wilt, 1974); a stage that was probably equivalent to XI or XII in the high resolution tables of Eyal-Giladi and Kochav (1976). These results can be interpreted as follows: The cells in this posterior horseshoe region of the marginal zone are already determined to form Hb (Wilt's view); or, alternatively, all the components required for later cell interactions are held within these isolated pieces, and the causal steps leading to the determination of Hb take place at a later stage.

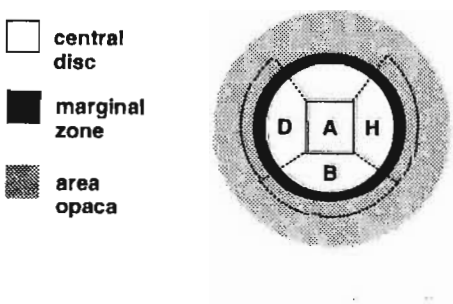

Fig. 1.6 The pieces, i.e. components, of the unincubated blastoderm which showed a high propensity to form Hb when isolated and grown in culture for 3 days, were found by Wilt (1974) to be situated in a posterior horseshoe-shaped area of the blastoderm. The pieces dissected from this horseshoe-shaped area were component B (of which 12 out of 20 tests formed Hb); component D (8 out of 20 tests formed Hb); and component H (10 out of 20 tests formed Hb). These three components (D, B and H) were composed of marginal zone and adjoining epiblast. The inner core of the central disc (component A) did not form Hb (0 out of 20 cases).

The marginal zone as the source of specified erythroid cells at this early stage has also been demonstrated by Zagris (1985). He found that wedge-shaped portions of the stage X blastoderm had the potentiality to form erythroid cells, only when part of the marginal zone was included in the portion. Notwithstanding, Zagris (1982) suggests that the unincubated blastoderm is induced to form erythroid cells by the hypoblast, as he found that supernumerary hypoblasts placed on a single pregastrula blastoderm led to the formation of multiple AOV foci, and excess Hb formation.

The current view held by recent investigators is that progenitor mesodermal cells in the epiblast become specified to form blood cells before the specification of the axial mesoderm. This is based on observations of blood island formation (assessed by histological evaluation rather than by Hb differentiation) in cultures of the stage XIII epiblast stripped of marginal zone and hypoblast (Azar and Eyal-Giladi, 1979; Mitrani and Eyal-Giladi, 1982). The primitive streak does not develop in these pieces unless the hypoblast or marginal zone is replaced. The presence of non-axial mesoderm but not axial mesoderm in these explants, therefore suggests that mesoderm induction is a sequential multistep process, with non-axial mesoderm being specified before the axial mesoderm (for reviews see Eyal-Giladi, 1991; Slack, 1991a; Stern, 1992; Khaner, 1993).

Wilt (1974) found that at the stages before this (i.e. stage XI and XII), the central disc of the area pellucida stripped of the marginal zone did not have the potentiality to form Hb. Contradicting this however, are reports that central discs explanted at stage X formed blood islands when grown in culture (Mitrani and Eyal-Giladi, 1981; Ginsburg and Eyal-Giladi, 1987). However, it should be noted that the components were cultured in media supplemented with serum, and the added extraneous factors may have stimulated blood island formation in these cases.

In summary, the various findings described above point to the non-axial mesodermal tissue being determined either at the same time, or before the axial mesoderm. However, the causal mechanisms effecting blood tissue specification, and the stage at which it occurs, await clarification.

1.4 MESODERM INDUCTION AND THE ROLE OF GROWTH FACTORS AS SIGNAL MOLECULES

Significant advances have been made recently to our current understanding of the causal mechanisms of the specification of axial and non-axial mesoderm in amphibian embryos. In amphibians, mesodermal tissue arises in the equatorial region of the animal hemisphere. It has been clearly shown to be formed by multistep inductive interactions beginning at the mid-cleavage stages. These mesodermal inducing interactions have been demonstrated experimentally by combining blastomeres from different regions of the midblastula stage and onwards, and analysing their developmental fate (Nieuwkoop, 1969; Dale et al., 1985; Dale and Slack, 1987). It was found that when cells from the animal hemisphere of the blastula were cultured in isolation, they formed epidermis. However, when cultured in combination with the vegetal blastomeres (presumptive endoderm) they formed mesoderm. Furthermore, the vegetal blastomeres dissected from the prospective ventral region induced ventral-type mesoderm (i.e. mesothelium, blood and some muscle); while the dorsovegetal cells induced dorsal mesoderm (i.e. notochord and somites). These two inductive signals are described as the first two "signals" in the three signal model that has been advanced to explain mesodermal patterning in the frog (Dale and Slack, 1987). In this model it has been proposed that the first mesoderm inducing signal is released uniformly from the vegetal hemisphere creating a ring of ventral-type mesoderm in most of the equatorial zone. A second signal localized in the dorsal vegetal region induces dorsal mesoderm in the dorsal equatorial zone. Finally, the third signal originating in the prospective dorsal mesoderm (known as the organizer), converts the initial ventral-type mesodermal cell types across the equatorial zone into a variety of different intermediate mesodermal types. The discovery that peptide growth factors act as the candidate signal molecules in these inductions has led to intensive studies which have established a contemporary model that elegantly describes the balanced and harmonious nature of mesodermal induction and anteroposterior body patterning in *Xenopus* (reviewed in Smith et al., 1989; Slack, 1990; Kimelman et al., 1992; Slack and Tannahill, 1992; Green et al., 1992; Jessell and Melton, 1992).

1.4.1 Evidence for growth factor signalling in *Xenopus*

The blastomere combination experiments mentioned above provided a bioassay with which to identify the signal molecules responsible for mesoderm differentiation. To date a number

of polypeptide growth factors have been provisionally identified as morphogens in the embryo. Members of the fibroblast growth factor (FGF) (also known as the heparin-binding growth factors) and transforming growth factor-β (TGF-β) families induce a variety of mesodermal tissues in vitro. The members of the FGF family that act as mesodermal-inducing factors (MIF) include FGF-1 (acidic or aFGF), FGF-2 (basic or bFGF), FGF-3 (int-2, the product of the murine *int*-2 oncogene), FGF-4 (a product of the human *ks* and *hst* oncogenes, kFGF), FGF-5, XeFGF (a novel FGF from *Xenopus* embryos), and embryonal carcinoma derived growth factor (Slack et al., 1987; Slack, 1990; Isaacs et al., 1992).

The basic fibroblast growth factor can mimic the ventral vegetal cell signal and induce the formation of ventral-type mesoderm in animal cap cells (Slack et al., 1987; Slack, 1990). The tissue in the animal cap that was induced by 2 to 30 ng/ml bFGF was identified by Slack et al., (1987) as "a concentric arrangement of loose mesenchyme, mesothelium and blood cells...", but, further explained that "... these blood cells, do not usually differentiate sufficiently to synthesize haemoglobin, but have a characteristic morphological appearance." However, "blood-like" cells induced by bFGF have not been clearly identified in *Xenopus* animal cap assays, unlike in the ventral vegetal cell-animal cap combination experiments, where genuine erythrocytes were identified (see Green et al., 1990).

The members of the TGF-β family that have shown mesodermal-inducing activity include a secretory product from a *Xenopus* tadpole cell line, the XTC mesodermal-inducing factor (XTC-MIF; also know as activin A, *Xenopus* activin, or erythroid differentiation factor), activin B, TGF β-2, TGF β-3, a secretory product from a mouse macrophage cell line P388D1 or PIF, and bone morphogenetic protein-4 (BMP-4) (Rosa et al., 1988; Smith et al., 1990; Sokol et al., 1990; Asashima et al., 1991; Jessell and Melton, 1992). These are potent inducers of dorsal mesoderm (notochord and somites), and therefore resemble the inductive interaction of the dorsal vegetal blastomeres on animal cap cells. Recent studies have shown that different mesodermal types are induced in response to variations in the concentration of these growth factors (Green et al., 1992). Low doses of activin or FGF induce posterior mesodermal structures and markers, eg. the expression of the homeobox-containing genes, *Xbra*, *X1Hbox6* and *Xhox3*, which are normally expressed in the posterior half of the embryo (Cho and De Robertis, 1990; Ruiz i Altaba and Melton, 1989; Smith et al., 1991). Activin at high doses induces notochord formation in animal caps, and can also increase the expression of anterior markers, eg. the *goosecoid* gene of the dorsal lip organizer and

XlHboxl (Ruiz i Altaba and Melton, 1990; Cho and de Robertis, 1990; Green and Smith, 1991; Jessell and Melton, 1992; Green et al., 1992).

Even at high doses bFGF only induces ventral mesoderm, however it does have a synergistic effect with activin or TGF-β, and potentiates the induction by these dorsal signals (Kimelman and Kirschner, 1987; Green et al., 1992). It is suggestive therefore that mesoderm specification and the anteroposterior body patterning is generated by gradients and thresholds of growth factors within the embryo (Green and Smith, 1991).

The localization of growth factor proteins and mRNAs that encode the FGF family (bFGF and XeFGF), and the TGF-β family (Vg 1, activin B and BMP-4) in the embryo at the time of mesoderm induction further implicates these molecules as the natural inducers of mesoderm in *Xenopus* (Kimelman and Kirschner, 1987; Weeks and Melton, 1987; Thomsen et al., 1990; Shiurba et al., 1991; Dale et al., 1992; Hemmati-Brivanlou and Melton, 1992).

Functional evidence that growth factors act as mesodermal-inducing factors (MIFs), is supported by recent studies made on the perturbation of the receptive pathways for the signal transduction. FGF receptor (FGFR) signalling can be disrupted by injecting a dominant mutant receptor (XFD) lacking the tyrosine kinase domain, or a non-functional *raf-1* RNA into the cleaving embryo (Amaya et al., 1991; Amaya et al., 1993; MacNicol et al., 1993). Explanted animal caps derived from embryos expressing the truncated receptor or with blocked Raf kinase activity fail to form mesoderm in response to FGF treatment, but can be rescued by a forced overexpression of the wild-type receptor or *raf-1* RNA. In whole embryos the XFD constructs inhibit the formation of muscle and notochord, and the expression of *Xbra* and *Xpo*, early markers of ventral and lateral mesoderm (Amaya et al., 1993). Similar studies using a truncated TGF-β receptor abolishes axial structures (Hemmati-Brivanlou and Melton, 1992). These results give compelling evidence that signal transduction by growth factor receptors influences mesodermal differentiation. Although the components of this signalling pathway are known, the mechanism of growth factor signalling in mesoderm induction is still not clear. The localization of the endogenous growth factors and receptors do not show great regional specificity. Cell-cell interactions of embryonic induction presupposes that a signal molecule secreted from the inducing cell binds to its specific receptor on the competent responding cell. This would make the bFGF molecule an unlikely candidate as it has no signal sequence for its release through a conventional secretory pathway. This is not the case for XeFGF (Isaacs et al., 1992) however. It may be possible

that the bFGF has another unorthodox secretory pathway (see Kandel et al., 1991), or is released by cell lysis (Jessell and Melton, 1992), or acts in an autocrine manner (Dickson et al., 1990).

Studies made by Slack (1991b) throw some doubt on activin and bFGF being rated as the signal molecules secreted by the vegetal cells in mesoderm induction. In his transmembrane filter apparatus experiments, the natural induction by vegetal cells on the animal cap could not be inhibited by placing antisera to bFGF or follistatin (an inhibitor of activin) between the vegetal and animal cap cells. However, heparin, a highly sulphated glycosaminoglycans which has a high affinity for bFGF (Gospodarowicz et al., 1987), can neutralize this induction (Slack et al., 1987; Slack, 1991b). All the above neutralizing agents block the inducing activity of these growth factors in the in vitro assays, however.

Other growth factor-like proteins of the *Wnt* family and *noggin* which are also present in early frog embryos, are thought to be competence factors that influence the type of mesoderm that is induced by growth factor signalling (Christian et al., 1991; Moon and Christian, 1992; Beddington and Smith, 1993; Lemaire and Gurdon, 1994).

1.4.2 **The role of growth factors in mesoderm induction in the chick embryo**

Recent studies have revealed that some growth factor signalling controls the specification of the embryonic axis in the chick, which is comparable to that found in the frog. In the chick embryo, the embryonic axis, i.e. the primitive streak, and axial mesoderm arise from one or possibly more inductions from the ventral hypoblast on the overlying competent epiblast at stage XIII. This interaction between the two germ layers of the chick can be compared to that demonstrated between the vegetal and animal hemisphere cells of the frog blastula. Also, as in the frog, it is members of the TGF-β family, namely XTC-MIF and activin B, that can imitate this dorsal mesodermal endogenous signal in the chick (Mitrani and Shimoni, 1990; Mitrani et al., 1990b). It was shown that isolated chick epiblasts at stage XIII will form blood islands, mesenchyme and muscle when cultured without a hypoblast (Azar and Eyal-Giladi, 1979; Mitrani and Eyal-Giladi, 1982); but will generate an embryonic axis with notochord and somites, when cultured with either a hypoblast, activin B, or XTC-MIF. Activin B can also induce an ectopic axis when placed at 90^{0} to the posterior marginal zone (Ziv et al., 1992). Furthermore, an endogenous activin B is transcribed in the hypoblast at the time that axial mesoderm is induced (Mitrani et al., 1990b). However, it is not yet clear

whether the induction of the primitive streak and the axial mesodermal derivatives in the epiblast are controlled by the same inducing factor (reviewed by Stern, 1992; Khaner, 1993).

Mitrani and his colleagues have also isolated FGF protein and its transcripts from the blastoderm at this stage (Mitrani et al., 1990a), but found that this growth factor could not induce axial structures in vitro (Mitrani and Shimoni, 1990). The bFGF protein was distributed throughout the blastoderm; while the bFGF RNA was localized in the marginal zone at stage XIII. It is interesting that heparin and suramin which blocks FGF action, can also inhibit the formation of the embryonic axis (Mitrani et al., 1990a).

It is therefore suggestive that FGF signalling may act at the pregastrula stage in the chick, as in *Xenopus*; and play a role in the specification of non-axial mesoderm which is equivalent to the ventral-type mesoderm in the frog. The in vitro effect of activin B on axial mesodermal induction, and the presence of activin and FGF in the chick embryo, justifies this notion.

1.5 AN OVERVIEW OF THIS STUDY

The present study investigates the determinative events which launch the development of the erythropoietic pathway in the chick embryo. The initial question asked in this study is whether a growth factor signalling mechanism occurs in the chick; particularly with regard to the specification of the erythropoietic cell lineage, i.e. a non-axial mesodermal tissue.

This could be tested experimentally by observing the effects of treatment of pregastrula stage embryos (see Fig. 1.7) with substances which neutralize FGF activity. After each treatment, the early morphogenetic changes were observed using conventional microscopic techniques on whole mounts and serial sections. Assessment of erythropoietic tissue differentiation in the explants was made cytochemically with a sensitive modification of the benzidine stain (O'Brien, 1960), which uses o-dianisidine to detect the pseudo-peroxidase activity of Hb (see also Wilt, 1965, 1967; Kessel and Fabian, 1986, 1987).

Initially, a non-specific approach was taken by treating unincubated embryo explants with heparin (as a FGF binding factor); and counteracting the inhibitory effects of heparin with the addition of recombinant bovine bFGF (Chapter Two), or with conditioned medium from XTC cells (Chapter Three).

In a more specific approach the embryos, or pieces of embryos, were cultured in antisera to bFGF (anti-FGF) and to the bFGF receptor. An in vitro assay was designed for

culturing small pieces of the blastoderm in a small volume of antisera in a neutral medium (i.e. economy of scale). Pieces of the unincubated blastoderm (posterior marginal zone - PMZ) which were found to have a commitment to form Hb when cultured in a neutral medium, were challenged with anti-FGF. Pieces of the epiblast (stage X inner core of the central disc - ICD) which were not committed to form Hb when cultured in a neutral medium, were stimulated to form Hb with the addition of recombinant bovine bFGF to the culture medium (Chapter Four).

The evidence from these experiments showing that the presence of bFGF is necessary for the specification of the erythropoietic sequence, allowed the following questions to be addressed:

(i) At what stage in development does a determinative event occur in the chick that directs a proportion of the epiblast cells to enter a Hb differentiative pathway?

This was elucidated by the observation of a window in development when the inhibitory effect of heparin, or anti-FGF, on erythropoiesis becomes refractory (Chapter 4).

(ii) Which tissue is the source of the FGF-like signal responsible for the specification of the erythropoietic cell lineage?

The results of tissue recombination experiments, and an immunocytochemical analysis to detect the endogenous bFGF, points to the hypoblastic tissue as a source of bFGF (Chapter 4 and 5).

(iii) How does the posterior horseshoe pattern of blood tissue which becomes specified during hypoblast formation arise in the chick?

Immunocytochemical analysis of whole mount preparations revealed a posterior predominance of bFGF in the developing hypoblast. This posterior area of high bFGF concentration lies within the field of the posterior and posteriolateral horseshoe-shaped area of prospective blood tissue at the stage when blood tissue becomes specified (Chapter 5). An immunocytochemical assay of the bFGF receptor was carried out to determine which cells in the blastoderm may be competent to respond to the FGF signal (Chapter Five).

In the concluding discussion (Chapter Six), a synthesis of the main findings of this study is presented; and some models are proposed to explain the causal basis of blood tissue specification in the chick embryo.

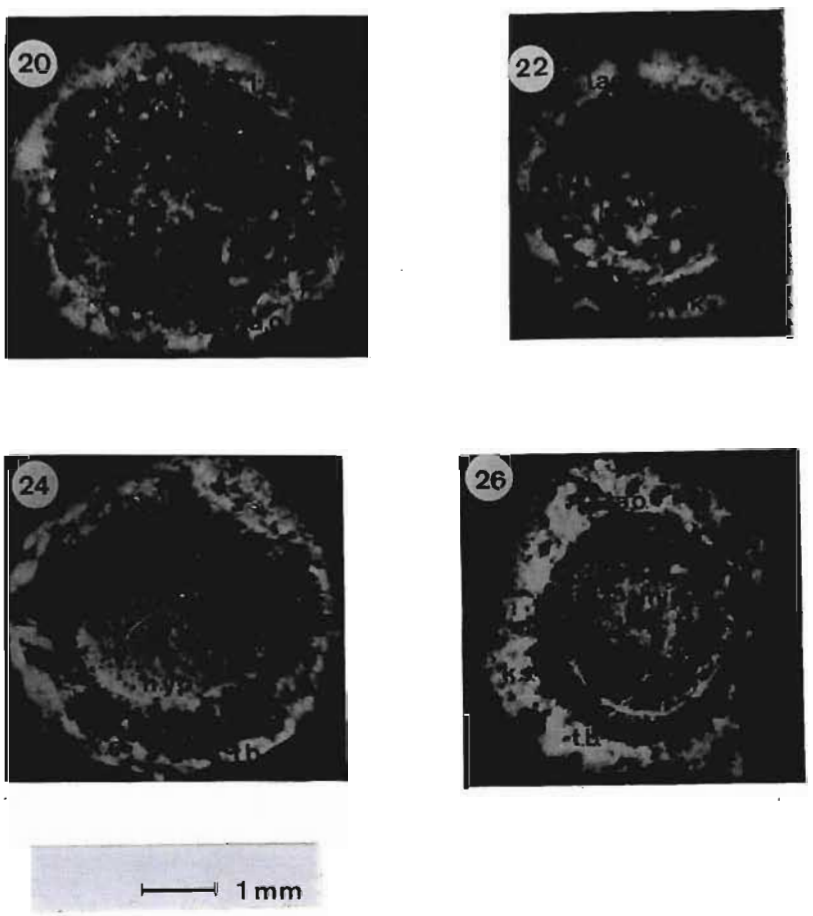

Fig. 1.7 Photomicrographs of the ventral surfaces of embryos at stages X to XIII from the tables of Eyal-Giladi and Kochav (1976). The stages used in the experiments described in this thesis were very carefully selected according to the ventral features of the blastoderms, as shown in the photomicrographs numbered 20, 22, 24 and 26 above.

(No. 20) **Stage X**: Note that the ventral surface of the area pellucida is covered with isolated cell aggregates (i.ag) known as polyinvaginated epiblast cells; the posterior marginal zone is indicated by a transparent belt (t.b) between the area opaca (a.o) and polyinvaginated epiblast cells at one end of the area pellucida.

(No. 22) **Stage XI**: The transparent belt of the posterior marginal zone (t.b) has become more defined with an anterior border demarcated by a horseshoe-shaped concentration of cells referred to as Koller's sickle (k.s). The hypoblast layer is beginning to form anterior to Koller's sickle.

(No. 24) **Stage XII**: The posterior marginal zone (t.b) is well demarcated by a transparent belt posterior to Koller's sickle (k.s), and the sheetlike hypoblast layer (hyp) covers half of the ventral surface of the area pellucida.

(No. 26) **Stage XIII**: The fully formed hypoblast layer (hyp) now covers a central disc-shaped area of the area pellucida. The well demarcated transparent belt (t.b) between the area opaca and the hypoblast is called the marginal zone. The horseshoe-shaped Koller's sickle (k.s) forms the posterior border of the hypoblast.

CHAPTER TWO[1]

FIBROBLAST GROWTH FACTOR CAN NEUTRALIZE THE INHIBITORY EFFECT OF HEPARIN ON ERYTHROPOIESIS BUT NOT ON PRIMITIVE STREAK FORMATION IN CHICK EMBRYOS

2.1 SUMMARY

There is increasing evidence that growth factors and growth factor-like substances act as morphogens in early amphibian development. For example, a heparin-binding growth factor, basic fibroblast growth factor (bFGF) has been shown to be involved in the induction of non-axial mesoderm, such as blood. This study examines whether bFGF plays a similar role in the development of blood tissue in the chick embryo. The approach is to culture undifferentiated chick blastoderms from stage X (the stage before the induction of the primitive streak) on vitelline membranes which rest on a medium containing heparin. Using the o-dianisidine staining reaction to identify haemoglobin-positive blood cells, it was found that heparin at concentrations of 3 mg/ml and higher has three effects on developing chick embryos: it inhibits haemoglobin-positive blood cell differentiation; it inhibits primitive streak formation - although "mesenchymal" cells are observed between the forming ectoderm and endoderm; and it causes the ventral layer to develop with large gaps, or holes. The addition of bFGF at doses of 5-500 ng/ml together with 3 mg/ml heparin to the embryos at stage X reverses the inhibitory effect of heparin on haemoglobin-positive blood cell differentiation; as well as preventing the formation of the holes in the ventral "endodermal" layer. However, bFGF is not able to reverse the inhibition of primitive streak formation under the conditions of this experiment.

These results support the involvement of bFGF in the induction of non-axial mesoderm, such as blood tissue, but not in the induction of primitive streak formation. Other possible effects of heparin are discussed.

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[1] The experimental work described in this chapter has been published in the *South African Journal of Science* **87**, 227 (1991). This chapter is transcribed from the manuscript, with minor alterations, in keeping with the style of the thesis.

2.2 INTRODUCTION

Recent findings have shown that a number of growth factors can act as mesodermal inducers in frog embryos (reviewed by Smith, 1989; Woodland, 1989). A family of heparin-binding growth factors (HBGF) to which bFGF belongs is able to induce the formation of small amounts of muscle and "blood-like" tissue in animal cap cells from *Xenopus* blastulas (Slack et al., 1987; Slack et al., 1989; Slack, 1990). Normally, these cells would develop into epidermis. Furthermore, the natural signal for ventral mesoderm induction in *Xenopus* can be neutralized by heparin suggesting that the natural morphogen is similar to a heparin-binding growth factor (Slack et al., 1987; Slack, 1991b). There is also evidence that bFGF is present in situ. A mRNA that codes for a protein homologous to bFGF, and also a protein with similar characteristics to bFGF, have both been detected in *Xenopus* (Kimelman and Kirschner, 1987; Kimelman et al., 1988; Slack and Isaacs, 1989) and chick (Mitrani et al., 1990a) embryos.

This preliminary investigation shows that heparin has an inhibitory effect on the formation of non-axial mesodermal derivatives particularly on the erythropoietic sequence in chick embryos; as well as effecting primitive streak and "endoderm" formation. The rescue effect of bFGF on embryos cultured in heparin, is also reported.

2.3 MATERIALS AND METHODS

The strategy was to culture the chick blastoderms of freshly laid hens' eggs (obtained from Rainbow Chicken Farms), on rafts made from the vitelline membrane over media containing heparin (which is used as a blocking agent) and bFGF. Under aseptic conditions, the blastoderms were dissected from the yolk and cleaned in Ringer solution (see Appendix 1) to remove excess yolk. Only blastoderms that were easily identified as stage X of the tables in Eyal-Giladi and Kochav (1976) were used in this study (refer to Fig. 1.7 in Chapter One). Each blastoderm was explanted ventral side up on its own vitelline membrane which was stretched over a glass ring (New, 1955; refer to Appendix 2), and transferred to a watch glass containing the fluid component of the egg albumen with the various additives (see Table 2.1). Each watch glass was placed on cotton wool moistened with Ringer solution in a Petri dish and incubated at 38°C in a humidified atmosphere for a period of 48-56 hours (referred to as day 2 from here on).

Preliminary experiments were undertaken to determine the effect of heparin on chick development. Concentrations ranging from 1-5 mg/ml of heparin (Na salt, Grade 1 : Sigma, USA) in albumen were used. Explanted blastoderms attached to vitelline membranes were cultured continuously over the medium containing the heparin. Control embryos (A and B in Table 2.1) were cultured on 1 part Dulbecco's minimum Eagles medium (DMEM) (obtained from Highveld Biological Supplies, RSA) with 19 parts albumen, or with the addition of 3 mg/ml chondroitin sulphate (Sigma) which is a glycosaminoglycan similar to heparin. Mesoderm formation was determined by the appearance of a primitive streak between 18 and 20 hours (day 1) of incubation. Differentiation of mesodermal tissue into blood tissue was determined after 48 hours (day 2) of incubation using the o-dianisidine test for haemoglobin (O'Brien, 1960; Kessel and Fabian, 1986, 1987; refer to Appendix 1). The stained whole mounts were examined by bright field light microscopy.

TABLE 2.1. Summary of the additives to culture media*

In determination of optimum heparin concentration:	
Experiment	1-5 mg/ml heparin
Control A	No additives
Control B	3 mg/ml chondroitin sulphate
In bFGF treatment:	
Experiment	5-500 ng/ml bFGF + 3 mg/ml heparin
Control C	No additives or 5-2000 ng/ml bFGF
Control D	3 mg/ml heparin

*The additives were initially dissolved in one part serum-free Dulbecco's minimum Eagle's medium (DMEM) (obtained from Highveld Biological Pty Ltd), and diluted further with 19 parts egg albumen to make up the required concentrations.

Rescue operations with bFGF were then performed by culturing stage X blastoderms over one of a number of increasing concentrations (5-500 ng/ml) of recombinant bovine bFGF (Amersham, UK) reconstituted in serum-free DMEM, together with the optimum dose of heparin found to inhibit blood formation, i.e. 3 mg/ml heparin (see Table 2.1). Each

blastoderm was dosed with an additional 5 ng/ml bFGF in serum-free DMEM (concentration = 10 ng/ml), which was distributed around the edge of the embryo with a micropipette to ensure bFGF reached the embryo. Positive control embryos were cultured over albumen with or without bFGF (C in Table 2.1), while blastoderms cultured over 3 mg/ml heparin in albumen were used as negative controls (D in Table 2.1). The embryos were incubated at 38°C, checked briefly on day 1 for a primitive streak, and the whole mounts stained with o-dianisidine on day 2, and examined.

Further histological analysis was made of the heparin-treated (3 mg/ml) embryos, the "rescued" embryos (50 ng/ml bFGF together with 3 mg/ml heparin), and control embryos as follows: scanning electron microscopy to resolve the cellular structure of the ventral surfaces of the embryos was made on day 1, as described previously (Gordon-Thomson et al., 1988). Briefly, the embryos were fixed in 2% glutaraldehyde in Ringer solution and processed conventionally, before viewing with a Hitachi S520 scanning electron microscope. Material for sectioning was prepared by fixing 1 and 2 day old embryos either in Smith's fixative (Humason, 1979), followed by routine dehydration, clearing in methyl benzoate and embedding in Paraplast; or in 2% glutaraldehyde in Ringer's solution at room temperature for 1 hour, followed by routine dehydration, clearing in propylene oxide and embedding in epoxy resin (Spurr, 1969). Wax-embedded embryos were serially sectioned (7 μm), and sections at every level of each embryo were stained with Mayer's haematoxylin and eosin. The resin-embedded embryos were sectioned (2μm), and sections at 20 μm intervals were stained with toluidine blue. The sections were all examined by bright field light microscopy.

2.4 RESULTS

2.4.1 The effect of heparin on erythropoiesis

Most of the two-day old control embryos (A and B of Table 2.1) showed a positive staining reaction with the o-dianisidine test. In the control series, 21 out of 25 embryos cultured without heparin or with the addition of chondroitin sulphate had blood islands and blood cells in the area vasculosa which stained light to dark brown (refer to Fig. 2.1A). O-dianisidine positive tissue was also found in a number of the embryos incubated with heparin at concentrations ranging from 1 to 2.5 mg/ml; while all 31 of the embryos (i.e. 100%) treated with heparin concentrations of 3 mg/ml and higher showed no staining reaction with o-dianisidine (Fig. 2.1B and Table 2.2).

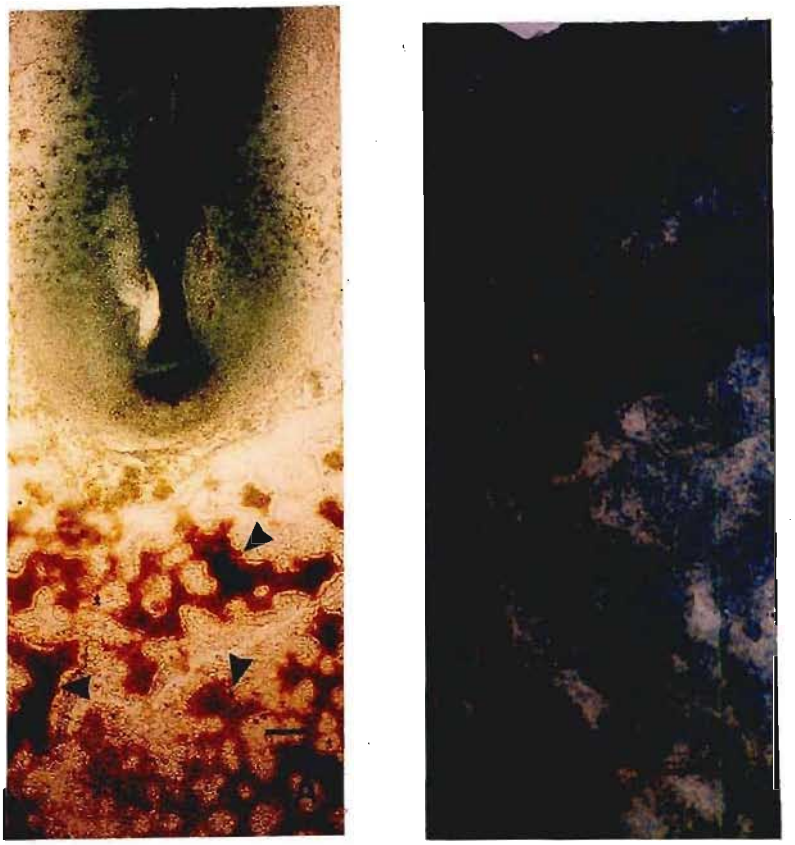


Fig. 2.1 Whole mounts of two-day embryos stained with o-dianisidine. The control incubated in albumen with DMEM (A) is noted for the dark brown patches of haemoglobin-positive cells in blood vessels and blood islands (arrows) in the area opaca vasculosa. Note also the presence of somites and neural tube in the area pellucida. The embryo treated with 3 mg/ml heparin (B) shows no reaction for haemoglobin and no recognizable primary organ rudiments characteristic for this stage. Bars, 200 μm.

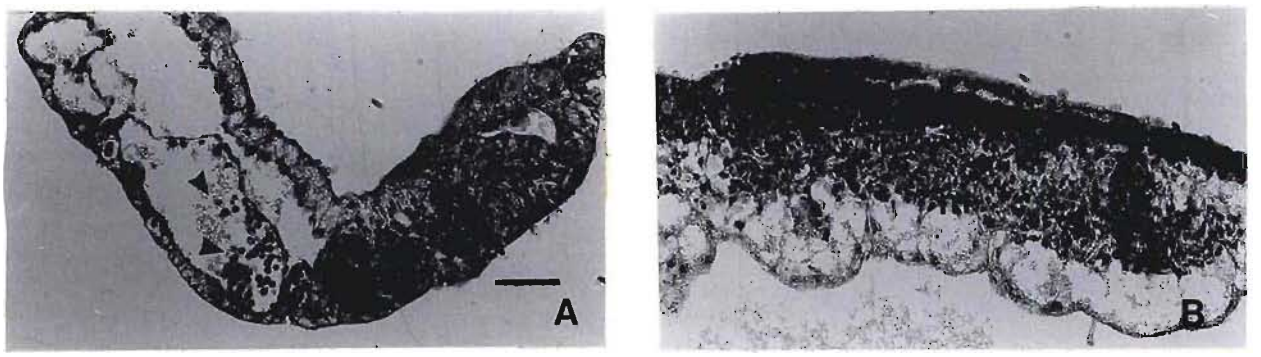


Fig. 2.2 Transverse sections of embryos after two days in culture. (A) Control cultured without heparin with red blood cells within blood vessels (arrows). (B) Blood tissue was not detected in any of the embryos cultured with 3 mg/ml heparin embryos. The mesenchymal tissue (m) in the heparin-treated embryo remained undifferentiated. A and B are at the same magnification. Bar, 100 μm.

Serial sections from four of the two-day embryos treated with 3 mg/ml heparin showed no histological evidence of red blood cells; and furthermore, no other tissue types could be recognized. On the other hand, the control embryos at this stage showed blood vessels containing blood cells, as well as primary organ rudiments such as somites, notochord and neural tube (Figs 2.2A and B).

TABLE 2.2 The effect of various concentrations of heparin on primitive streak (PS) formation and development of haemoglobin-positive tissue on stage X embryos. (Treatment with or without the addition of chondroitin sulphate is represented as 0 mg/ml heparin).

Heparin Concn: (mg/ml)	No. of Tests:	Percentage with Primitive Streak:	Percentage Erythropoiesis:
0	25	96	84
1-1.5	11	64	82
2-2.5	9	22	33
3	26	0	0
4	3	0	0
5	2	0	0

2.4.2 **Heparin treatment eliminates the formation of a primitive streak**

The formation of a definitive primitive streak at day 1 was inhibited in 100% of the embryos (n= 31 embryos) treated with heparin at concentrations of 3 mg/ml and higher, while lower doses of heparin showed a lower percentage of embryos lacking a primitive streak (see Table 2.2). Heparin's inhibition of primitive streak formation and subsequent erythropoiesis was dose dependent (Fig. 2.3).

While formation of a primitive streak was inhibited in the heparin-treated embryos, a middle layer of mesenchymal cells equivalent to a mesodermal layer was noted in the serial sections of nine embryos treated with 3 mg/ml heparin after 18-24 hours incubation (Fig. 2.4A). These mesenchymal cells appeared to have originated by a diffuse polyingression from an extensive area of the posterior region of the epiblast layer rather than collectively from a median primitive streak, as seen in the controls (Fig. 2.4B). Observations of the embryos growing in the presence of 3 mg/ml heparin showed a distinct thickening in the posterior region of the area pellucida (Figs 2.5A and B). In sectioned material, this posterior region of the embryo corresponded to the area where polyingression appeared to have taken

place, and it consisted of a thicker layer of mesenchymal cells (Fig. 2.5C). The layer of mesenchymal cells became thinner anterior to this thickened region (Fig. 2.5D), and was absent in the anterior area pellucida (Fig. 2.5E).

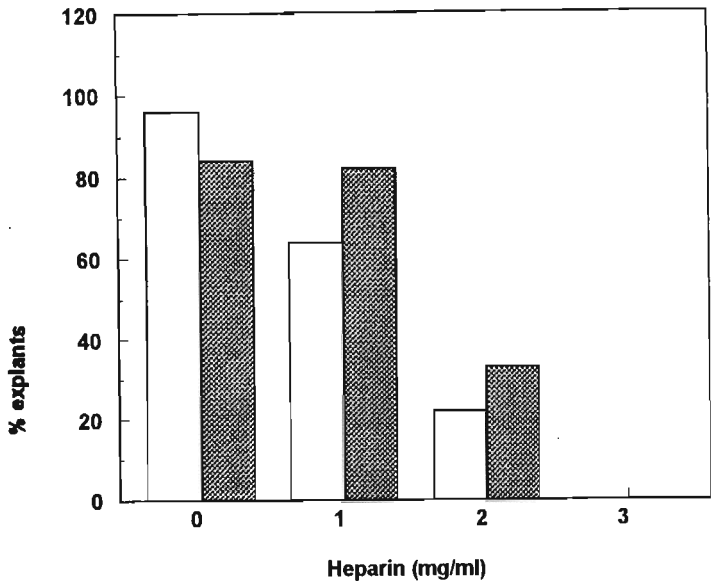


Fig. 2.3 Histogram showing the inhibitory effect of various concentrations of heparin on primitive streak formation at 1 day (blank columns), and blood tissue differentiation (as determined by a negative o-dianisidine staining reaction for haemoglobin) at 2 days (shaded columns).

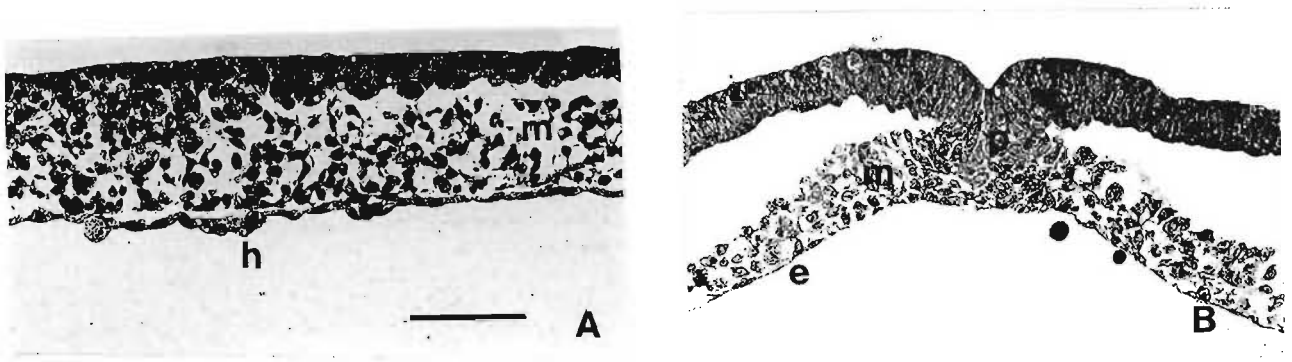


Fig. 2.4 Transverse sections through comparable levels in the germ layers of one-day embryos (A) treated with 3 mg/ml heparin, and (B) an untreated control. The middle layer of mesenchymal cells (m) beneath the dorsal epiblast appears to have been derived by cells polyingressing from a wide area of the epiblast in the posterior region of the heparin-treated embryo (A), rather than a migration from the central primitive streak (p) seen in the control (B). Some of the cells in the ventral layer of heparin-treated embryos are large yolk-filled cells (h) characteristic of hypoblast cells, which are different from the flatter cells (e) characteristic of endodermal cells seen in the ventral layer of the controls. A and B are at the same magnification. Bar, 100 μm.

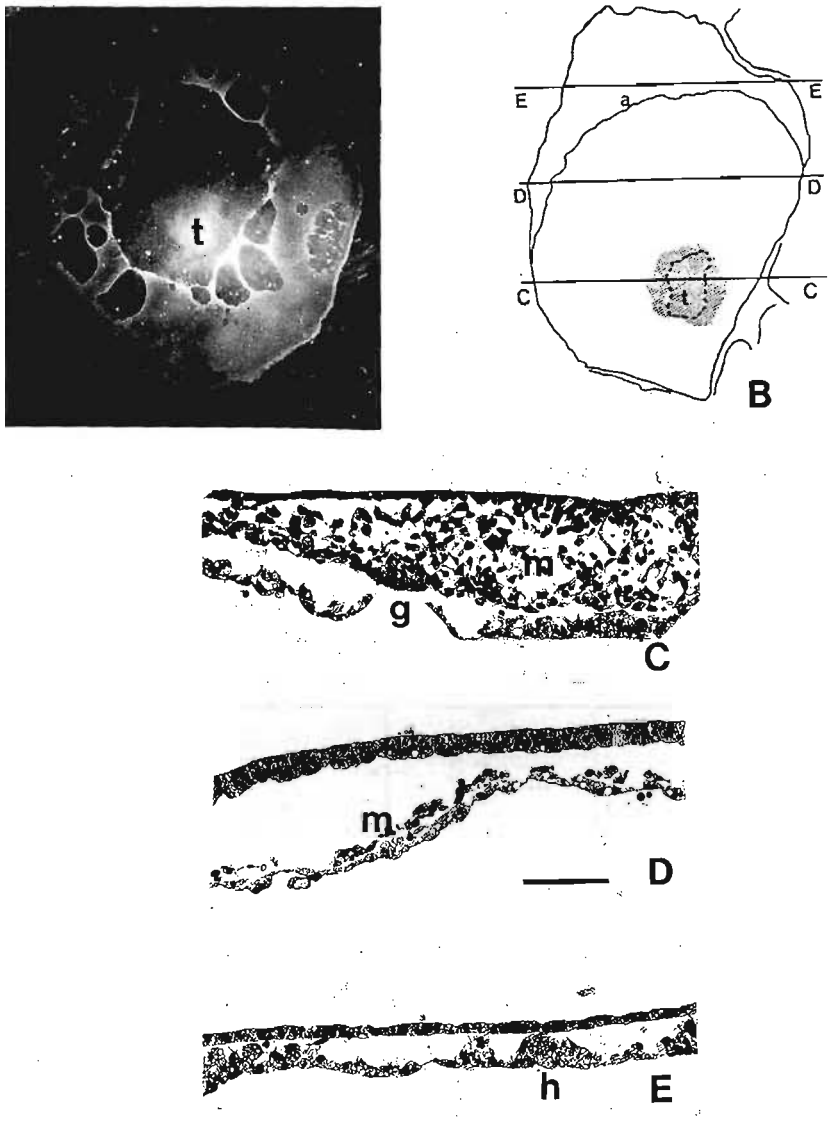


Fig. 2.5 One-day embryo treated with 3 mg/ml heparin. (A) Photomicrograph of the ventral surface of the embryo on the vitelline membrane while in culture, showing the anterior edge of the mesoderm, i.e. mesodermal mantle (a), and the opaque thickening of tissue (t) in the posterior region of the area pellucida. (B) Tracing of the embryo in photomicrograph A, with lines drawn to indicate the planes through which transverse sections in Figs 2.5C-E have been cut. Shaded area represents opaque area (t). (C) Posterior region of the area pellucida through the opaque thickening, which consists of a thick layer of mesenchymal cells (m) between the dorsal epiblast and ventral layer. The mesenchymal cells appear to have originated by a delamination process from the basal surface of the epiblast. A gap (g) can be seen in the ventral "endodermal" layer. (D) Anterior to the thickened region, the mesenchymal layer spreads out between the epiblast and ventral layer, and consists of one layer of cells (m). (E) In the anterior area pellucida only the dorsal epiblast and ventral layer can be seen. Cells of the ventral layer are large and yolk-filled, characteristic of hypoblast-type cells (h). Bars, in A = 500 μm, in C = 100 μm. (C,D and E are at the same magnification).

2.4.3 The effect of heparin treatment on the ventral endoderm

The ventral layer in the heparin-treated embryos develops one or more extracellular gaps (or holes) ranging in size from 10-30 μm in diameter, at the time when endoderm is forming in the controls, as has been briefly reported (Gordon-Thomson et al., 1988). These gaps in the ventral surface could be seen in sectioned material (Figs 2.5C, 2.6A and B), and in scanning electron micrographs of the ventral surfaces of the heparin-treated embryos (Figs 2.7A and B).

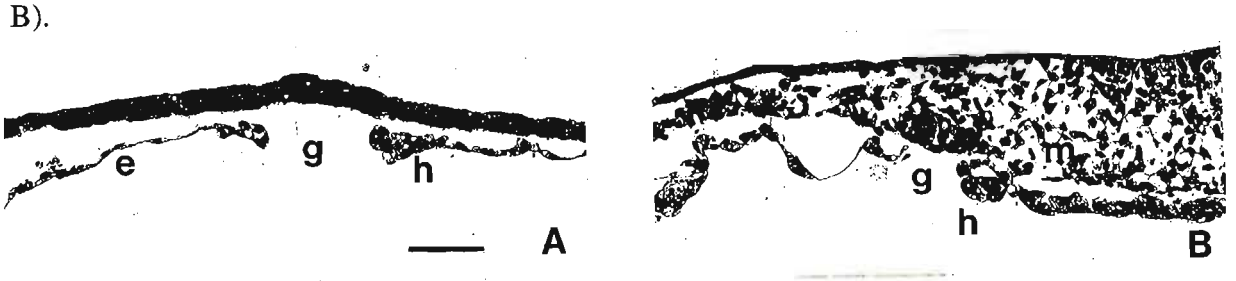


Fig. 2.6 Transverse sections through one-day heparin-treated embryo showing gaps (g) in the ventral layer in the anterior region of the area pellucida (A) where mesoderm is not present, and in the posterior region (B) where mesenchymal cells (m) have accumulated. Note the two types of cells in the ventral layers, the large yolk-filled cells characteristic of hypoblast cells (h), and the flatter cells (e) characteristic of endodermal cells. A and B are the same magnification. Bar, 100 μm.

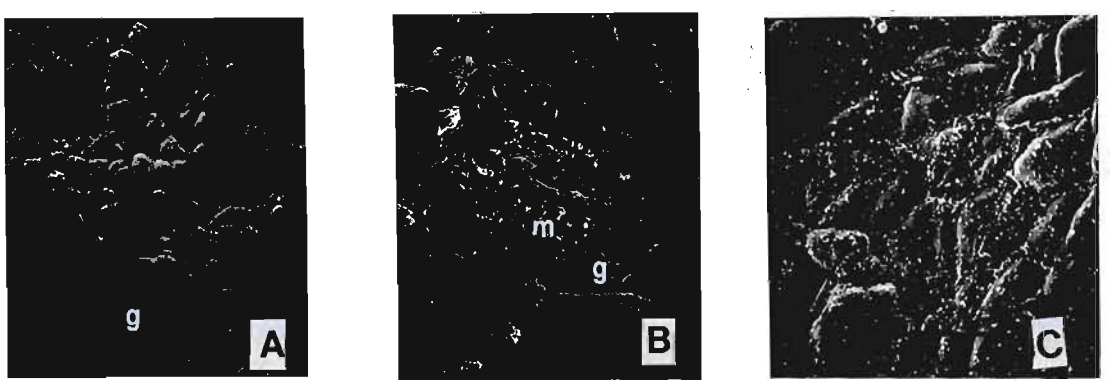


Fig. 2.7 Scanning electron micrographs of the ventral surfaces of one-day embryos showing large gaps (g) in the incomplete ventral surface of the heparin-treated embryos (A and B), but not in the untreated embryos (C). In the heparin-treated embryos the gaps in the anterior region of the embryo reveal the underlying epiblast layer (see A); while in the posterior region (B) the ruffled edges of the mesenchymal cells (m) can be seen through the gaps. (Refer to Fig. 2.6 for the respective transverse views through two similar areas). Some of the cells (arrow) of the ventral layer in A are rounded and do not appear to be adhering to each other, unlike cells on the ventral surface of controls (C) which all appear to adhere to each other, making up a cohesive sheet of flattened endodermal cells. Bars, 30 μm.

Two types of cell were observed in the ventral layer of heparin-treated embryos, namely large yolk-filled cells similar to hypoblast cells which were commonly found on the margins of the holes (Figs 2.6A and B), and flat cells characteristic of endodermal cells. In the control embryos only the latter endodermal-type cells could be seen in the ventral layer at this stage (Fig. 2.4B).

2.4.4 **The effect of the addition of bFGF to heparin-treated embryos**

Stage X embryos cultured in the optimum concentration of heparin that inhibits erythropoiesis (i.e. 3 mg/ml) together with increasing concentrations of bFGF, were examined to determine whether the inhibitory effect of heparin could be blocked by the addition of excess exogenous bFGF. A total of 25 out of 55 embryos incubated with 3 mg/ml heparin together with bFGF stained positively for haemoglobin with the o-dianisidine test on day 2 (see Table 2.3, and Fig. 2.8A). Although haemoglobin-positive tissue was noted in "rescued" embryos at 2 days (Fig. 2.8B), other mesodermal derivatives such as somites, notochord and neural tube were not seen in the serial sections of "rescued" embryos. Interestingly, a higher percentage of embryos showed a "rescue" response (i.e. a positive o-dianisidine reaction) with the lower doses of bFGF used, namely 5-50 ng/ml, than by a concentration of bFGF higher than 50 ng/ml (Fig. 2.9).

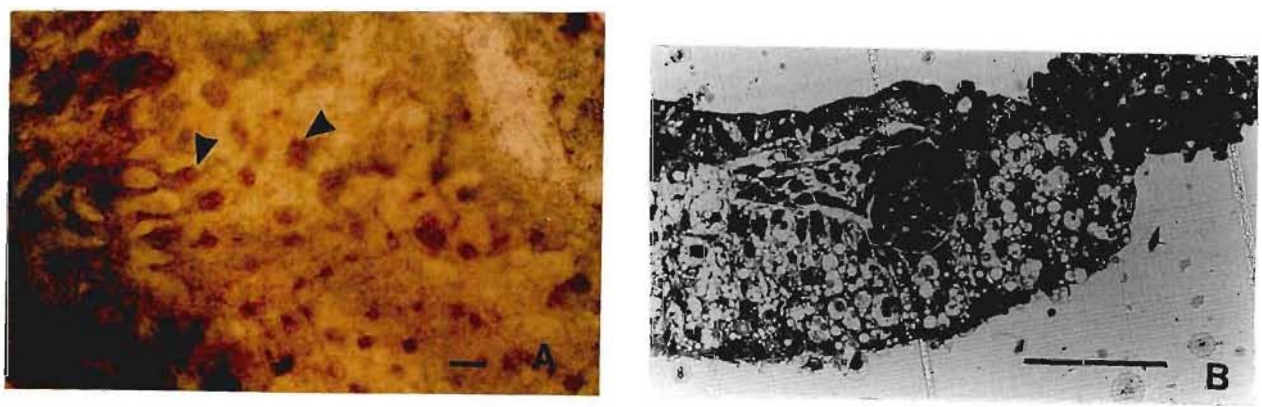


Fig. 2.8 "Rescued" embryos cultured with 3 mg/ml heparin together with 50 ng/ml bFGF. (A) Whole mount of two-day embryo showing light brown patches of haemoglobin-positive tissue (arrows). Other organ rudiments were not detected. (B) Transverse section of two-day embryo showing a cluster of cells resembling erythrocytes surrounded by endothelial cells (arrow).
Bars, 100 μm.

TABLE 2.3. Neutralization of the inhibitory effect of heparin on erythropoiesis by the addition of various concentrations of bFGF

	bFGF Concn: (ng/ml)	**No. of Tests:**	**Percentage Erythropoiesis:**
Experimental cultures:	5	9	78
	10	4	50
	50	7	71
Subtotal	5-50	20	70
Experimental cultures:	100	11	46
	250	12	17
	400	9	33
	500	3	33
Subtotal	100-500	35	31
*Control cultures** :			
Control C	0-500	16	81
Control D	0	11	18

*See Table 2.1 for media used for controls C and D. All experimental embryos and control D were cultured with 3 mg/ml heparin.

Thirteen out of 16 control embryos (i.e. 81%) cultured over medium containing varying concentrations of bFGF (5-500 ng/ml) gave a positive reaction with the o-dianisidine stain (see Table 2.3, Control C). Of the 11 embryos cultured in heparin alone in this series (see Table 2.3, Control D) only 2 embryos showed a positive reaction with o-dianisidine.

Besides the rescuing effect of bFGF on erythropoiesis, the addition of bFGF to the heparin-treated embryos also suppressed the formation of the holes in the ventral layer at 24 hours of incubation. Thirty-two out of 39 embryos examined (i.e. 82%) did not have holes in the ventral layer. On the other hand, bFGF did not obviate the inhibitory effect of heparin on primitive streak formation. Although a primitive streak was not present in any of the "rescued" embryos, a mesenchymal layer of cells was observed between the epiblast and ventral layer. This mesenchymal layer appeared to have originated by a polyingression process, as found in the heparin-treated embryos.

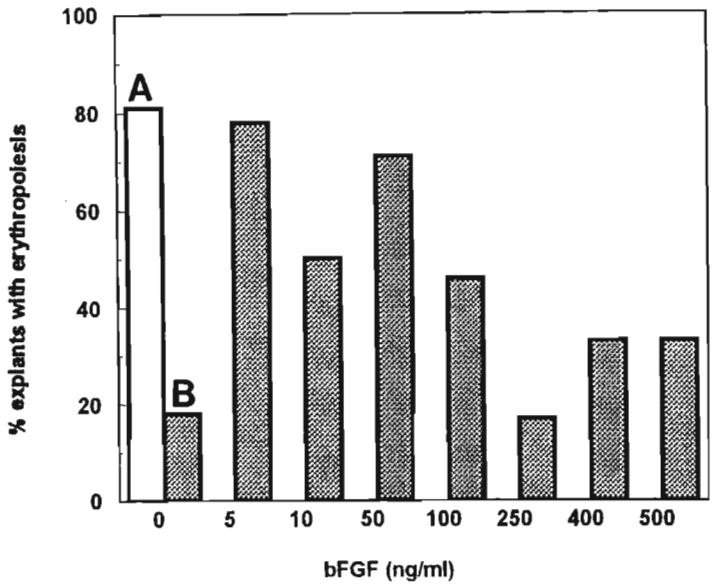


Fig. 2.9 Histogram showing the percentage of embryos in which the inhibition of blood tissue development by heparin was obviated by bFGF. Blood tissue differentiation was determined by the o-dianisidine test on whole mounts. Control embryos were cultured without heparin (control A, unshaded column), and with 3 mg/ml heparin alone (control B, shaded column). Experimental embryos were treated with 3 mg/ml heparin together with increasing concentrations of bFGF. Results from embryos which were treated with 3 mg/ml heparin (i.e. control B and the experiment) are represented in the shaded columns.

2.5 DISCUSSION

2.5.1 The effect of heparin on early chick development

Heparin administered to undifferentiated single-layered blastoderms (stage X), was found to inhibit primitive streak formation and subsequent erythropoiesis, as well as causing holes to appear in the developing ventral "endodermal" layer. The inhibitory effect of heparin is concentration dependent. At heparin concentrations of 1-1.5 mg/ml, 64% of the embryos developed a primitive streak and 82% displayed haemoglobin-positive tissue; whereas at 2-2.5 mg/ml heparin only 22% developed a primitive streak and 33% were positive for haemoglobin. At concentrations of 3 mg/ml and higher none of the embryos developed a primitive streak or showed haemoglobin-positive tissue (refer to Fig. 2.3). As heparin has been reported by others (Neufeld and Gospodarowicz, 1985; Kardami et al., 1988) to prevent FGF binding to its cell surface receptors, these results may well suggest that heparin is binding to an endogenous bFGF. This conclusion is supported by a recent finding which

describes the presence of bFGF in chick embryos from stage XI onwards (Mitrani et al., 1990a).

Despite the absence of a primitive streak after heparin treatment, mesenchymal tissue was still seen to have accumulated between the dorsal epiblast and ventral hypoblast layers (Fig. 2.4A). This mesenchymal layer may be derived from a delamination or polyingression from the epiblast, which according to Eyal-Giladi and colleagues is part of the normal gastrulation process in chick embryos, separate from the migration of cells through a primitive streak (Eyal-Giladi and Wolk, 1970; Azar and Eyal-Giladi, 1979). They also report that these cells possibly gives rise to non-axial mesoderm, such as blood tissue. It is interesting that in the present study, heparin inhibits the movement of cells that form the primitive streak, but does not block the polyingression process. Possibly, more than one mechanism or inducing factor could be involved in mesoderm formation in the chick. Substantial evidence is accumulating which suggests that there are at least two factors involved in the induction of mesoderm in frogs (Woodland, 1989; Smith et al., 1989; Slack, 1990; Green et al., 1990). In *Xenopus*, these two factors are both present in the embryo in sufficient amounts to be considered as the candidate signal molecules responsible for the induction of all the mesodermal derivatives (Green et al., 1990). The one signal molecule, bFGF, has been found when used at low concentrations to induce animal cap cells to form non-axial ventral-type mesodermal derivatives consisting of mesenchyme, mesothelium and cells resembling erythrocytes; and more dorsal-type mesodermal derivatives e.g. muscle, at higher concentrations; but it very rarely induced dorsal notochord (Slack et al., 1987). Another signal molecule which is structurally related to the transforming growth factor (TGF-ß) family is a soluble factor secreted by the *Xenopus* XTC-2 cell line (Smith, 1987; Smith et al., 1988; Smith, 1989). This factor which is called XTC-mesoderm inducing factor (XTC-MIF) can induce animal cap cells to form dorsal-type mesoderm such as muscle and notochord. Exogenous TGF-ß2 is also capable of inducing notochord in animal cap cells (Rosa et al., 1988).

In the present investigation it is possible that heparin is also binding to a specific factor which is responsible for primitive streak formation, as well as the bFGF. The formation of a primitive streak is believed to be induced by a diffusible substance of unspecified nature emanating from the hypoblast cells that originate from the posterior marginal zone (Waddington, 1933; Eyal-Giladi and Wolk, 1970; Azar and Eyal-Giladi, 1981).

It appears from this study that the diffusible substance is not bFGF itself, as bFGF did not obviate the inhibitory effect of heparin on primitive streak formation. It is interesting that an equivalent system in which the hypoblast and marginal zone are removed to leave a central disc of the blastoderm, XTC-MIF, but not bFGF, was found to induce primitive streak formation (Mitrani and Shimoni, 1990).

Heparin also had an effect on the integrity of the ventral layer. In the one-day heparin-treated embryos a defective ventral layer of cells with large gaps (Figs 2.6, 2.7A and B) was formed. These gaps may arise from the inability of mesenchymal cells to migrate into the hypoblast layer, which is the normal mechanism for the formation of endoderm proper (Rosenquist, 1971; Stern and Ireland, 1981). A reduction in the number of migrating cells integrating into the hypoblast, coupled with the active expansion of the rest of the blastodisc, could result in the disruption of adhesion between hypoblast cells, initiating extracellular clefts which would be enlarged during the expansion process. The separation of cells would also be assisted by the lack of stickiness of hypoblast cells which are known to have poor adhesive properties, unlike endodermal cells which have close intercellular junctions (Sanders et al., 1978). It is of interest that this study showed that the gaps are generally surrounded by typical hypoblast cells (Fig. 2.6), which would suggest that these clefts are formed in the ventral layer at places where cells do not adhere strongly.

While the appearance of gaps in the ventral layer during gastrulation may arise from the affinity of heparin for a particular inducing substance; it could also be considered that the effect of heparin may be to cause defects in the extracellular matrix. Heparin belongs to the glycosaminoglycan group (one of the three main components of the extracellular matrix in embryos) which is involved in directing the behaviour of cells during development (Toole and Underhill, 1983), and the addition of heparin in excess to the blastoderm could change the composition of this extracellular environment. In this context it should be noted from a histological analysis of serially-sectioned embryos that the epiblast layer, which was in closer contact with the heparin-containing medium than the ventral layer formed intact epithelial tissue without gaps.

2.5.2 The inhibition on red blood cell formation by heparin is blocked by the inclusion of bFGF in the medium

The inclusion of bFGF in the medium containing 3 mg/ml heparin allows red blood cell differentiation to proceed (Fig. 2.8). We found that lower concentrations of bFGF (i.e. 5-50 ng/ml) gave a greater percentage of embryos with haemoglobin-positive tissue than did concentrations of 100 ng/ml and higher (see Fig. 2.9). This could represent a similar ventral-to-dorsal response to low and high concentrations of bFGF as found in *Xenopus* (Slack et al., 1987), although it would need a suitable marker to verify the early presence of muscle tissue in the embryos treated with the higher concentrations of bFGF. Although bFGF is not able to restore primitive streak formation, a mesenchymal layer of cells corresponding to a mesodermal layer nevertheless still forms by polyingression. The formation of red blood cells in this mesenchymal layer is in accord with the findings of others who found that cells destined to form blood tissue need not necessarily arise from cells that migrate through the primitive streak. For example, blood tissue differentiation has been found in explants in which primitive streak formation had been inhibited by the removal of the marginal zone (Azar and Eyal-Giladi, 1979); as well as in cases where the primitive streak has been inhibited by mechanical suppression (Zagris, 1980).

There is little doubt that bFGF cancels out the inhibitory effect of heparin on the development of haemoglobin-positive tissue in these experiments. A key interpretation of this finding is that bovine bFGF can mimic the natural "inducer" of erythropoiesis. It would be of interest to know which cells synthesize this "inducer". In this regard there is evidence that the endodermal cells which normally migrate into the area opaca may contain a substance that is "salutary" to erythropoiesis (Wilt, 1965). If the normal spread of endodermal cells from the centre of the embryo to the area opaca could be interfered with, then the mesoderm of the area opaca will not be stimulated to undergo erythropoiesis. This may be precisely what is happening in this investigation where heparin causes defects in the form of gaps in the central "endoderm". The formation of these gaps may prevent the spreading of endoderm into the area opaca. It has been shown that the addition of bFGF to the heparin-treated embryos eliminates these gaps, and the inhibition of erythropoiesis is neutralized.

CHAPTER THREE

XTC-MIF CAN NEUTRALIZE THE INHIBITORY EFFECT OF HEPARIN ON PRIMITIVE STREAK FORMATION AND ERYTHROPOIESIS

3.1 SUMMARY

The present chapter describes the rescue effect of a mesodermal-inducing factor (MIF) derived from the *Xenopus* tadpole cell line (XTC) on the blockade on primitive streak formation and on blood tissue (haemoglobin) differentiation caused by heparin treatment in the chick. This factor can induce different mesodermal tissue types in the frog animal cap, depending on the dose. Chick embryos treated with heparin were cultured with the addition of XTC-MIF, or XTC-MIF together with bFGF. The morphology of the embryos on the first day of culture was assessed by a histological analysis for the presence of a primitive streak. The presence of blood tissue was determined by the cytochemical detection of haemoglobin in whole mounts using the o-dianisidine test, after two days in culture.

Treatment with XTC-MIF neutralized heparin's inhibitory effect on primitive streak formation in 25% of the treated embryos. It also neutralized heparin's inhibitory effect on blood tissue formation. However, no axial structures or other mesodermal derivatives, such as somites, were recognized in the heparin-treated explants rescued with either XTC-MIF, or a cocktail of XTC-MIF and bFGF. Interestingly, the reversal of heparin's inhibitory effect on erythropoiesis with bFGF and XTC-MIF, coincided with the development of an intact ventral layer, without the formation of gaps caused by heparin treatment.

3.2 INTRODUCTION

Early treatment of chick embryos at stages X and XI (Eyal-Giladi and Kochav, 1976) with heparin was found to have an inhibitory effect on primitive streak formation, and on blood mesodermal tissue differentiation (Gordon-Thomson et al., 1988; or refer to Chapter Two). Heparin is known to bind specifically with members of the FGF family (Lobb et al., 1985) preventing the binding of bFGF to its receptor (Neufeld and Gospodarowicz, 1985). However, there is some evidence that heparin shows variable binding power with other

growth factors belonging to the TGF-β2 family. In the frog animal cap bioassays, it was found that the induction of dorsal mesoderm by TGF-β2 could be neutralized by heparin in 50% of the cases (Slack and Isaacs, 1989). While another homologue, Tiedemann's vegetalizing factor extracted from the 9 - 14 day chick embryo, which induces ventral mesodermal structures in the frog, is also known to have an affinity to heparin (see Godsave et al., 1988).

In Chapter Two it was proposed that heparin could be binding to one or more growth factors in the embryo that play a role in the determination of mesodermal tissue. It was suggested that bFGF could be one of these determinants as the addition of a recombinant bFGF to the heparin-treated embryos at stage X and XI neutralized the inhibition of erythropoiesis (Gordon-Thomson et al., 1991; or refer to Chapter Two). This indicated that the recombinant bFGF had replaced the heparin-bound endogenous factor; and that the specification of blood tissue requires the natural activity of a bFGF-like factor at these early stages of development. As bFGF did not neutralize the inhibition of the primitive streak and axial mesodermal tissues, it appears that there may be other factors inactivated by the heparin-treatment, that have a role to play in the specification of the primitive streak and the axial mesodermal derivatives.

At present the general consensus with regard to the frog embryo is that two or more factors may act synergistically in the specification of the full spectrum of mesodermal types (Kimelman and Kirshner, 1987; Kimelman et al., 1992; Moon and Christian, 1992); two of which belong to the FGF and TGF families. It has been demonstrated that bFGF can induce ventral mesoderm in uncommitted animal cap cells that would normally form epidermis; thus mimicking the natural induction by the ventral blastomeres. Furthermore, it has been shown that heparin blocks the natural induction of the ventral blastomeres, inhibiting the formation of ventral mesoderm (Slack et al., 1987; Slack, 1990, 1991b). As members of the TGF-β family act as dorsal mesodermal-inducing factors, yielding notochord and muscle at high doses (Smith, 1987, 1989; Smith et al., 1990; Jessell and Melton, 1992; Green et al., 1992), it was pertinent to ask whether the addition of a member of the TGF-β family could rescue the heparin-treated chick embryos and reinstate the formation of a primitive streak and the mesodermal derivatives.

In this study a factor derived from the *Xenopus* tadpole cell line (XTC) which is a potent mesodermal-inducing factor (MIF) belonging to the TGF-β family, and a homologue

of activin A (Smith et al., 1990; van den Eijnden-Van Raaij et al., 1990) was used. Its rescue effect on heparin-treated chick embryos was analyzed by microscopic observations of the embryos in culture and by histological analysis of sectioned material at one and two days of incubation. The condition of the ventral layer of the one day embryo in culture was also noted. The level of erythropoiesis was measured by testing for haemoglobin using the o-dianisidine test after 2 days' incubation. The results show that XTC-MIF can "rescue" erythropoiesis in the heparin-treated embryos, as did bFGF; but in addition, it can also reinstate the formation of a primitive streak.

3.3 MATERIALS AND METHODS

3.3.1 Preparation of the mesodermal-inducing factor (XTC-MIF)

The mesodermal-inducing factor was derived from the conditioned medium of a *Xenopus* tadpole cell line, XTC. The *Xenopus* tadpole cell line was kindly donated to B. C. Fabian by Dr. E. A. Jones of the Department of Biological Sciences, University of Warwick, Coventry, UK. The XTC-MIF was extracted according to Smith (1987). The cells were maintained at 25°C in Liebovitz L15 medium diluted to 61% and supplemented with 10% foetal calf serum (purchased from Highveld Biological Supplies, RSA). All procedures were carried out under aseptic conditions. The cells were subcultured weekly at a 1:4 split ratio in 25 cm^3 Nunclon tissue culture flasks, and fed with fresh medium once a week. Conditioned medium was prepared from the confluent XTC cells. The confluent cells were rinsed three times with serum-free L15 diluted to 65%, and incubated in 4 ml of the serum-free medium. After 48 hours the conditioned medium was poured into test tubes and centrifuged to remove any loose and dead cells. The supernatant was heat-shocked in a water bath at 100°C for 5 minutes (according to Smith et al., 1988); and stored at -20°C until required.

3.3.2 Culture methods

Stage X and XI embryos (staged according to the tables of Eyal-Giladi and Kochav, 1976; refer to Fig. 1.7) were explanted from unincubated eggs under aseptic conditions, and cultured on vitelline membrane rafts (refer to Appendix 2). The pool of egg albumen beneath the vitelline membrane contained the appropriate doses of the growth factors and heparin. The control blastoderms were cultured on a pool of egg albumen with or without the addition of

3 mg/ml heparin (Na salt, Grade 1 supplied by Sigma, USA). The optimum concentration of heparin needed to inhibit primitive streak formation and erythropoiesis in 100% of the embryos in this experimental design was determined earlier to be 3 mg/ml heparin (see Fig. 2.3 in Chapter Two).

Experimental series 1: Each blastoderm on its vitelline membrane raft was cultured on a pool of egg albumen with the addition of heparin together with XTC-MIF (final concentration of the heparin being 3 mg/ml; and the XTC-MIF ranged from 10% to 40% of the medium beneath the vitelline membrane). A 5 µl dose of the full strength heat-shocked XTC-MIF was added by micropipette to the edge of the blastoderm on the vitelline membrane before incubation. The embryos were incubated at 38°C in a humidified atmosphere.

Experimental series 2: Each blastoderm on its vitelline membrane raft was cultured on a pool of egg albumen with 3 mg/ml heparin, 25% XTC-MIF, and 50-100 ng/ml recombinant bovine bFGF (supplied by Amersham, UK) in the egg albumen. A dose of 5 µl full strength XTC, and 5 µl of 50-100 ng/ml bFGF in L15 medium was added to the edge of the blastoderm before incubation, as above.

3.3.3 **Analysis of axis formation and other morphogenetic changes**

Stereomicroscopic examination of the embryos in culture were made at day one and at day two. At day one, the cultures were examined for the presence of a primitive steak, and the morphology of the ventral layer was noted. At day two, the cultures were examined for the presence of a body axis. Some of the embryos were fixed at 20 hours and 48 hours incubation in Smith's fixative (Humason, 1979), rinsed, dehydrated in ethanol, and embedded in wax. Serial sections of 7 µm were stained conventionally with Mayer's haematoxylin and eosin. A histological examination of the sections were made with a Nikon photomicroscope; Fujicolour Super HG II film was used for the photomicrographs.

3.3.4 **The o-dianisidine test for haemoglobin**

Blood mesodermal tissue was identified by O'Brien's (1960) o-dianisidine test for Hb, by staining the whole mounts after 48 hours in culture (refer to Appendix 1).

3.4 RESULTS

3.4.1 The effect on axis formation after the addition of XTC-MIF to heparin-treated embryos.

In the previous chapter it was shown that primitive streak formation was inhibited by the treatment of stage X embryos with heparin. The optimum concentration that inhibited primitive streak formation in 100% of the cases was 3 mg/ml heparin; which is the same optimum concentration of heparin needed for 100% inhibition of erythropoiesis (refer to Fig. 2.3 in Chapter Two).

In experimental series 1 of the present chapter, the addition of XTC-MIF to the heparin-treated embryos resulted in the formation of a structure resembling a primitive streak in 25% of the cultures observed during the first day of the incubation period (refer to Table 3.1, and Fig. 3.1). In experimental series 2, the addition of a cocktail of XTC-MIF and bFGF also rescued primitive streak formation, and at the same rate as with XTC-MIF alone (refer to Fig 3.1). Histological examination of serial sections of "rescued" one day embryos which appeared to have an axis when observed in culture, were found to have structures resembling a primitive groove in 3 of the 6 embryos examined (refer to Fig. 3.2). The two day "rescued" embryos did not appear to have any recognizable axial structures, and histological examination revealed the presence of clusters of mesenchymal cells with similar characteristics to blood islands, which were equivalent to the structures seen in the bFGF-rescued embryos (refer to Fig. 2.8 in Chapter Two).

In the controls that were treated with a high dose of XTC-MIF the embryos developed normally. It is noteworthy that incubation in the high concentration of XTC-MIF in the present study did not lead to the deviation from normal development, that has been reported by Cooke and Wong (1991).

TABLE 3.1 The neutralization of heparin's inhibitory effect on primitive streak formation by the addition of XTC-MIF (final concentration ranging from 10-40% of the culture medium), or XTC-MIF together with bFGF (the final concentration of bFGF ranged from 50-100 ng/ml). All the embryos (Control 1 and experimental series) were incubated with 3mg/ml heparin, except Control 2 which was treated with 50% XTC-MIF only.

Treatment:	No. of Tests:	Percentage with Primitive streak:
Heparin (Control 1)	19	0
Heparin + XTC-MIF	48	25
Heparin + XTC-MIF + bFGF	27	26
XTC-MIF (Control 2)	25	100

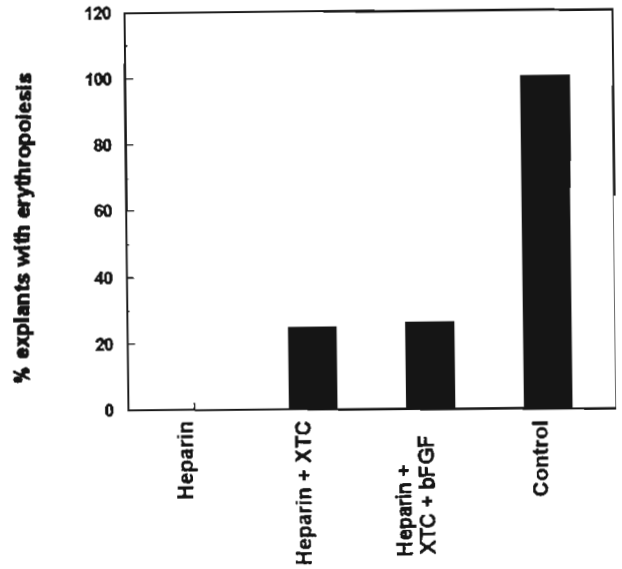


Fig. 3.1 Histogram showing the percentage of embryos in which the inhibition of the primitive streak by heparin was neutralized by the addition of XTC-MIF (10-40%), or XTC-MIF together with bFGF (50-100 ng/ml medium). Control embryos were cultured without heparin, or with 50% XTC-MIF. Negative controls were cultured with heparin alone.

3.4.2 The rescue effect on erythropoiesis after the addition of XTC-MIF to heparin-treated embryos

Heparin treatment was found to inhibit the formation of o-dianisidine positive tissue (Hb formation) at doses of 3 mg/ml and higher (refer to Fig. 2.3 in Chapter Two). The addition of XTC-MIF at concentrations of 10-40% of the culture medium together with heparin was found to neutralize this inhibition (refer to Table 3.2). Although 16 out of 28 (57%) of the

treated embryos formed o-dianisidine-positive tissue (Hb); no other recognizable mesodermal structures were observed at the level of analysis used in this study. Moreover, the "rescued" embryos appeared to consist mostly of blood islands and erythropoietic foci (Fig. 3.3).

TABLE 3.2 The neutralization of heparin's inhibitory effect on erythropoiesis by the addition of XTC-MIF (final concentration being from 10-40% of the culture medium). All the embryos (Control 1 and experimental series) were incubated with 3mg/ml heparin, except Control 2 which was cultured with 50% XTC-MIF.

Treatment:	No. of Tests:	Percentage Erythropoiesis:
Heparin (Control 1)	25	12
Heparin + XTC-MIF	28	57
XTC-MIF (Control 2)	20	90

Fig. 3.2 Transverse section of one day embryo cultured with 3 mg/ml heparin together with 10% XTC-MIF. Note primitive groove-like structure (arrow) in the central blastoderm, and mesenchymal cells (m). The epiblast (ep) appears as a single layer of epithelial cells. Bar, 25 μm. (The morphological characteristics of the negative controls treated with heparin and the untreated controls were equivalent to those seen in the experiments described in Chapter Two; refer to Figs 2.4A and B for representative figures of the morphology of the heparin-treated and control embryos respectively, as seen in transverse section.)

While it was not possible to quantify the concentration of the crude extract of XTC-MIF used in the experiments of the present study, the neutralization of heparin's inhibitory

effect on erythropoiesis and primitive streak formation with XTC-MIF did not show any dose response. XTC-MIF at concentrations as low as 10% and as high as 40% rescued both erythropoiesis and primitive streak formation.

Fig. 3.3 The o-dianisidine reaction in a whole mount preparation of embryos after two days in culture. (A) The control embryo is noted for the dark brown patches of Hb-positive cells in blood vessels and blood islands (arrows). (B) An embryo treated with 3 mg/ml heparin showing no reaction with the o-dianisidine stain. (C) An embryo cultured with the XTC-MIF (10%) and 3 mg/ml heparin, showing many blood island-like foci (arrows), some of which stained positively for Hb, but no recognizable axial structures. Bars, in A = 1 mm, in B = 100 μm; (B and C are the same magnification).

3.4.3 **The morphology of the ventral layer in normal and treated embryos**

In the control embryos cultured for one day, the ventral layer appeared intact with closely-packed cells; while the addition of heparin to stage X and XI embryos caused gaps to appear in the ventral layer of the one day embryo in 64% of the cases (refer to Fig. 2.6 and 2.7 in Chapter Two). It is significant that heparin treatment of embryos of stage XII and later, resulted in the formation of an intact ventral layer in most of the cases observed (5 out of six embryos).

The addition of XTC-MIF to the embryos treated with heparin at stages X and XI, decreased the incidence of gaps forming in the ventral layer by 63%, as did bFGF; while treatment with the cocktail of XTC-MIF and bFGF prevented gaps forming in the ventral layer of 89% of the cases (refer to Table 3.3).

TABLE 3.3 Morphology of the ventral layer after treatment with 3 mg/ml heparin; and 3 mg/ml heparin together with various combinations of XTC-MIF (10-40%) and 100 ng/ml bFGF. Controls were cultured over egg albumen, alone or with the addition of 3 mg/ml chondroitin sulphate, or with 50% XTC-MIF. Treatment was commenced at stage X or XI in A; and at stage XII or XIII in B. Observations made by stereomicroscopy of explants at one day in culture.

Treatment:	**No. of tests:**	**Percentage embryos with holes in ventral layer:**
A. *Stage X-XI*		
Control	36	0
Heparin	28	64
Heparin + XTC-MIF	19	37
Heparin + bFGF	14	30
Heparin + XTC-MIF + bFGF	18	11
B. *Stage XII-XIII*		
Heparin	6	17

3.5 DISCUSSION

In this chapter, the rescue effect of XTC-MIF on heparin-treated embryos has been evaluated. The inhibition of primitive streak formation by heparin, and the formation of a structure resembling a primitive streak after the addition of XTC-MIF, suggests that there is an endogenous factor that acts as a determinant which is possibly a homologue of XTC-MIF. Mitrani and his colleagues have recently shown that activin, a homologue of XTC-MIF, could clearly be one of the natural factors responsible for primitive streak formation in the chick. They have reported that XTC-MIF and activin B can induce a primitive streak in the isolated central disc of the stage XIII epiblast (Mitrani and Shimoni, 1990; Mitrani et al., 1990b; Ziv et al., 1992; reviewed in Chapter One, section 1.4.2). This central disc would otherwise have

been deprived of this capacity, as the natural source of this induction, the hypoblast, had been removed (refer to Eyal-Giladi, 1991). They not only showed that activin can mimic the natural inducer of primitive streak formation; but also that activin B is expressed in the hypoblast at stage XIII, which is the time this induction is thought to take place (refer to Eyal-Giladi, 1991).

It is interesting that in the chick embryo activin is expressed in the hypoblast at the time the induction presumably takes place (Mitrani et al., 1990b); whereas in the frog, activin is only expressed after the time of mesoderm induction. Although another TGF-β member, known as *Xenopus* bone morphogenetic protein 4 (XBMP-4), has recently been found to be present in the frog embryo at the time the natural inducer is seen to act, it can only induce ventral mesoderm (Dale et al., 1992). Unlike activin which can induce nearly the full spectrum of dorsal and ventral mesodermal tissue types in a dose-dependent manner (Green et al. 1992; Umbhauer et al., 1992).

In the chick experiments, the XTC-MIF also neutralized the inhibitory effect of heparin on blood tissue formation. However, at the level of the analysis made in this study, it did not seem to "rescue" any other mesodermal derivatives, although a primitive streak was reinstated in some of these embryos. In the frog animal cap bioassays, XTC-MIF and activin do not induce blood tissue; although, low doses of activin A have been found to induce the expression of the posterior spectrum of mesoderm-specific genes, eg. *Xbra* (reviewed in Green et al., 1992). It is reasonable to expect XTC-MIF to elicit an erythropoietic response in the heparin-treated chick embryos, as various homologues of XTC-MIF are known to regulate blood tissue development in other systems. For example, an angiogenesis factor regulating vascular endothelial cell differentiation in the adult mammalian system, is TGF-β (Folkman and Klagsbrun, 1987; Klagsbrun and Amore, 1991). Another homologue, secreted by several human transformed cell lines called erythroid differentiation factor (EDF), induces Friend erythroleukemia cells and human K-562 cells to differentiate into mature erythrocytes (Murata et al., 1988). Activin A also induces the K-562 cells to differentiate into erythrocytes (see Smith et al., 1990). Most interestingly, another homologue of XTC-MIF, Tiedemann's vegetalizing factor extracted from the 9 - 14 day chick embryo can induce "blood-like" cells in the frog animal cap, and this factor also has a binding affinity with heparin (Grunz, 1983; Asashima et al., 1991). It would be of interest to determine whether Tiedemann's vegetalizing factor is also present in the pregastrula stages of the chick; and whether it could

act as one of the morphogenetic determinants in the specification of mesodermal tissues in the chick.

Heparin treatment also disrupted the normal development of the ventral layer during the stages of gastrulation, with the formation of large gaps in this germ layer. Both bFGF and XTC-MIF prevented these gaps forming, and blocked heparin's inhibitory effect on erythropoiesis. This infers that erythropoiesis is dependent on the normal morphogenesis of the ventral layer. It is of significance that the extraembryonic endodermal cells that arise from this ventral layer, are permissive inducers of Hb formation in the overlying extraembryonic mesoderm at a later stage (Wilt, 1965, 1967; Kessel and Fabian, 1986, 1987).

The neutralization of the inhibitory effects of heparin by XTC-MIF and by bFGF, suggests that homologues of these factors may act in the chick embryo by inducing primitive streak formation and specifying the blood mesodermal tissue line. It is possible that other factors in the chick embryo are responsible for the induction of the axial mesodermal structures; as has been found in the frog embryo. The evidence from the frog investigations has led to the proposal that members of the FGF, TGF and *Wnt* families act synergistically in mesodermal induction and patterning (refer to Kimelman and Kirshner, 1987; Kimelman et al., 1992; Moon and Christian, 1992).

The present study shows that XTC-MIF, a homologue of activin A, can neutralize heparin's inhibitory effect on the formation of a primitive streak and on erythropoiesis. It supports the work of others which has shown that activin is a candidate morphogenetic determinant necessary for the induction of primitive streak formation (Mitrani et al., 1990b).

CHAPTER FOUR[2]

HYPOBLASTIC TISSUE AND FIBROBLAST GROWTH FACTOR INDUCE BLOOD TISSUE (HAEMOGLOBIN) IN THE EARLY CHICK EMBRYO

4.1 SUMMARY

The temporal and the causal basis of blood tissue specification in the chick embryo has been investigated and the results are reported in this chapter. The prospective blood forming area is specified in a horseshoe-shaped area at the posterior side of the embryo (Settle, *Carnegie Inst. Wash. Publ.* **35**, 221-235, 1954; Wilt, *Ann. N.Y. Acad. Sci.* **241**, 99-112, 1974). It was 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 antisera against bFGF or the bFGF receptor, or with heparin.

In another series of experiments, it was 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 could however 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 firmed by the finding that antibody to bFGF inhibited the formation of Hb in these hypoblast sandwich explants.

It is proposed that the erythropoietic cell lineage, i.e. blood tissue, is determined in the chick embryo between stages XI and XII by a bFGF-type of inductive signal from the emerging hypoblast adjacent to Koller's sickle, in the area encompassing the posterior

[2]The experimental work described in this chapter has been transcribed from a manuscript that has been reviewed and accepted for publication in *Development*. Minor alterations have been made in keeping with the style of this thesis.

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.

4.2 INTRODUCTION

The determinative events which 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 which 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 which 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 (refer to Fig. 1.6 in Chapter One). 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 which 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 integrate with the islands of polyingressed epiblast cells as it migrates anteriorly to form the fully formed hypoblast layer by stage XIII (reviewed by Eyal-Giladi, 1991; Stern, 1991; and Khaner, 1993; and in Chapter One, Section 1.2.2). It is proposed 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 which overlaps the marginal zone and Koller's sickle in the posterior and posterolateral area pellucida. The 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 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, it has been 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 it was 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. It was shown 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 antisera to bFGF or the bFGF receptor inhibited erythropoiesis, as well as the formation of other posterior structures, namely somites and heart). It was also shown that the addition of a hypoblast or the addition of bFGF to a stage X ICD leads to the formation of Hb; and that anti-bFGF is able to inhibit this response.

Based on these findings it is proposed 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.

4.3 MATERIALS AND METHODS

4.3.1 Culturing methods for blastoderm and blastodermal pieces

Freshly laid hens' eggs were collected, 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), (see Fig. 1.7. in Chapter One).

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. 4.1), 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. 4.1) 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; or (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. 4.1). All cultures of blastodermal pieces were incubated for three 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.)

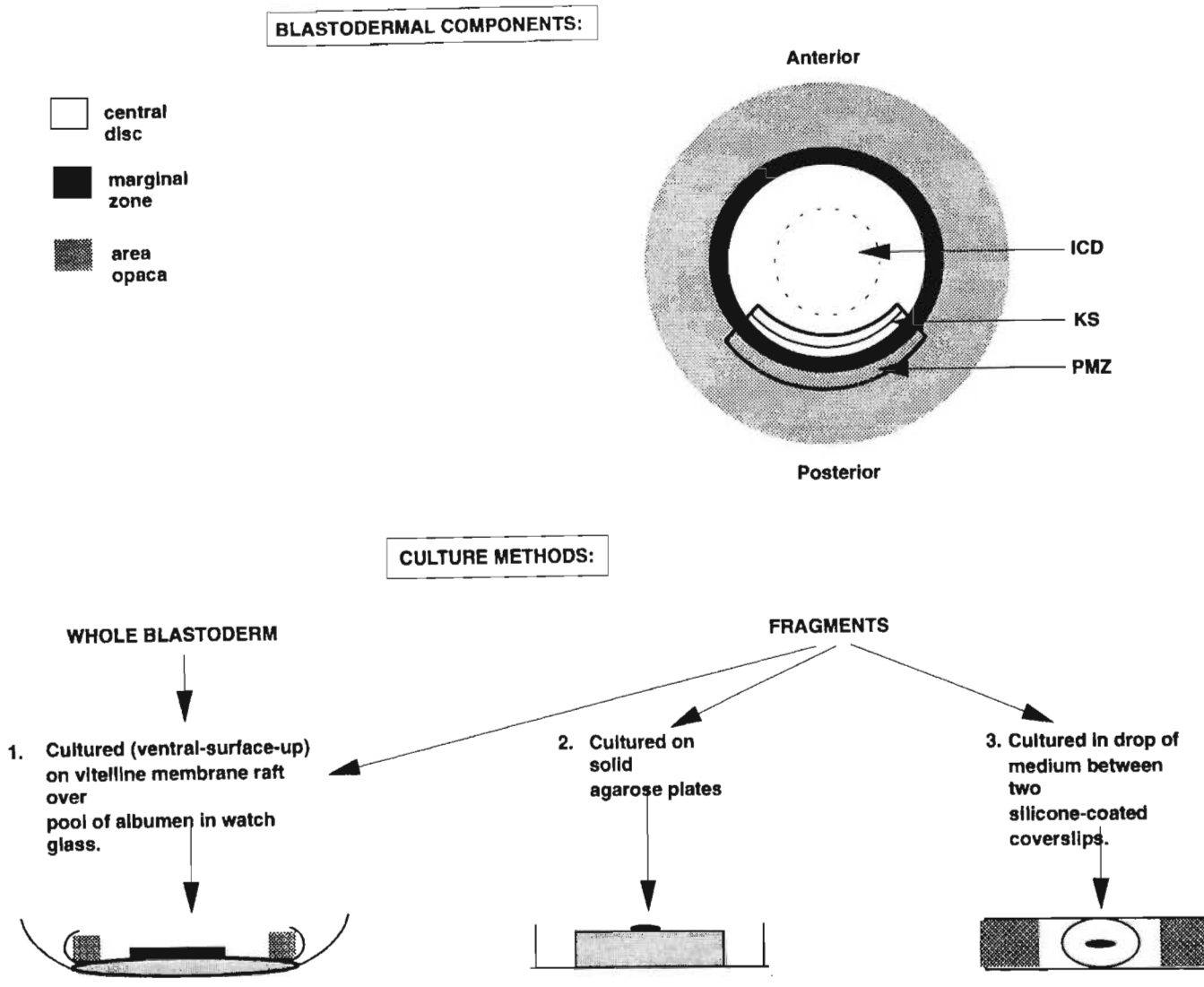


Fig. 4.1 Diagram outlining the pieces (described as components or fragments in the figure) dissected from the blastoderm; and the culturing procedures used. (Abbreviations: ICD = inner core of the central disc of the area pellucida. The ICD from the stage X embryo consisted of the epiblast and islands 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.

4.3.2 **Treatment of whole blastoderms with antisera 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. A rabbit polyclonal antibody raised against the human bFGF receptor (anti-FGFR; supplied by Promega, USA) was used, which is reported to recognize a peptide corresponding to the deduced cDNA sequence of the putative FGF receptor (*flg*, *bek*, and *cek* gene products), and to have specificity for chicken FGF receptors (see Lee et al., 1989). Whole blastoderms were treated with the receptor antibody at concentrations of 32.5 to 130 µg/ml anti-FGFR in phosphate buffered saline.

Embryo explants of stages X and XI were treated with anti-FGF or anti-FGFR 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 two 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 to 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 IgG's 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 these 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.

4.3.3 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 IgG's (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.

4.3.4 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.

4.3.5 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 IgG's (as described above) were substituted for the anti-FGF in the controls.

4.3.6 **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 0.1M 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.

4.4 **RESULTS**

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

After two 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

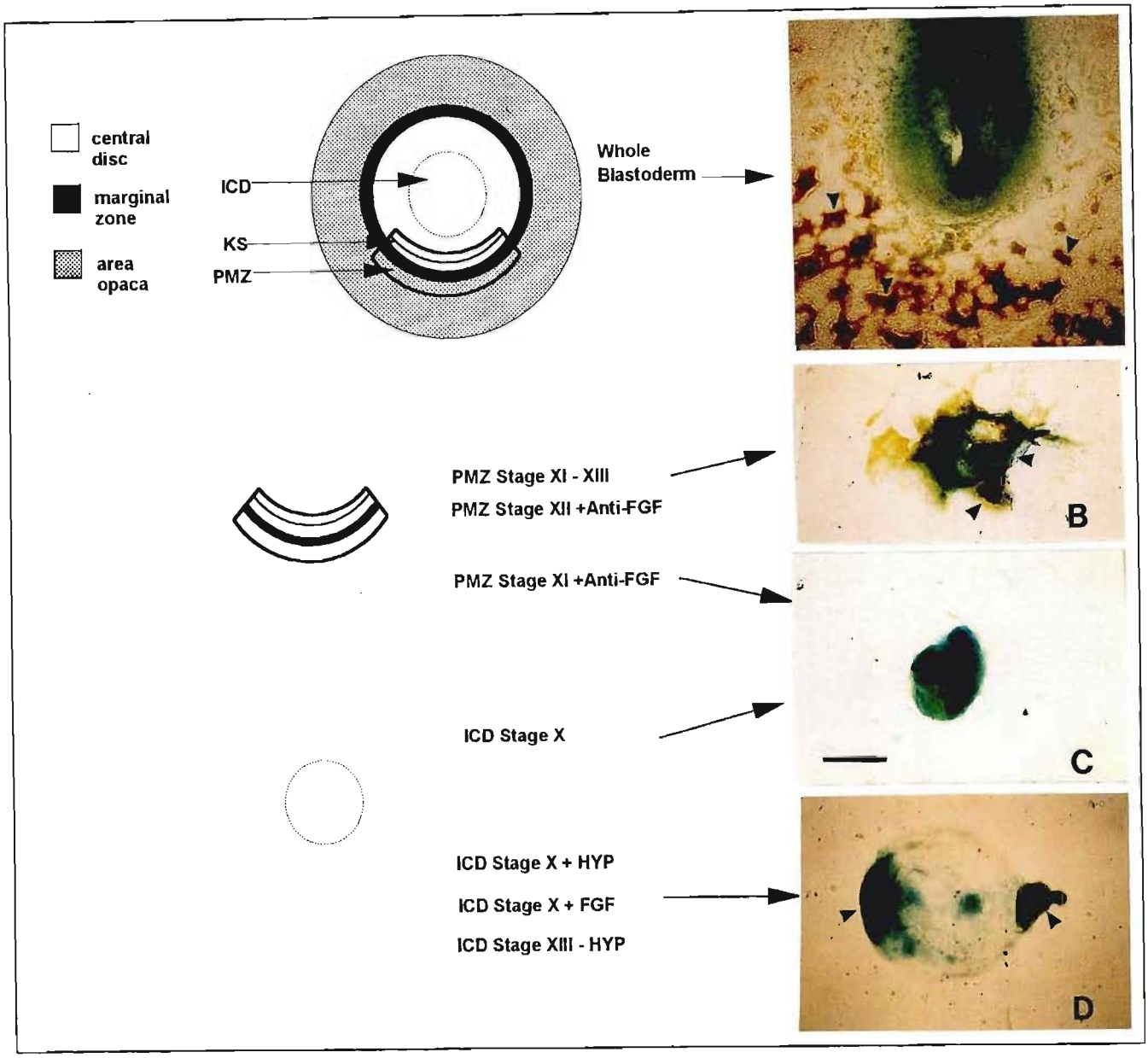
Fabian, 1986, 1987). In the present investigation the differentiating red blood cells in blood islands, and the erythrocytes within vascular channels of the control whole mount preparations stained brown, while all other tissues stained green with the counterstain (Fig. 4.2A).

The blastodermal pieces after three 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. 4.2B, and Table 4.1). 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. 4.2C). 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. 4.2D). Erythropoiesis in stage XIII ICD components was at a lower percentage (58%) after removal of the hypoblast; whereas the isolated hypoblasts formed loose sheets of cells which dissociated easily and showed no positive o-dianisidine staining (refer to Table 4.1).

TABLE 4.1. 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



Figs 4.2A-D The o-dianisidine reaction in whole mount preparations. Bar, 1mm.
(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.

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

Treatment of whole embryo explants at stages X and XI with anti-FGF, anti-FGFR, or heparin inhibited the development of erythropoietic tissue; while erythropoietic tissue developed in approximately 80% of the controls (refer to Fig. 4.3, and Table 4.2.) 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 anti-FGF or anti-FGFR, development was seen to be abnormal. These embryos had well developed head structures; while the posterior trunk region appeared defective as heart, somites, and blood tissue were absent.

TABLE 4.2. 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. (Controls were treated with Ringer's solution, chondroitin sulphate, or other irrelevant rabbit IgGs.)

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
32.5 µg/ml anti-FGFR	11	73
65 "	11	45
130 "	12	17
3 mg/ml heparin	10	0
Stages XII and XIII:		
Controls	15	80
3 mg/ml heparin	14	64

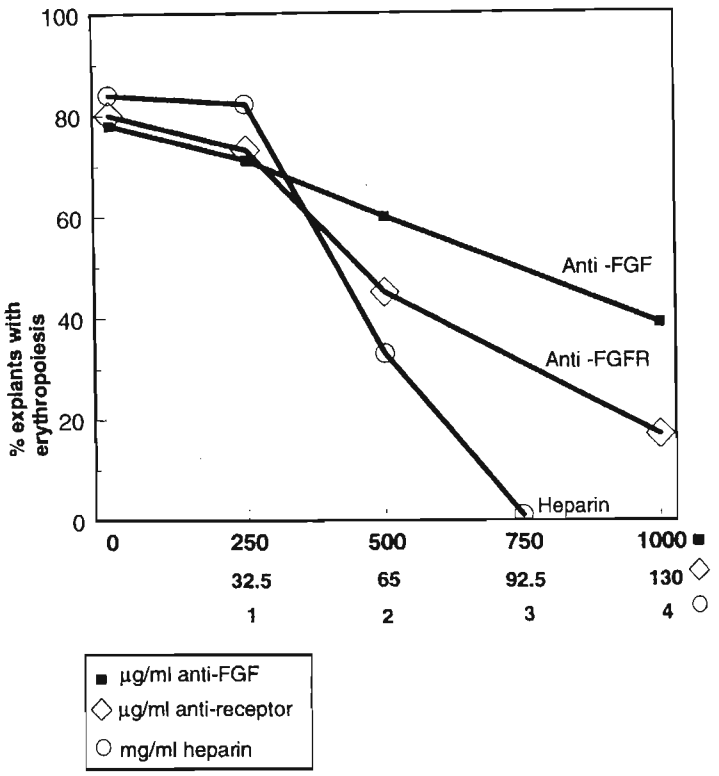


Fig. 4.3 Inhibition of erythropoiesis by the administration of antisera to bFGF (anti-FGF) or the bFGF receptor (anti-FGFR), or heparin, to whole blastoderms at stages X and XI, as determined by the o-dianisidine test for haemoglobin after 48 hours' incubation. Controls were treated with either Ringer's solution, other irrelevant rabbit IgGs, or chondroitin sulphate.

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. 4.4, and Table 4.1). 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.

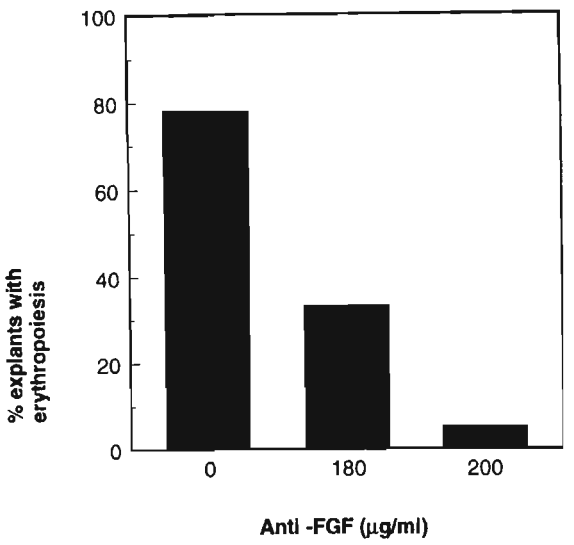


Fig. 4.4 The percentage of stage XI posterior marginal zone components (PMZ) in which erythropoiesis was inhibited by the addition of different concentrations of anti-FGF to the culture medium. Controls were treated with other irrelevant rabbit IgGs.

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

In the earlier investigations it was noted that heparin treatment of whole embryos at the later stage of XII and XIII, did not inhibit erythropoiesis (refer to Table 4.2); 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. 4.5, and Table 4.1).

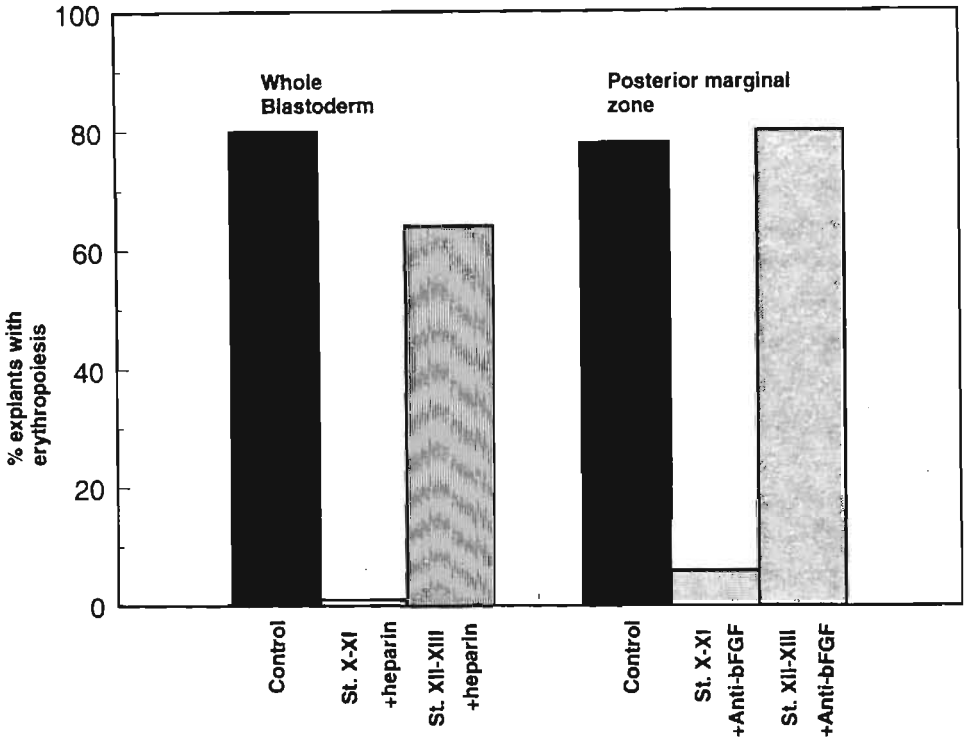


Fig. 4.5 The percentage of whole blastoderms or posterior marginal zone (PMZ) components that developed erythropoietic tissue after early (stages X and XI) or later (stages XII and XIII) treatment with antisera to bFGF or heparin. Controls were treated with chondroitin sulphate in the heparin series; or with other irrelevant rabbit IgGs in the anti-FGF series.

4.4.4 **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 three 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 Fig. 4.2D, and Fig. 4.6). This response was abolished when 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 these 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 the present investigation the explants were cultured in a neutral medium, i.e. without serum; thereby eliminating the extraneous introduction of unknown growth factors.

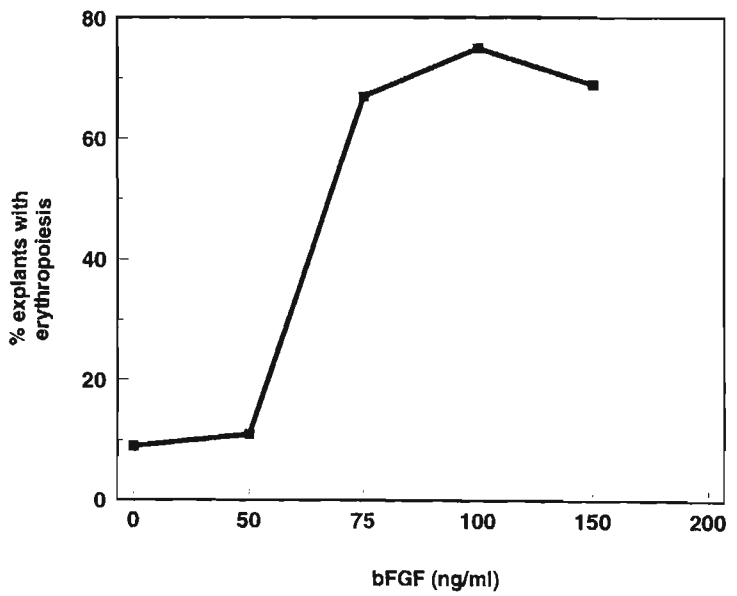


Fig. 4.6 Induction of erythropoiesis by bFGF in ICD components of stage X and XI embryos. Controls were cultured in serum-free medium without bFGF.

4.4.5 **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 which stained positively for Hb with o-dianisidine were seen in 75% of these sandwich explants (refer to Fig. 4.2D, and Table 4.1). 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 4.1.)

4.5 DISCUSSION

4.5.1 The early determination of the blood (Hb) differentiative pathway

It was shown that the formation of haemoglobin in whole embryos is inhibited when embryos at stages X and XI were cultured in the presence of antisera to bFGF or its receptor, or with heparin. However, similar treatments at the next stages, i.e. XII and XIII, do not inhibit Hb formation, suggesting that a commitment to the Hb differentiative pathway takes place in whole embryos prior to these stages. In order to narrow down the field of Hb commitment in the embryos it was demonstrated firstly, in agreement with Wilt (1974), that explants of the posterior marginal zone (PMZ) have a high propensity to form Hb at stages X to XIII, while the inner core of the epiblast does not make Hb. It was then shown, most interestingly, that anti-bFGF is able to inhibit Hb formation in PMZ explants at stage XI, but not in explants from stage XII and onwards.

These findings suggest that there are uncommitted cells in the PMZ of stage X-XI embryos which can be induced by bFGF into a Hb differentiative pathway. In support of bFGF's role as an inducer of blood mesoderm it was found that the treatment of an explant of uncommitted cells from a stage X blastoderm with bFGF (at a concentration of 75-150 ng/ml) results in the formation of Hb. The explant of uncommitted cells was taken from the inner central disc (ICD) of stage X embryos, which does not form blood in culture, as compared to ICDs from stage XII and XIII, which do. It is presumed that the uncommitted cells in the ICDs are part of, or equivalent to, the population of uncommitted cells extending 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 complex morphological changes which take place between stages X and XIII. These morphological changes involve 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). As mentioned previously, the horns of the sickle have extended more laterally by stage XII, and form the posterior and lateral borders of the emerging hypoblast (Khaner, 1993).

It is not possible at present to ascertain which cells in the PMZ are emitting and which are responding to a bFGF-type signal. This investigation provided evidence to show that

there are equivalent uncommitted cells in stage X ICDs which can respond to an FGF induction. Likewise, by using cultured sandwich experiments it was shown that the hypoblast, which arises in part from the area demarcated by the sickle at the PMZ, can induce Hb in uncommitted ICD cells. No blood was formed in control experiments when hypoblasts were cultured alone. Furthermore, and most relevantly, the addition of anti-bFGF to the sandwiches (hypoblast plus ICD tissue) inhibited the formation of Hb.

Finally it should be noted that these results are supported by a related experiment carried out at a later stage of development by Zagris (1982), who showed that grafting supernumerary hypoblasts from stage XIII embryos onto pregastrula blastoderms induces the formation of multiple erythropoietic sites.

4.5.2 **The posterior horseshoe of blood forming tissue**

How does the induction of the Hb differentiative pathway, as mediated by the hypoblast or by exogenous bFGF in these experiments, give rise to the horseshoe-shaped PMZ area which is committed to form blood by stage XII? It is appropriate to recall that the horseshoe-shaped area at stage XII persists through the definitive primitive streak stage (Settle, 1954) and is thereafter projected onto the AOV at the stage of Hb differentiation (Wilt, 1967). As noted above, Wilt found that explants of the posterior marginal zone (approximately at stage XI-XII of EG-K) has the highest propensity to form blood. The propensity dropped off in the lateral horns of the horseshoe while no activity was recorded in explants from the inner central disc (Wilt, 1974). It was noted that this horseshoe-shaped area of Hb positivity is roughly demarcated by Koller's sickle, from which area the posterior wave of the hypoblast emerges.

It is suggestive that the horseshoe pattern of blood tissue specification becomes committed following the continuous induction by cells of the (posterior) hypoblast with cells in the epiblastic layer along the Koller's sickle area, with which they are in intimate contact. This induction would not be effective in the most central part of the epiblast which becomes separated from the hypoblast with the formation of the blastocoelic space. While the central disc cells would not therefore normally receive an FGF-type signal, it was shown here that these cells are in fact competent to respond, as at least some of them can be induced to produce Hb, if the appropriate contact is made with the hypoblast, or in response to bFGF. The width of the horseshoe area of blood forming tissue in the posterior blastoderm could be limited 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 restriction 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. This would be in accordance with the specification map of Wilt, and would allow for a gradient-threshold mechanism to be set up, as has been proposed in *Xenopus* (Green and Smith, 1991; Green et al., 1992).

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

These experiments support the classic posterior "horseshoe" map of blood specification (see Wilt, 1974) and the neutralizing studies using antibodies show that the commitment to this pathway in the PMZ is taking place at stages X - XI. The specified "horseshoe" retains its relative position as development proceeds in the early chick embryo. This posterior to posterolateral "horseshoe" of Hb commitment can be equated with the ventral mesoderm of the amphibian blastula, with tissues from both species being induced by bFGF into a blood line pathway; although the level of the pathway appears to have only been minimally expressed in the frog (see Slack, 1990; Green et al., 1990; Isaacs et al., 1992). The PMZ thus anchors the blood forming end of the mesodermal spectrum in the chick, as in the frog. The other end of the mesodermal spectrum, i.e. the axial end, has been shown to be induced by activin (Mitrani et al., 1990b) in agreement with the frog model (see reviews by Ruiz i Altaba and Melton, 1989; Jessell and Melton, 1992; Green et al., 1992). The fate map of the chick, prior to gastrulation is consistent with these findings, 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 (see Balinsky, 1975; Gilbert, 1991; Stern et al., 1992). The effects of bFGF and activin should, of course, be in harmonious balance in order to generate the range of mesodermal tissue which results in the normal pattern of morphogenesis.

In further support of the role of bFGF in the balanced patterning of mesodermal morphogenesis in chick development it was found that the development of stage X and XI embryos was abnormal after treatment with antiserum to bFGF. The anti-FGF blocked the formation of Hb, but did not effect the development of head structures, as might perhaps be

expected; however, and most interestingly, the somites and heart were missing. This should be compared to the results of the 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. This supports the existence of a delicate balance between the effects of the growth factors at the two ends of the mesodermal spectrum, resulting in the harmonious development of the embryo.

In conclusion, the determination of the erythropoietic sequence in the chick is induced between stages XI and XII by cells of the hypoblast and a bFGF-like molecule is the causal trigger of this event.

CHAPTER FIVE

IMMUNOLOCALIZATION OF A bFGF-LIKE PROTEIN AND EVIDENCE FOR AN ACTIVE FGF RECEPTIVE SYSTEM DURING HYPOBLAST FORMATION

5.1 SUMMARY

The present chapter reports the results of an immunocytochemical analysis of bFGF distribution during the period of hypoblast formation, i.e. during the stages in development that both exogenous bFGF and hypoblastic tissue have been seen to have an effect on blood mesodermal tissue specification. In a parallel experiment an assay on the distribution of bFGF receptors was carried out. Immunocytochemistry was performed on whole mounts and serial sections of stage X to XIII embryos, using commercial polyclonal antibodies raised against bFGF or the bFGF receptor, coupled with a peroxidase anti-peroxidase (PAP) detection assay. In addition, a pilot study of the effect of lithium (which has been reported by others to disrupt tyrosine kinase phosphorylating pathways) on blood tissue formation after treatment at stage X and XI, was examined.

Cells that were immunoreactive to anti-FGF were located predominantly within the developing hypoblast from stage X onwards, which is consistent with the findings (Chapter Four) that the addition of a hypoblast or exogenous bFGF can induce erythropoiesis in isolated central discs. Immunostaining of bFGF was also seen in the cells (middle mesodermal cells) that accumulate between the epiblast and hypoblast in the posterior region of the area pellucida from stage XI onwards; while a less intense immunostaining was seen in some cells in the epiblast layer. This distribution of cells immunoreactive to the bFGF antibody was predominantly in the posterior region during the time window when blood tissue is seen to be specified in a posterior horseshoe-shaped area of the embryo.

Immunostaining with the bFGF receptor antibody was detected in the embryo from stage XII onwards in cells of the hypoblast and within the nuclei of cells in the epiblast layer. Interestingly, treatment with lithium, which has been proposed to effect signal transduction pathways in other systems, was found to inhibit blood tissue formation. The presence of the

bFGF receptors, and the inhibitory effect of lithium on erythropoiesis, lend further support for the importance of a FGF signal transduction for blood tissue specification.

5.2 INTRODUCTION

The hypoblast arises between stages X and XIII (reviewed in Chapter One, Section 1.2.2; and refer to Eyal-Giladi, 1991; Stern, 1991). There is evidence that the fully formed hypoblast at stage XIII induces primitive streak formation in the epiblast layer (Waddington, 1933; Azar and Eyal-Giladi, 1979); but it is not known whether this induction, which is activin driven (Mitrani et al., 1990b; Ziv et al., 1992), is also responsible for the specification of the mesodermal derivatives which arise from the primitive streak.

Evidence that blood mesodermal tissue is determined before primitive streak induction, has been provided by the earlier experiments of this study (Chapter Four). It was found that uncommitted cells in explants of stage X embryos can respond to an induction from a transplanted hypoblast, or exogenous bFGF, and form haemoglobin. It was also demonstrated that the antibody to bFGF and heparin can inhibit erythropoiesis, but this inhibitory effect becomes refractory from stage XII onwards, suggesting that a bFGF driven event before stage XII plays a role in blood tissue specification. It was proposed in Chapter Four that the emerging hypoblast was the source of the bFGF signal which commits cells in the epiblast before stage XII towards a haemoglobin differentiative pathway. This hypothesis also rests on the fate maps of Settle (1954) and Wilt (1974) who demonstrated that prospective blood tissue arises in a posterior horseshoe-shaped region of the embryo, which is projected from the region where the coherent hypoblast layer first appears.

For bFGF to be the putative signal molecule for the induction of blood mesodermal tissue, it is presumably localized within the inducing tissue, i.e. the hypoblast, before stage XII. Mitrani et al. (1990a) have reported that bFGF is distributed in both epiblast and hypoblast at this stage; although they did find that some cells in the sections of stage XI - XIII blastoderms gave a stronger signal with the immunofluorescence staining than others. As this distribution of bFGF in all the layers of the embryo is therefore greater than the area of the posterior horseshoe pattern where blood tissue is specified, it raises the possibility that bFGF activity functions over a wider field. To begin to address how bFGF functions during the determination of the erythropoietic cell line, it is essential to know both the spatial and temporal distribution of bFGF and its receptor during the time window when blood tissue

appears to be specified. In this study, an immunocytochemical analysis was used to detect the expression patterns of the bFGF-like ligand and its putative receptor in whole mounts and sections of the chick blastoderm between stages X and XIII.

It is generally accepted that the mode of action of bFGF involves receptor binding and the activation of the highly conserved tyrosine kinase contained in the cytoplasmic domain of the receptor , which in turn activates the phosphatidylinositol signalling pathway (Ullrich and Schlessinger, 1990; Friesel and Brown, 1992). Lithium has been shown to interfere with the phosphoinositide cycle which is important for the normal functioning of this signal transduction pathway; and has been used previously as a tool to perturb mesodermal inductions in the frog embryo (reviewed by Berridge et al., 1989). Treatment of early frog embryos with lithium leads to a dorsalization of the embryo, at the expense of posterior and ventral structures (Kao and Elinson, 1988; Elinson and Kao, 1993). As this effect can be blocked by the addition of myo-inositol, which is thought to be depleted by lithium, it has been proposed that the phosphoinositide cycle plays a role in early patterning by FGF signalling in the frog (Busa and Gimlich, 1989). It was therefore of interest to determine whether inhibiting FGF signalling at the level of the signal transduction with lithium could block erythropoiesis in the chick embryo. In a pilot study it was found that lithium treatment has a similar effect on chick development to that with anti-FGF and anti-FGFR (refer to Chapter Four). Lithium treatment at stage X and XI inhibited blood tissue (o-dianisidine positive tissue) formation and other posterior structures, although normal head structures were seen to arise in some cases.

5.3 MATERIALS AND METHODS

5.3.1 Preparation of embryos

Stage X - XII blastoderms (according to the tables of Eyal-Giladi and Kochav, 1976; see Fig. 1.7) were explanted from freshly laid unincubated eggs; the stage XIII blastoderms were explanted from eggs incubated for 4-8 hours at 38^{o}C. The embryos were cleaned of yolk in Ringer's solution under aseptic conditions, as described in the first three steps in Appendix 2. Embryos were either fixed and processed for immunocytochemistry (refer to Sections 5.3.2 to 5.3.4 below); or treated with lithium, cultured on vitelline membrane rafts for two days, and stained with o-dianisidine (refer to Section 5.3.5 below).

5.3.2 Tissue preparation

The explanted embryos used for the immunocytochemical analysis were fixed immediately in either 4% paraformaldehyde in phosphate buffered saline (refer to Appendix 1.3) for 1-4 hours, and rinsed in PBS overnight; or, in alcoholic or Hollande Bouin fixative (Humason, 1979) overnight, and rinsed in a number of changes of distilled water over a 5 hour period. The embryos destined to be sectioned were dehydrated in ethanol, cleared in methyl benzoate, and embedded in Paraplast. Serial sections of 7μm thickness were made through either the longitudinal or transverse plane of the blastoderm, and were mounted on slides pre-coated with poly-L-lysine (Sigma Diagnostics, USA). The slides and whole mounts were processed for either bFGF or bFGF receptor detection by indirect immunoperoxidase staining using primary rabbit IgGs.

5.3.3 Primary antibodies

Anti-FGF: A series of whole mounts and sections were incubated in a rabbit polyclonal antibody raised against bovine brain bFGF (supplied by Sigma Immunochemicals, USA) at a concentration of 300 μg IgG/ml phosphate buffered saline containing 0.1% bovine serum albumen (PBS/BSA; see Appendix 1.5), i.e. a 1:50 dilution of the IgG.

Anti-FGFR: A series of whole mounts and sections were incubated in one of two different primary antisera raised against the human bFGF receptor, which were stated to have specificity for chicken FGF receptors by the supplier (see also Lee et al., 1989). Two different anti-FGFR products were used in this investigation, due to changes being made by the supplier (Promega, USA) between purchases. The anti-FGFR used at the beginning of this investigation had specifications to recognize a peptide corresponding to the deduced cDNA sequence of the putative FGF receptor (*flg*, *bek*, and *cek* gene products). The concentrations used were 26 μg IgG/ml and 13 μg IgG/ml PBS/BSA containing 5% dimethyl sulphoxide; i.e. a 1:5 and a 1:10 dilution of IgG respectively. The addition of 5% DMSO was used as it is reported to improve antibody penetration (Klymkowsky and Hanken, 1991). The anti-FGFR purchased at a later date, had specifications to recognize the extracellular domain of purified, human recombinant FGF receptor. This was used at a concentration of 333 μg IgG/ml in PBS/BSA containing 5% DMSO; i.e. a 1:4 dilution of IgG.

The optimum dilution for each of the IgGs above, was determined by immunocytochemical analysis of head and yolk sac tissues dissected from six day chick

embryos. The six day chick embryo has been reported to have abundant bFGF and bFGF receptors by Lee et al. (1989).

Controls: The above primary antibodies were substituted with normal rabbit serum, or with the blocking serum (normal sheep serum provided in the staining kit; see Appendix 3). Some controls were incubated with anti-FGF serum that had been depleted of anti-FGF activity by adsorption with an excess of recombinant bovine bFGF (Boehringer Mannheim, Germany) that was added to the anti-FGF before processing.

5.3.4 **Immunocytochemical procedure**

Sectioned material and whole mounts were processed using the peroxidase anti-peroxidase (PAP) method with one of the above primary antibodies and a Universal immunoperoxidase staining kit (Signet Laboratories, USA). The chromogen used was 3-amino-9-ethylcarbozole which gave a red reaction product. The staining procedure was done at room temperature, and followed that given in the kit with some variation in the incubation periods (see Appendix 3). The whole mounts were incubated in the primary and secondary antibodies over an extended period of 16-24 hours to ensure antibody penetration. The primary antibody incubation times with the sectioned material was one hour in anti-FGF, or overnight in anti-FGFR.

5.3.5 **Lithium treatment**

Stage X and XI embryos were explanted and cleaned of yolk under aseptic conditions. Each blastoderm was immersed in a solution of lithium chloride (125 mM - 500 mM LiCL in one part Dulbecco's modified Eagle's medium to one part Ringer's solution) and incubated at 38°C for 10 minutes. The high level of concentrations used in these experiments were based on the range of concentrations that produced teratogenic effects, and brought about the inhibition of inositol phosphatases in frog embryos (refer to Berridge et al., 1989). Control chick blastoderms were treated similarly, except that 500 mM NaCl was substituted for the LiCl. Thereafter, the embryos were rinsed in three changes of Ringer's solution, placed on vitelline membrane rafts, and cultured over a pool of albumen, according to New (1955) as described in Appendix 2. Stereomicroscopic observations were made of the embryos in culture at day one. After 48 hours incubation the cultures were removed from the vitelline membrane rafts, and stained for the presence of haemoglobin, using the o-dianisidine test (refer to Appendix 1.2).

5.4 RESULTS

5.4.1 Specificity of the antibodies

The specificity of the rabbit polyclonal bFGF and bFGF receptor antibodies (anti-FGF and anti-FGFR respectively) was tested on whole mount and sectioned material of six day old chick embryos. Immunostaining was evident in the whole mount preparations and sectioned tissues of six day old chick embryos with the antisera to both bFGF and the bFGF receptor. Immunoreactivity to the anti-FGF was located in neural tissue of the eye, the lens, some mesenchymal cells, developing cartilage, and in the yolk sac endothelial and endodermal cells. The immunostaining was abolished in the controls incubated with bFGF antiserum pretreated with recombinant bovine bFGF (refer to Fig. 5.1). Cells immunoreactive to anti-FGFR in the six day embryo showed a similar pattern of distribution to the cells showing immunoreactivity to anti-FGF. As the appropriate immunizing peptide antigens for the bFGF receptor antisera were not available, preabsorbtion control tests were not done in this preliminary investigation. However, immunostaining was eliminated when the primary antibody was substituted with normal serum (refer to Fig. 5.1).

The influence of different methods of fixation on the patterns of immunostaining in the stage X to XIII embryos was also examined. Immunostaining with anti-FGF showed the same distribution pattern in material fixed in either paraformaldehyde or Bouin fixatives; whereas immunostaining with anti-FGFR gave inconsistent results. The Bouin fixed material gave poor immunostaining with the anti-FGFR in the whole mounts, but allowed for nuclear staining in the sectioned material; compared to the paraformaldehyde fixed material that gave clear immunostaining in whole mount preparations, but gave poor immunostaining of sectioned material. The reason for this different pattern of immunostaining with the anti-FGFR is not clear; and did not appear to be related to the different bFGF receptor antisera used in this investigation. A further immunocytochemical analysis with the anti-FGFR therefore needs to be undertaken using other methods of fixation, eg. cryopreservation, to ensure that the antigens are not lost during the fixation procedure.

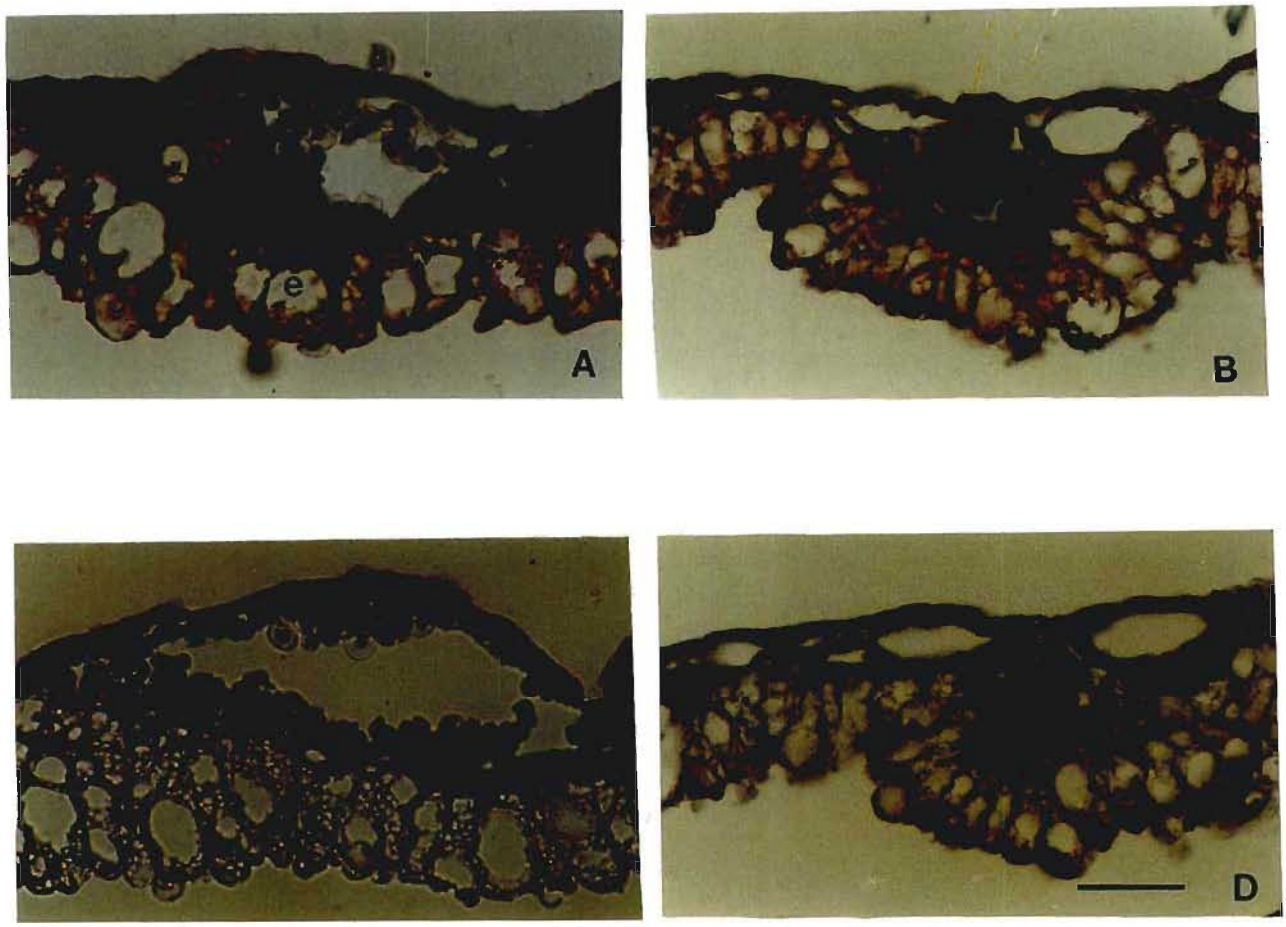


Fig. 5.1 Bright field photomicrographs of immunostaining (seen as red-stained areas) in the yolk sac of the six day old chick embryo. Sections A-D paraformaldehyde fixed; counterstained with haematoxylin; Bar, 50 μm. (A) Anti-FGF immunoreactivity was located in the extraembryonic endoderm cells (e), and the endothelial cells (arrows) of the blood vessel. Erythrocytes show no immunoreactivity. (B) Anti-FGFR immunoreactivity was localized in the endodermal cells (e), and in the endothelial cells (arrows) of the blood vessels. (C-D) Control sections of yolk sac incubated with preabsorbed anti-FGF (C), or with normal rabbit serum (D), showed no immunostaining.

5.4.2 **Immunolocalization of anti-FGF**

Cells immunoreactive to the anti-FGF were present at all stages of hypoblast formation (stages X - XIII), with the developing hypoblast showing the most intense immunostaining. At stage X, cells immunoreactive to the bFGF antibody were only located in amongst the islands of polyingressed epiblast cells (PIGs) of the developing hypoblast. These immunoreactive cells were distributed evenly over the ventral surface of the area pellucida, and the immunostaining was intracellular and intense (Fig. 5.2). No other cells in the area pellucida, including Koller's sickle, showed immunostaining at stage X. From stage XI the immunostaining increased significantly. By focusing through the different levels of the whole mounts of stage XI and XII blastoderms, it was found that the immunostaining was distributed predominantly in the cells of the emerging hypoblast (Figs 5.3 and 5.4). By focusing at the level of the epiblast it was found that some cells within the epiblast layer showed a low level of immunoreactivity with the anti-FGF. This less intense staining in the epiblast was clearly seen in the sectioned material to be due to immunostaining being restricted to the surfaces of certain epiblast cells (Fig. 5.5). A few cells in the epiblast, which appeared to be in the process of invaginating from the epiblast layer, however, showed a more intense intracellular staining (refer to Fig. 5.5A). The middle mesodermal cells could be observed by focusing at a level between the epiblast and hypoblast layers in the whole mounts, or seen more clearly in the sectioned material, and were present from stage XI onwards. These middle mesodermal cells showed intense intracellular staining (Figs 5.4 and 5.5).

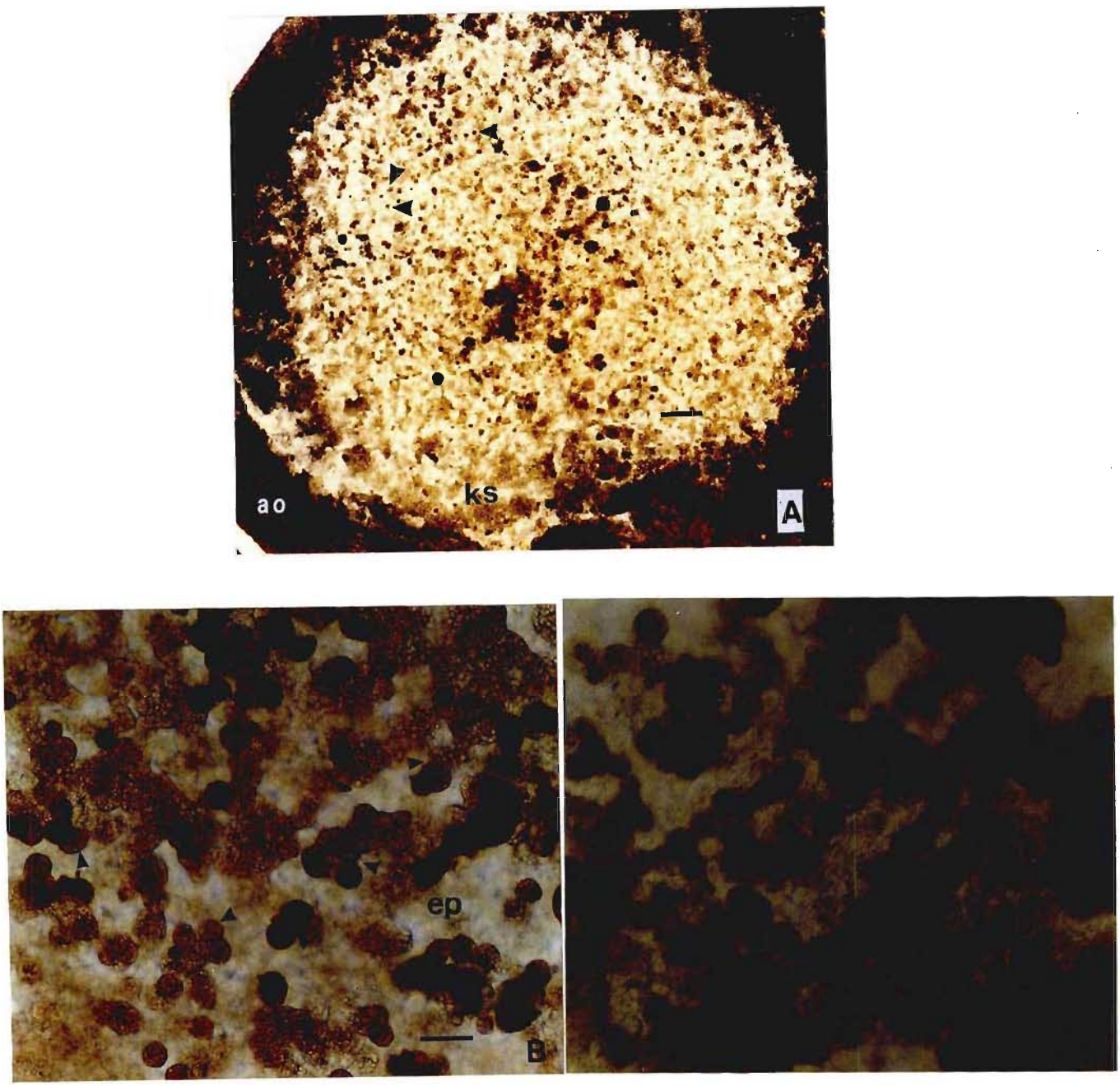


Fig. 5.2 Bright field photomicrographs of immunostaining with anti-FGF in stage X blastoderms. Whole mounts fixed in alcoholic Bouin solution, and viewed from the ventral surface. Bars, in A = 250 μm, in B = 25 μm; (B and C are the same magnification). (A) Low power micrograph of the ventral surface of the area pellucida showing the clusters of polyingressed epiblast cells (arrows), and the small brown dots (arrows) which represent the immunostained cells shown in B. The posterior Koller's sickle region (ks) showed no immunostaining. The area opaca (ao) was immunostained. (B) By focusing on the polyingressed epiblast cells (PIGs; arrows), some cells were seen to be immunoreactive to anti-FGF (red stain). The remaining PIGs were unstained, as was the underlying epiblast (ep; out of focus in this micrograph). (C) Control incubated with preabsorbed anti-FGF showed no staining reaction; note the PIGs (arrows).

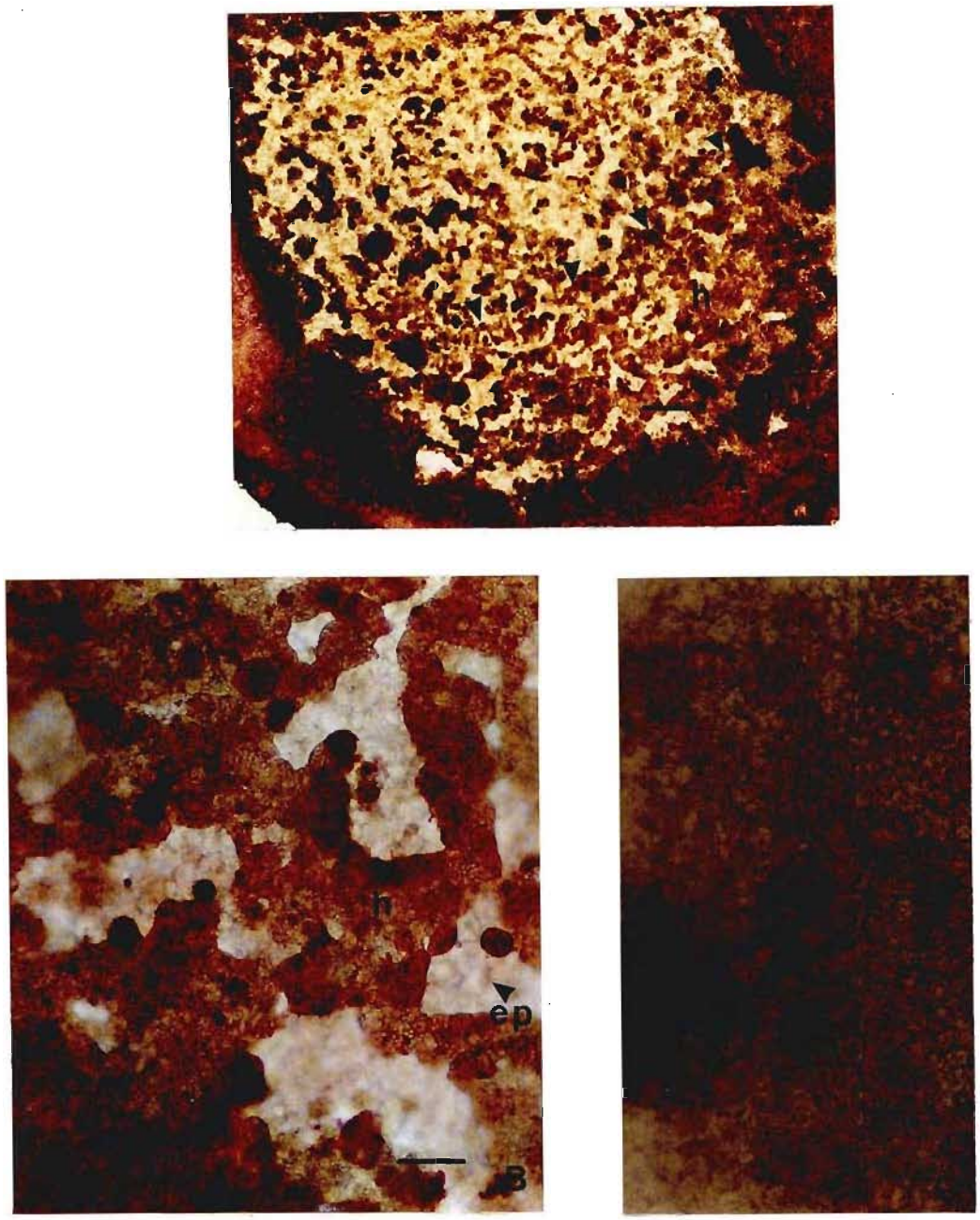


Fig. 5.3 Bright field photomicrographs of immunostaining with anti-FGF in stage XI embryos. Whole mounts fixed in alcoholic Bouin solution, and viewed from the ventral surface. (The photomicrographs are aligned with the posterior end directed to the bottom of the page.) Bars, in A = 250 μm, in B = 25 μm; (B and C are the same magnification. (A) Low power micrograph of the ventral surface of the area pellucida showing the emergence of the hypoblast (h) in the posterior region (arrows point to the anterior edge of the hypoblast). The hypoblast showed immunostaining (appears as a reddish-brown colour at this magnification). (B) Anti-FGF immunoreactivity (red stain) predominates within the developing hypoblast layer (h) in the posterior region of the area pellucida. A lower level of immunostaining (pink stain) was observed in some cells in the underlying epiblast layer (ep; out of focus in this micrograph). (C) Control incubated with preabsorbed anti-FGF showed no staining reaction.

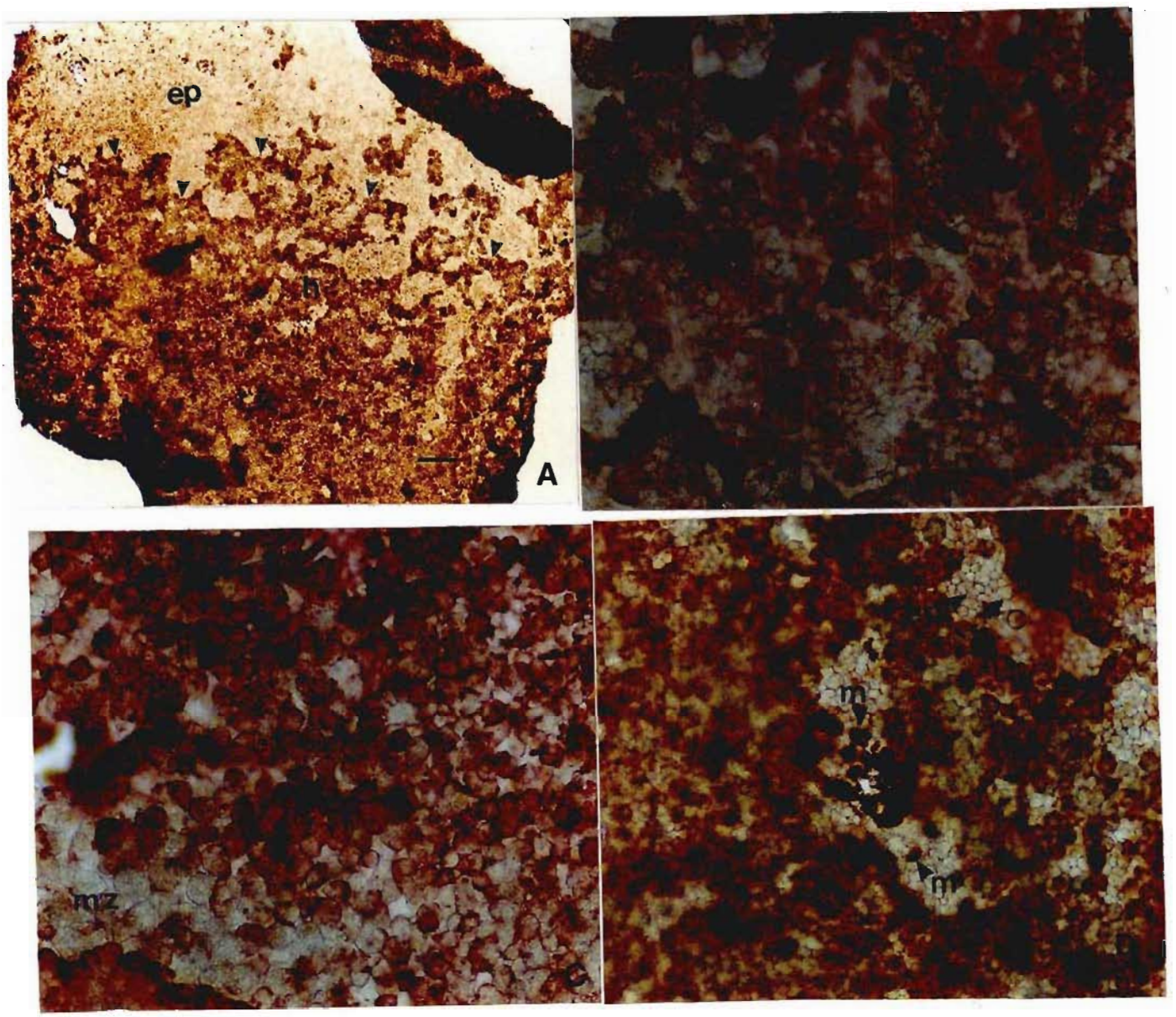


Fig. 5.4 Bright field photomicrographs of immunostaining with anti-FGF in the stage XII embryo. Whole mounts (A,B,C and D) fixed in paraformaldehyde, viewed from the ventral surface. Bars, in A = 250 μm, in B = 25 μm; (B,C and D are the same magnification). (The photomicrographs are aligned with the posterior end directed to the bottom of the page.) (A) Low power micrograph showing the immunoreactive hypoblast (h) in the posterior half of the area pellucida (arrows point to the anterior edge of the hypoblast). In the anterior area pellucida the epiblast (ep) appeared unstained at this magnification. (B) Anterior area pellucida showing anti-FGF immunoreactivity (red stain) within a few polyingressed epiblast cells (arrows) which have been focused on in this micrograph. A number of the epiblast cells (ep) showed low level immunostaining (pink stain); (these epiblast cells are slightly out of focus in this micrograph). (C) Posterior area pellucida, showing intense immunostaining (red stain) in the hypoblast layer (h) which has been focused on in this micrograph. Immunostaining was not evident in the posterior marginal zone (mz). (D) By focusing at the level between the hypoblast and epiblast at a region where the ventral hypoblast was torn away, groups of intensely stained middle mesodermal cells (m) could be seen. The epiblast cells (ep) appeared to be stained on their cell surfaces only. (The hypoblast (h) is out of focus in this micrograph).

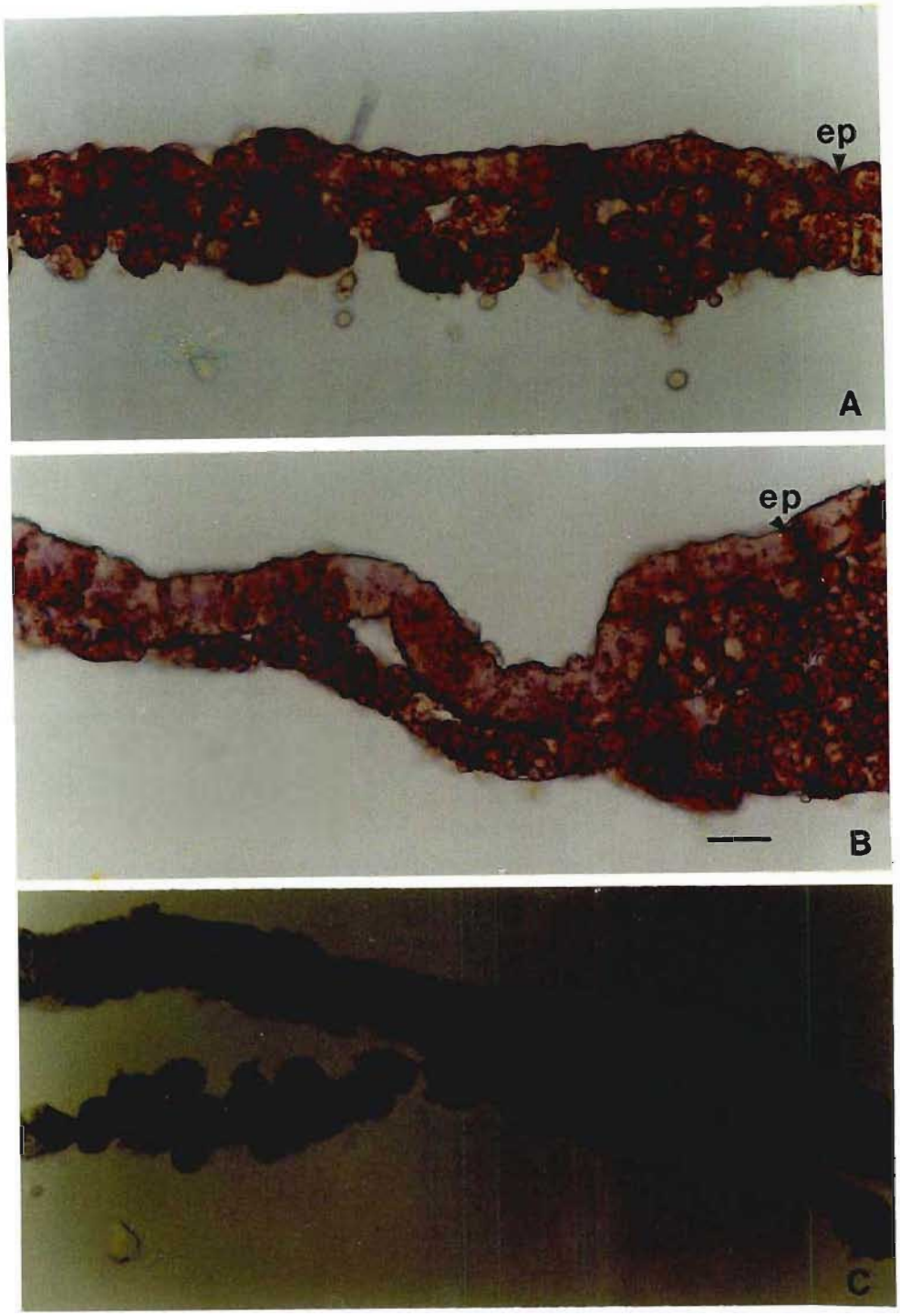


Fig. 5.5 Bright field photomicrograph of immunostaining with anti-FGF in stage XI and XII embryos. Longitudinal sections fixed in paraformaldehyde; sections B and C counterstained with haematoxylin. (The posterior end of the blastoderm is to the right of each photomicrograph.) Bar, 25 μm. (A) In the stage XI embryo, immunostaining was seen on the surface domains of the epiblast cells (ep), and within cells that appeared to be invaginating from the epiblast (i), and the middle mesodermal cells (m) and hypoblast cells (h). (B) In the stage XII embryo, immunostaining was seen within the hypoblast (h) and middle mesodermal cells (m), and on the surfaces of some epiblast cells (ep). (C) Control section incubated with preabsorbed anti-FGF showed no staining reaction.

5.4.3 Immunolocalization of anti-FGFR

The localization of cells immunoreactive to the anti-FGFR is not conclusive, as the distribution pattern of the immunoreactive cells was inconsistent with the different fixation protocols used. However, in all the trials (n=7) it was noted that cells immunoreactive to anti-FGFR were only present in blastoderms from stage XII onwards. Cells immunoreactive to anti-FGFR were distributed in the hypoblast and in the middle mesodermal cells at stage XII (Fig. 5.6). A faint intranuclear immunostaining developed in the cells of the epiblast layer from stage XII, which increased in intensity at stage XIII (Figs 5.6 and 5.7). Although, immunostaining was not apparent in stage X and XI embryos; it is not clear whether at these earlier stages the bFGF receptors are (1) not normally expressed; or (2) present in another form, as there are several known members of the FGF receptor family (see Johnson and Williams, 1993; Patstone et al., 1993; Marcelle et al., 1994); or (3) lost during the fixation procedure. This immunocytochemical analysis of the bFGF receptor distribution must therefore be considered provisional until further work has been done.

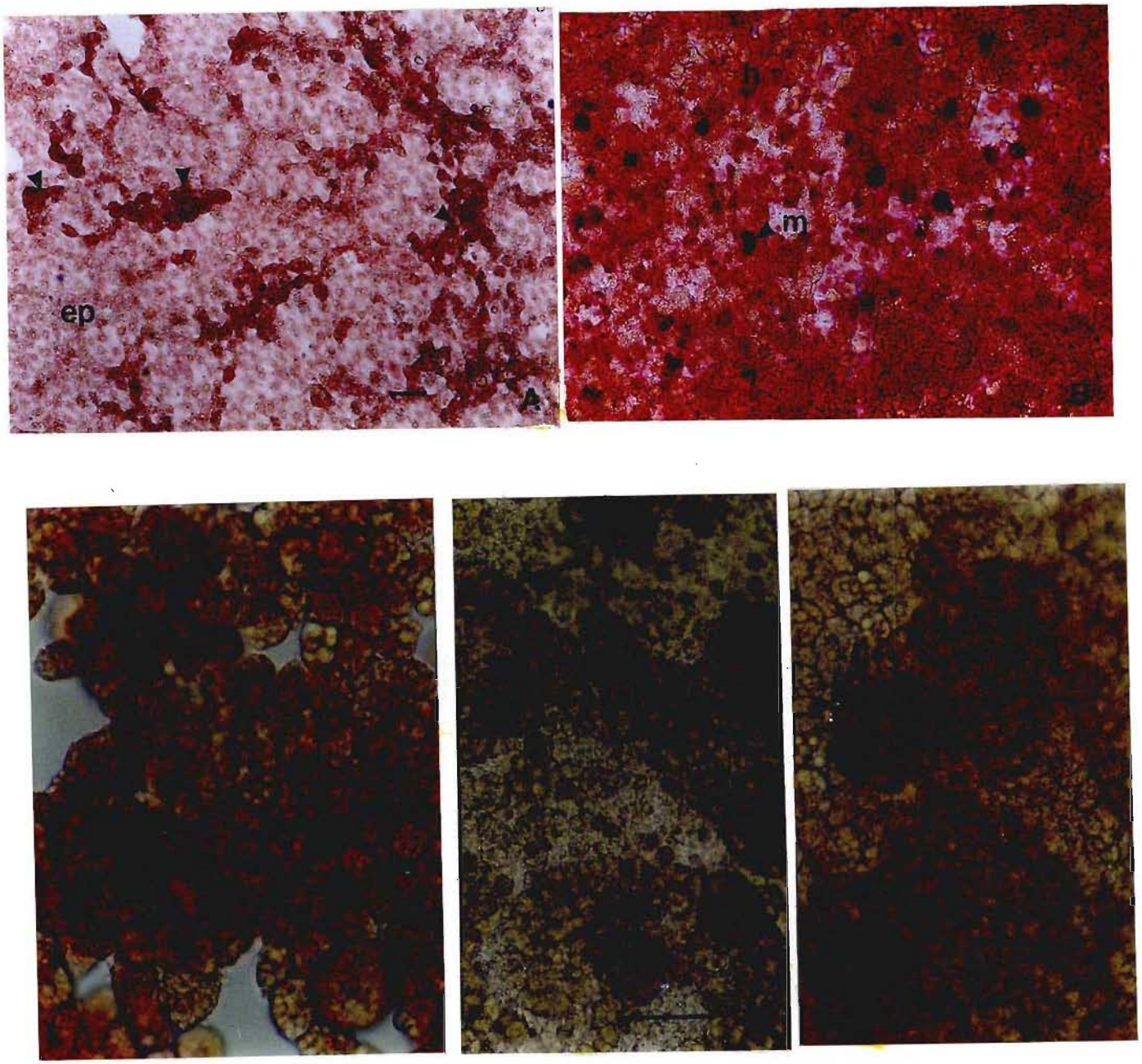


Fig. 5.6 Bright field photomicrographs of immunostaining with anti-FGFR in stage X, XII, and XIII embryos. Whole mounts fixed in paraformaldehyde (A,B,C, and E); or alcoholic Bouin solution (D), and viewed from the ventral surface. Bars, 25 μm; (A and B, and C,D and E are the same magnification). (A) In the anterior area pellucida of a stage XII embryo anti-FGFR immunoreactivity was located in the islands of polyingressed cells (arrows) and in the nuclei of cells in the epiblast layer (ep). (B) In the posterior area pellucida immunostaining was intense (red stain), and appeared to be distributed in the hypoblast (h) and middle mesodermal cells (m), which could be seen by focusing at a level between the hypoblast and epiblast layers. (C) An isolated hypoblast layer from a stage XIII blastoderm showing anti-FGFR immunoreactivity in some cells (arrows). (D) A stage X embryo showed no immunoreactive tissue after incubation in anti-FGFR. (E) Control embryo (stage XII) incubated with normal rabbit serum showed no immunostaining.

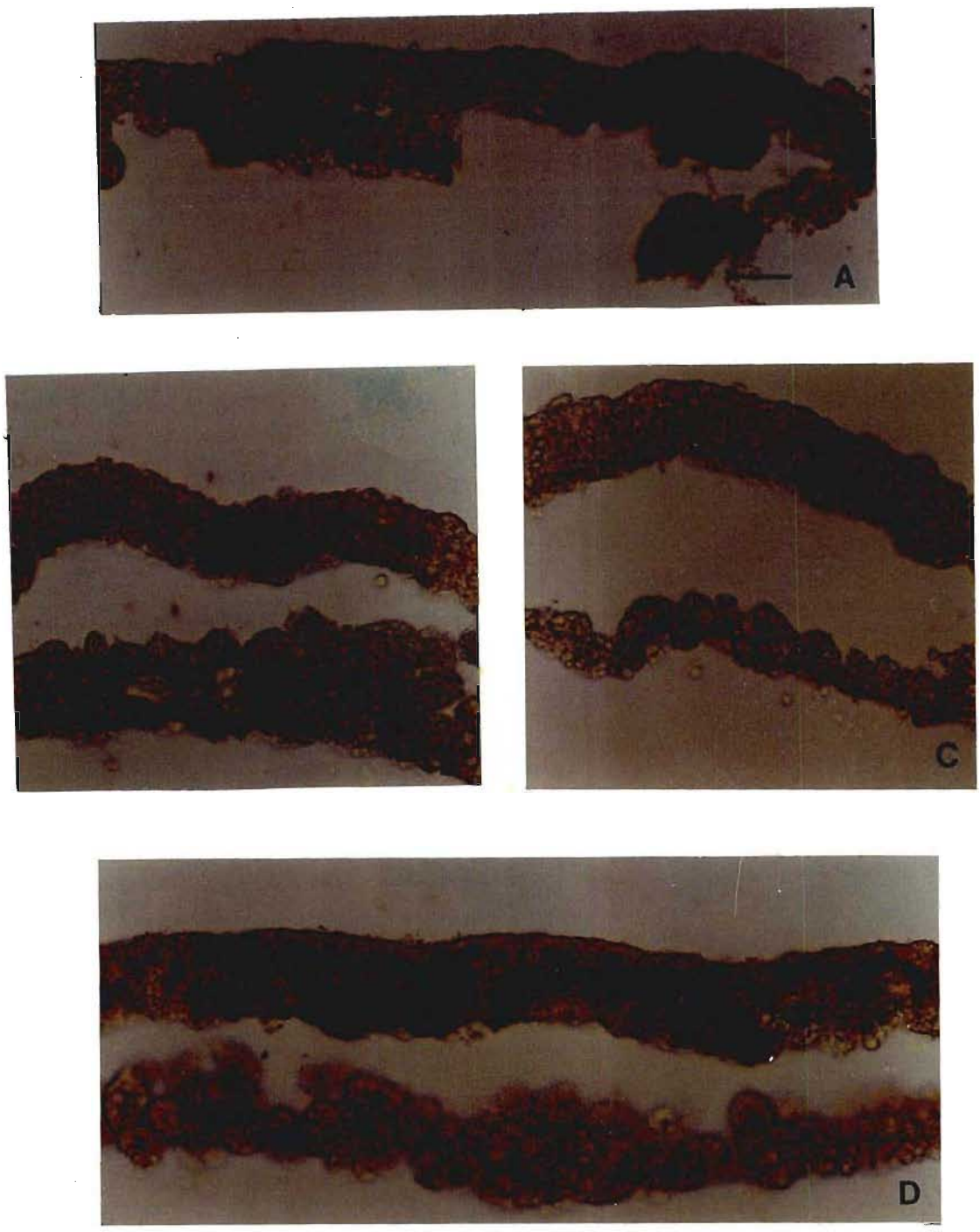


Fig. 5.7 Bright field photomicrographs of immunostaining with anti-FGFR in stage X, XII, and XIII embryos. Transverse sections; fixed in Hollande Bouin solution. Bar, 25 μm. (A-B) Low level immunostaining was seen in nuclei of the epiblast layer (ep) in stage X and XII embryos. (C) Control section (stage XII) incubated with normal serum showed no staining reaction. (D) Intranuclear immunostaining with anti-FGFR was intense (red stain) in the nuclei of the epiblast layer (ep) at stage XIII, with low level staining in the hypoblast (h). (The hypoblast is out of focus in this micrograph.)

5.4.4 Lithium effect on primitive streak formation and erythropoiesis

The embryos treated with 500 mM NaCl or 125 mM LiCl for ten minutes at stage X and XI, developed normally. These embryos formed a primitive streak by day one; and after 48 hours in culture, blood tissue (indicated by positive staining with the o-dianisidine test for haemoglobin) had developed in the posterior region of the embryos. Embryos treated briefly with 250 mM LiCl at stage XI formed a primitive streak in 4 out of the 5 embryos. However, two out of the four embryos that had developed a primitive streak, subsequently formed abnormally along the anteroposterior axis. These embryos had a normal head but posterior structures, such as heart, somites and blood tissue did not develop. Erythropoiesis (o-dianisidine-positive tissue) was inhibited in 3 of the 5 embryos. The embryos treated with 500 mM LiCl had no primitive streak by day one, and after two days in culture o-dianisidine-positive tissue and axial structures had not developed (refer to Table 5.1, and Fig. 5.8).

TABLE 5.1 The effect of brief lithium chloride treatment at stage X and XI on axis formation and erythropoiesis. (The controls were treated with 500 mM NaCl instead of lithium.)

Treatment:	No. of Tests:	No. with Primitive Streak:	Percentage Erythropoiesis:
500 mM NaCl	5	5	100
125 mM LiCL	1	1	100
250 mM LiCl	5	4*	40
500 mM LiCl	5	0	0

* Two of the embryos that formed a primitive streak, developed head structures, but no somites or other posterior structures including blood, by day two.

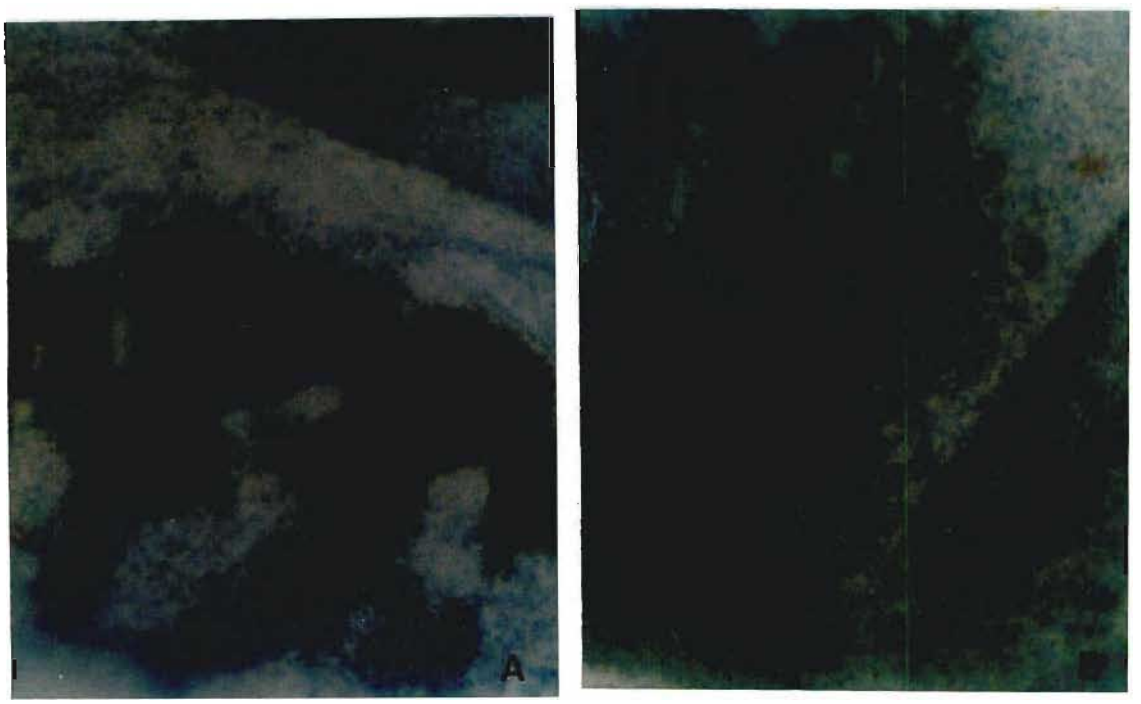


Fig. 5.8 Whole mounts of two day embryos stained with the o-dianisidine test for haemoglobin. Bar, 50 μm. (A) The control embryo incubated in 500 mM NaCl developed normally and shows dark brown patches of Hb (arrows) in the area opaca vasculosa. (B) The embryo treated briefly with 500 mM LiCl at stage XI shows no reaction for Hb.

5.5 DISCUSSION

5.5.1 The posterior dominance of a bFGF-like protein

In the present investigation bFGF-like proteins were localized by immunocytochemistry in the chick blastoderm at stages X - XIII, i.e. during the stages of hypoblast formation and the time window when both hypoblast and bFGF have been shown to have an inductive effect on Hb formation. The presence of bFGF in the chick blastoderm at this appropriate stage in development lends further support for it playing a role in blood tissue specification. Analysis of whole mount preparations revealed a temporal and spatial distribution pattern of the bFGF-like protein. Initially at stage X, the immunostaining was seen in cells distributed amongst unstained groups of cells in the islands of polyingressed epiblast cells (PIGs). Although the stages earlier than stage X have not been analyzed, it appears that bFGF begins to accumulate at stage X within certain cells that take part in the initial formation of the hypoblast (i.e. the PIGs). Thereafter from stage XI, the number of cells immunoreactive to anti-FGF increased significantly. These immunoreactive cells were distributed in all parts of the blastoderm, which confers with the earlier report of Mitrani et al. (1990a). However, this present study shows that there were different levels of staining intensity in different parts of the embryo. This was seen particularly in the whole mounts by focusing up and down through the different germ layers. It was clearly evident that the immunostaining was predominantly in the emerging hypoblast cells anterior to Koller's sickle and in the middle layer of mesodermal cells that had accumulated between the epiblast and hypoblast from stage XI. This middle layer of cells consists of a population of mesenchymal cells which arise before primitive streak formation during the formation of the hypoblast, by a de-epithelialization from the epiblast (reviewed in Chapter One, Section 1.2.3; and also in Stern, 1991).

As the developing hypoblast and middle mesodermal cells are situated in the posterior domain of the area pellucida during stages XI and XII, it results in a polarized distribution of bFGF with a distinct anteroposterior asymmetry. This predominant posterior expression of bFGF is coincident with the time window in development when blood tissue is seen to be specified in a posterior and posterolateral horseshoe-shaped area of the blastoderm. The generation of the horseshoe pattern therefore could be brought about not only by the intimate contact that the posterior hypoblast has with the overlying epiblast in this region, but also by the higher concentration of bFGF located in the posterior region at this time. A higher level of bFGF in this posterior region may function by setting up a concentration gradient whereby

cells in the overlying epiblast respond to threshold concentrations of the bFGF, as has been proposed in the frog (see Green and Smith, 1991; Green et al., 1992; Slack and Tannahill, 1992). It is also suggestive that the early expression of a bFGF-like protein in the posterior blastoderm of the chick may be equivalent to the proposed first signal of the "three signal model" used to explain mesodermal patterning in the frog. It is generally accepted that bFGF, which has been found to be present in the early frog embryo at the stages of mesodermal induction (Kimelman et al., 1988; Slack and Isaacs, 1989; Shiurba et al., 1991; Isaacs et al., 1992), is the putative first signal that induces ventral-type mesoderm (refer to Dale et al., 1985; Dale and Slack, 1987; Kimelman et al., 1992).

In the present immunocytochemical analysis it was not possible to determine whether the low level of immunostaining on the surfaces of the epiblast cells was as a result of the bFGF being expressed by these epiblast cells; or whether bFGF had diffused from some other source (i.e. hypoblast or middle mesodermal cells) into the epiblast layer. Although Mitrani et al. (1990a) have reported that transcripts of the bFGF-RNA are not present in the hypoblast and epiblast at this time, this result may have been due to low levels of transcript not being detectable in their Northern blot analysis. These workers reported that bFGF transcripts first appear at stage XIII in the marginal zone. The origin of the bFGF protein is therefore unclear. Perhaps an in situ hybridization analysis of the distribution of bFGF transcripts during the earlier stages of cleavage to stage XIII would possibly provide some answers to this problem.

It is interesting that the cells showing intense intracellular immunostaining with anti-FGF were located primarily in the regions of the blastoderm where morphogenetic processes occur during these stages, namely in the developing hypoblast and the middle mesenchymal layer of cells migrating between the hypoblast and epiblast; and also in the invaginating epiblast cells (progenitor mesodermal cells). Some of the cells within the epiblast also become dislocated from the posterior and posterolateral regions and migrate anteriorly along the median line within the epiblast layer itself (refer to Stern, 1990, and Eyal-Giladi et al., 1992). This could explain the presence of the low level of bFGF seen in some epiblast cells at this time. It cannot be excluded therefore, that bFGF may have an important role in modulating cell migratory processes.

5.5.2 Evidence for a functioning FGF receptive system during hypoblast formation

If bFGF is acting as an inducer of the erythropoietic cell line before stage XII, then one would expect to find particular cells in the blastoderm that contain bFGF receptors at this time, indicating their competence to mediate the morphogenetic response. In this preliminary investigation, cells that were immunoreactive to anti-FGFR appeared in the blastoderm at stage XII, and showed a similar distribution pattern as the cells expressing the bFGF-like protein, i.e. in the middle mesodermal cells and hypoblast. It would be interesting to determine whether the cells of the hypoblast and the middle mesodermal cells are co-expressing bFGF and its putative receptor, or if their expression occurs in distinct subpopulations of cells which have different developmental potentials. Assuming that there is a co-expression of bFGF and its receptor, it suggests that bFGF activity is functioning through an intracellular autocrine loop. This would be consistent with the reports that bFGF lacks the signal sequence necessary for its secretion from cells (refer to Slack, 1991b); although there is some debate with regard to bFGF acting as a secretory molecule with reports that it can be exported from cells by a novel mechanism (refer to Kandel et al., 1991). Most interestingly, nuclei of the epiblast cells were seen to develop an intense immunoreactivity to the anti-FGFR by stage XIII. This analysis is to be followed further.

It is plausible that some of these middle mesodermal cells could be the progenitor blood cells, as these cells were seen to be the earliest mesodermal cells to express the bFGF ligand and bFGF receptor. However, it should be noted that the isolated hypoblasts of stage XIII embryos, which inadvertently may have had mesodermal cells attached to them, did not form Hb-positive tissue when cultured alone (refer to Chapter Four).

Lithium was used as a non-specific tool to perturb FGF signalling during the time window when bFGF appears to affect the specification of blood tissue in this study. This was based on the proposal that lithium has an inhibitory effect on tyrosine kinase signal transductions by blocking inositol phosphate metabolism (Berridge et al., 1989; Maslanski et al., 1992). This proposal has led to the hypothesis that the lithium syndrome of dorsalization seen in *Xenopus* embryos, is a result of blocking inductive interactions mediated by second messenger pathways (reviewed in Berridge et al., 1989). Furthermore, this lithium effect in frogs also shows some similarity to the effects of treatment with a dominant negative mutant of the FGF receptor lacking the tyrosine kinase domain. Treatment with this truncated receptor blocked the inducing response to bFGF, and caused defects in the posterior body

pattern (Amaya et al., 1991). In the present study, lithium treatment of chick embryos at stage X and XI briefly for ten minutes was found to cause similar abnormalities as that seen in the neutralization experiments of whole chick embryos with anti-FGF and anti-FGFR (refer to Chapter Four). The lithium disrupted the normal development of blood tissue, but in some cases did not affect anterior head structures, which possibly correlates with the dorsalization effect seen in frogs. The inference from these preliminary results is that lithium could be affecting a tyrosine kinase signalling mechanism, as has been proposed in the frog.

In conclusion, these findings suggest that the initial steps in the specification of the erythropoietic cell lineage is through a bFGF receptor mediated pathway; and the high concentration of the bFGF-like molecule in the developing hypoblast and/or mesodermal cells in the posterior region of the embryo induces the horseshoe pattern of presumptive blood tissue mesoderm in the overlying epiblast.

CHAPTER SIX

CONCLUDING DISCUSSION

6.1 AN OVERVIEW

Mesodermal patterning in vertebrates is thought to be accomplished in a highly co-ordinated and sequential manner (see recent reviews by Wolpert, 1989; Green and Smith, 1991; Stern, 1992; Khaner, 1993). One of the first steps in the patterning of mesodermal tissue in the chick embryo is the determination of the anteroposterior axis before the egg is laid (reviewed by Eyal-Giladi, 1991; Khaner, 1993). This step establishes the caudal end of the embryo from which complex morphogenetic movements originate as waves of anteriorly migrating cells. Between stages XI and XIII, cells arising from the region of the posterior marginal zone migrate beneath the epiblast and become integrated with the polyingressed epiblast cells to form the ventral hypoblast layer. From stage XII onwards, epiblast cells dislocate from the posterior end and move anteriorly within the epiblast layer along its median line into the region that subsequently forms the primitive streak (Stern, 1990; Eyal-Giladi et al., 1992). It is generally believed that during these stages the various mesodermal tissue types become specified. It has been proposed that the primitive streak and axial structures are induced at stage XIII (reviewed by Eyal-Giladi, 1991; and in Chapter One, Section 1.1); and activin has been shown to be responsible for this induction (Mitrani and Shimoni, 1990; Mitrani et al., 1990b).

This study was aimed at determining whether growth factor signalling plays a role in the specification of the erythropoietic tissue, a non-axial mesodermal tissue that arises in a posterior horseshoe-shaped area of the chick embryo. Firm evidence has been reached from this study that bFGF is a candidate molecule that plays a role in the specification of the blood mesodermal tissue line. This study has also demonstrated that the source of the bFGF signal is in the ventral hypoblastic layer (i.e. in the hypoblast and/or in cells closely associated with the hypoblast). These findings suggest that the erythropoietic cell lineage is determined before stage XII, which is before the induction of the primitive streak at stage XIII. The evidence of this study that bFGF induces blood tissue, the non-axial mesoderm, together with the findings by Mitrani and his colleagues that activin induces primitive streak and axial mesoderm, which is at the other end of the mesodermal spectrum, correlates well with the

compelling evidence that bFGF and activin induce ventral and dorsal mesoderm respectively in *Xenopus* (see recent reviews by Kimelman et al., 1992; Slack and Tannahill, 1992; Jessell and Melton, 1992; Beddington and Smith, 1993). This is indicative that the cues for mesodermal patterning by growth factor signalling is conserved in amphibian and bird embryogenesis.

6.2 THE TIMING AND CAUSAL BASIS OF BLOOD MESODERMAL TISSUE SPECIFICATION IN THE CHICK

In the present analysis (summarized in Table 6.1), the key question asked was whether bFGF plays a role in the specification of the erythropoietic cell line in the chick. This was initially addressed by determining whether various agents known to block bFGF activity had an effect on Hb formation. Heparin inhibited erythropoiesis, and also suppressed primitive streak formation and the differentiation of other mesodermal tissues, when treatment was administered at the stage when the hypoblast begins to emerge from the posterior marginal zone at stage XI, but not thereafter. As the inhibitory effect on erythropoiesis could be neutralized by the addition of bFGF or XTC-MIF (activin A), it suggested that heparin could be binding to, and inactivating one or more endogenous growth factors that play an important role in the specification of the blood mesodermal tissue line before gastrulation. The role of bFGF in the determination of erythropoietic tissue was confirmed by employing other neutralizing agents to block bFGF activity; namely, the specific antisera to bFGF and the bFGF receptor, and also lithium. Treatment of stage X and XI embryos with these neutralizing agents blocked erythropoiesis, and most interestingly also inhibited the formation of other posterior body structures, such as heart and somites, although normal head structures were formed.

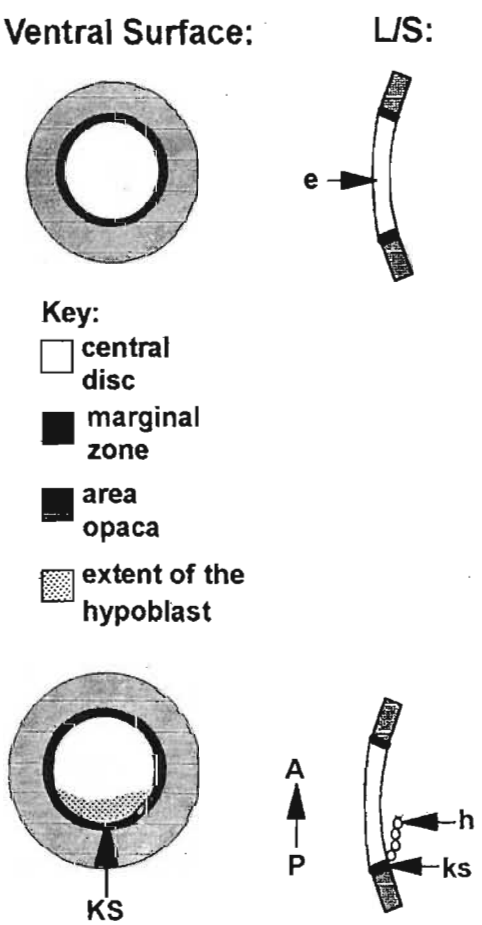
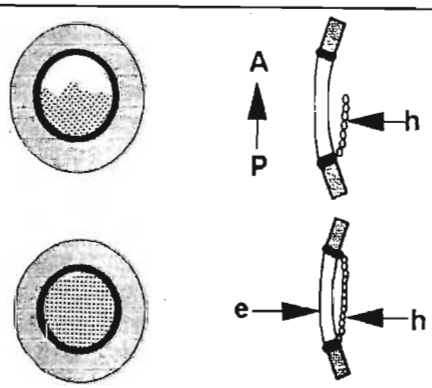
An early commitment to form Hb had been noted in pieces of the blastoderm dissected from the posterior marginal zone (PMZ) at stages XI and XII when cultured in a neutral medium, in accord with the findings of Wilt (1974). It is suggestive that this commitment to form Hb was a result of the erythropoietic cell line having already been determined in this part of the blastoderm before stage XI; a view that is held by Wilt and others (see Wilt, 1974; Eyal-Giladi, 1991; Slack, 1991a). The results of this study however, indicate that the specification of blood tissue had not occurred at stage XI, as anti-FGF had an inhibitory effect on erythropoiesis in the PMZ explanted from stage XI embryos. As the anti-FGF did not

inhibit erythropoiesis at the next stage, it was suggestive that a determinative event occurs between stages XI and XII. Moreover, a similar stage specific inhibitory effect on erythropoiesis with heparin was also noted in the whole embryo explants, with a refractory phase occurring at stage XII onwards.

The presence of bFGF therefore appears to be responsible for the determination of the Hb differentiative pathway between stages XI and XII. In further support of this, it was found that bFGF can induce a precocious commitment to form Hb in pieces of the central epiblast (the ICD components) dissected from embryos at stages X and XI. Normally, the ICD component only manifests a commitment to form Hb when it is dissected from embryos at stages XII and XIII, and not before. A possible reason for the cells in the ICD region of the embryo becoming committed to form Hb from stage XII onwards, is either as a result of an inducing substance diffusing from the posterior region reaching the ICD cells, or a population of cells from the PMZ migrating to this central position, by this stage.

One of the populations of cells originating from the PMZ that migrates to this central position are the cells that form the ventral hypoblast layer. It was pertinent therefore, to ask whether the hypoblast could act as the source of the induction. This indeed was found to be the case. The isolated ventral layer of the stage XIII embryo was able to elicit an erythropoietic response in the uncommitted epiblast of the stage X ICD; and furthermore anti-FGF blocked this induction. It was not conclusive however, whether the ventral layers of hypoblastic tissue used in these experiments were made up entirely of hypoblast cells, as it is possible that some middle mesodermal cells may have inadvertently been attached to the isolated stage XIII hypoblasts used in these experiments. Therefore, the particular cells in the ventral layer responsible for the induction of the Hb differentiative pathway have not been clearly identified in this study. The role of bFGF in the specification of other mesodermal tissues was not fully investigated. The addition of bFGF to the heparin-treated embryos did not rescue primitive streak formation, although it allowed for the formation of an intact ventral layer, followed by erythropoiesis. However, XTC-MIF (activin A) was seen to rescue primitive streak formation in a small percentage (25%) of the heparin-treated embryos; but subsequent body axis formation did not follow.

TABLE 6.1 Summary of experimental parameters affecting haemoglobin formation (determined by the o-dianisidine test). Direction of the spreading hypoblast over the ventral surface of the epiblast is from posterior to the anterior region (P → A). (Abbreviations: CD = central disc; ICD = inner core of the central disc; e = epiblast; h = hypoblast; KS = Koller's sickle; PMZ = posterior marginal zone, consisting of part of Koller's sickle, posterior marginal zone and adjoining area opaca.)

Stage	Morphology of blastoderm (ventral aspect and longitudinal section)	Explant type	Treatment	Hb response (+ or -)
X-XI		whole embryo	none	+
		whole embryo	anti-FGF anti-FGFR heparin	-
		whole embryo	heparin + FGF or XTC	+
		PMZ	none	+
		PMZ	anti-FGF	-
		ICD	none	-
		ICD	bFGF or hypoblast	+
XII-XIII		whole embryo	heparin	+
		PMZ	anti-FGF	+
		ICD	none	+

6.3 THE SOURCE OF THE bFGF SIGNAL

To verify that bFGF plays an active role in the embryo during blood mesodermal tissue specification, it was important to demonstrate that bFGF is not only a normal component in the embryo at the appropriate time (as previously shown by Mitrani et al., 1990a); but also to show that its distribution is spatially significant for the development of blood mesodermal tissue in the posterior horseshoe pattern described earlier by Settle (1954) and Wilt (1974). The present immunocytochemical analysis showed that the concentration of bFGF increases significantly at stages XI, the stage before erythropoiesis becomes determined. The bFGF is distributed mainly in the hypoblast and middle layer of mesodermal cells, in the posterior domain of the area pellucida. It is suggestive that these anti-FGF-immunoreactive cells are the source of the signal molecule that induces the formation of erythropoietic tissue; as the addition of bFGF or the ventral layer (which may be composed of both hypoblast and middle mesodermal cells) are potent inducers of Hb formation in the in vitro assays of uncommitted stage X ICDs.

It was proposed (see Section 4.2) that the salutary effect of the AOV endoderm on Hb formation in the overlying AOV mesoderm at stage 4 (shown by Wilt, 1965; Kessel and Fabian, 1986, 1987) may represent the end point of an earlier instructive induction that set up a continuing supply of inducing factor to complete the Hb differentiative pathway. To answer this question, the origin of the AOV mesoderm and endoderm needs to be identified, this could be done by following the migratory pathways of the hypoblast and middle mesodermal cells by cell lineage analysis. In this regard, it was of interest that heparin treatment disrupted the formation of the ventral layer; and that bFGF and XTC-MIF added to the heparin-treated embryos prevented the gaps forming in this layer, and erythropoiesis was reinstated. This indirectly suggests that the ventral layer is important for the induction of Hb. As was mentioned in Section 6.2 above, the particular cells of the ventral layer that are responsible for the induction have not been identified.

The elucidation of a bFGF-dependent determinative event in the chick opens up areas for further analysis, such as:

(i) The identification and isolation of early mesoderm specific genes in the chick that can be used as markers. This would allow us to determine which genes are influenced by bFGF signalling in vivo. For example in the frog, *Xpo* gene expression, a posterior marker gene, has been found to be activated by bFGF (Sato and Sargent, 1991).

(ii) The quantification of the levels of bFGF and its receptor in the embryo during the earlier stages of cleavage and gastrulation.

(iii) It would be interesting to determine whether a vegetalizing factor that has been extracted from the later stage chick embryo, and used extensively in the amphibian assays (refer to Grunz, 1983), can also induce erythropoiesis in the chick; and whether it is present in the chick blastoderm before gastrulation.

6.4 HOW DOES bFGF FUNCTION?

The experimental evidence of this study suggests that bFGF acts as a signal molecule that induces blood tissue formation, as it can induce an erythropoietic response in vitro, and erythropoiesis can be blocked with agents that neutralize bFGF's activity. These characteristics, together with the presence of bFGF in the hypoblast and middle mesodermal cells at the appropriate time, and its polarized distribution in the embryo, satisfy some of the criteria listed by Slack and Isaacs (1989) which would allow bFGF to qualify as a morphogen. The distribution of bFGF at stages XI and XII is predominantly in the posterior domain of the area pellucida, as a result of the developing hypoblast and middle mesodermal cells emerging from the posterior end of the area pellucida. This polarized distribution would allow for a gradient field of bFGF to be set up at stages XI and XII, that is maximal in the posterior region and declining anteriorly. Such gradient fields have been proposed by Mitrani et al., 1990; Green and Smith, 1991; Green et al., 1992. The posterior horseshoe pattern of blood tissue specification in the chick could therefore be generated in the overlying posterior epiblast cells which respond to a bFGF concentration threshold that yields an erythropoietic response.

It is unclear however, whether bFGF acts as an intercellular inductive signal in vivo, as it was not possible to determine whether bFGF immunolocalized in this study is a secretory product. The cells that would be competent to respond to the bFGF signal (i.e. immunoreactive to the bFGF receptor antibody) were localized within the hypoblast and middle mesodermal layers from stage XII onwards; and most interestingly, an intranuclear localization of the bFGF receptors emerged in the nuclei of the epiblast at stage XIII. Although the distribution pattern of the bFGF and the bFGF receptors suggest that these two proteins are co-expressed in the same cells of the hypoblast and middle mesodermal cells at stage XII, it is not known whether the bFGF ligand and receptor act within the same cells

(through an intracellular autocrine loop), or by an interaction between subpopulations of cells. There has been some debate with regard to the status of bFGF as an intercellular inductive signal molecule in the frog, because bFGF lacks the required secretory sequence to allow it to be exported from the cell. Nevertheless, a novel FGF molecule has recently been extracted from *Xenopus* embryos (XeFGF) which has the required secretory sequence, and shows similar mesodermal-inducing activity to bFGF (Isaacs et al., 1992). It has also been proposed that bFGF may be released from the cells by other mechanisms (Dickson et al., 1990; Kandel et al., 1991).

It is possible that other complex interactions could modulate FGF activity so that its effect is restricted to certain cells in the embryo, eg. gradients of "inhibiting" factors as proposed by Mitrani et al. (1990b), or by the interaction of bFGF with the extracellular matrix (see Yayon et al., 1991; Adams and Watt, 1993). It is suggestive that bFGF may be multifunctional, as bFGF has been found to play a role in a number of different processes at different stages in chick development (see Joseph-Silverstein et al., 1989; Kalcheim and Neufeld, 1990; Parlow et al., 1991; Funakoshi et al., 1993; Fallon et al., 1994). It has been also proposed that bFGF regulates migratory processes by promoting adhesive properties in chick neuroepithelial cells (Kinoshita et al., 1993). In this regard, it is of particular interest that the bFGF-immunoreactive cells localized by the immunocytochemical analysis of the present study were concentrated in the regions where migratory processes were taking place (i.e. in the hypoblast and middle mesodermal cells). It would be of interest to determine the morphogenetic effects after blocking bFGF activity with antisera to bFGF at stage XI in a cell lineage analysis.

In conclusion, the presence of the bFGF ligand and the bFGF receptors in the chick embryo at the stage of blood tissue specification, and the inhibition of blood tissue with an antibody to the bFGF receptor as well as with lithium, suggests that a bFGF signal transduction is functional at the stage of blood tissue specification, however, this function is still not fully understood.

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APPENDIX ONE

1.1 HOWARD'S RINGER SOLUTION (from DeHaan, 1967):

0,7% NaCl
0,018% $CaCl_2$
0,037% KCl in distilled water, and autoclaved.

1.2 O-DIANISIDINE STAINING PROCEDURE (O'Brien, 1960):

1. 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.

2. Explants were immersed in freshly prepared solution for 15 minutes, and then rinsed three times with distilled water.

3. The explants were counterstained with 1% aqueous methyl green for 1 minute, and rinsed three times.

4. An upgrade series of p-dioxane solutions (10%; 70%; 100% twice) was used to dehydrate the specimens.

5. The specimens were cleared in two changes of xylol, and mounted on slides with DePeX (BDH Chemicals, UK).

1.3 4% PARAFORMALDEHYDE FIXATIVE:

2 gm paraformaldehyde
5 ml distilled water
The solution was heated in a water bath to 60°C, cleared with approximately 5 drops 2 N NaOH, and made up to 50 ml with phosphate buffered saline (PBS), pH 7.2.

The explants were fixed at room temperature for 2-4 hours, and washed three times in PBS overnight.

1.4 AQUEOUS MOUNTANT, KAISER'S GLYCEROL JELLY (refractive index 1.47):

10 g gelatine
60 ml distilled water
70 ml glycerol
0.25 g phenol

The gelatine was dissolved in the distilled water in a water bath, and the remaining ingredients added to the dissolved gelatine. (The prepared mountant was melted in a water bath before use).

1.5 PHOSPHATE BUFFERED SALINE (PBS) with 0.1% BOVINE SERUM ALBUMEN (BSA), pH 7.4, used with the Signet Immunoperoxidase staining kit:

8.0 g NaCl
1.15 g Na_2HPO_4
0.2 g KCl
0.2 g KH_2PO_4
1.0 g BSA crystalline grade
1000 ml distilled water

APPENDIX 2

PROCEDURE USED TO EXPLANT THE BLASTODERM; AND ITS CULTURE ON A VITELLINE MEMBRANE RAFT (according to New, 1955):

1. The explantation procedure was carried out under aseptic conditions.

2. The egg was opened and the albumen poured off. The yolk was immersed in Ringer's solution (Appendix 1) in a dish, and the embryo orientated to the uppermost position. The vitelline membrane was cut along a line passing through the equator of the yolk, and peeled away from the yolk using fine forceps. The vitelline membrane was transferred in a soup spoon to a Petri dish containing Ringer's solution.

3. Invariably the embryo of the unincubated egg did not peel away with the vitelline membrane. To free the blastoderm from the yolk, watchmakers forceps were used to cut the yolk around and beneath the blastoderm. The embryo was then transferred in a spoon to a Petri dish containing Ringer's solution. The excess yolk was removed using watchmakers forceps followed by sweeping motions with hair loops. A fine jet of Ringer's solution gently squirted from a fine-mouthed pipette was employed to remove all traces of yolk. Each embryo was then carefully staged using the tables of Eyal-Giladi and Kochav (1976) for the earlier stages (see Fig. 1.7); and the tables of Hamburger and Hamilton (1951) for the later stages.

4. The vitelline membrane was cleaned of yolk, and stretched over a glass ring to form a raft, with the inner surface of the vitelline membrane forming the floor of the raft. The embryo was transferred to the raft with a wide-mouthed pipette and orientated ventral side up in the centre of the floor of the raft. Excess Ringer's solution was removed by pipette, and the embryo allowed to settle firmly on the surface of the vitelline membrane.

5. The raft with its embryo was settled over a pool of the fluid component of the egg albumen in a watch glass, placed inside a Petri dish containing cotton wool swabs soaked in sterile water, and incubated for 48 hours at 38°C.

APPENDIX 3

IMMUNOCYTOCHEMISTRY PROCEDURE:

The following procedure was used to detect the polyclonal rabbit IgGs (anti-FGF and anti-FGF receptor) using a universal immunoperoxidase staining kit supplied by Signet Laboratories, USA:

1. Sectioned material was deparaffinized in toluene, rehydrated through a graded ethanol series, and rinsed in phosphate buffered saline containing 0.1% bovine serum albumen (PBS/BSA, see Appendix 1.5) for 5 minutes.

   Whole mounts and rehydrated sections were then processed at room temperature as follows:

2. Endogenous peroxidase in the whole mounts and sections was quenched by incubating the specimens in 3% hydrogen peroxide in distilled water (supplied in Vial 1 of the kit) for 2 hours. The specimens were kept in a humidity chamber during this, and the following incubations. The specimens were then washed in PBS/BSA twice for 5 minutes (sections) or for 30 minutes (whole mounts).

3. The specimens were incubated in the blocking reagent, normal sheep serum in 0.01 M PBS (Vial 2), for 2 hours (sections) or 5 hours (whole mounts). The excess serum was then removed from the specimens by blotting.

4. The specimens were incubated in the primary antibody, anti-FGF or anti-FGFR (at the concentrations recorded in Chapter Five). The incubation times for the anti-FGF were one hour for the sections, and overnight for the whole mounts. The incubation time for the anti-FGFR was overnight for the sections and whole mounts. Controls are incubated with normal serum, or with anti-FGF serum preincubated with excess recombinant bovine bFGF (Boehringer Mannheim, Germany).

5. The specimens were rinsed three times in PBS/BSA for either 1 hour (the sections) or 5 hours (the whole mounts).

6. The specimens were incubated in the linking secondary antibody, i.e. sheep antiserum to rabbit IgG in 0.01 M PBS (Vial 4). The incubation time was 20 minutes (the sections) or overnight (the whole mounts). The specimens were rinsed 3 times with PBS/BSA for either 5 minutes (the sections) or 30 minutes (the whole mounts).

7. The specimens were incubated in the labelling reagent, peroxidase-antiperoxidase in 0.01 M PBS (Vial 5) for 20 minutes. The specimens were then rinsed twice with PBS/BSA for either 5 minutes (the sections) or 30 minutes (the whole mounts).

8. The specimens were incubated in the substrate solution for 40 minutes, prepared as follows:

   One drop of the substrate-chromogen, 2% 3-amino-9-ethylcarbozole (AEC) in organic solvent (Vial A) was added with one drop of the substrate reagent, 1% hydrogen peroxide in water (Vial B) to 2 ml of the substrate buffer, 0.1 M acetate buffer (Vial C).

9. The specimens were rinsed with three changes of distilled water for 10 minutes.

10. Counterstaining was carried out in some cases. The specimens were stained with Mayer's haematoxylin for 1 minute; and rinsed briefly in warm water, followed by cold water for 5 minutes.

11. Coverslips were mounted on the sections and whole mounts with an aqueous mounting medium (Appendix 1.4). The whole mount specimens were orientated on the slides with their ventral surface facing upwards.