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Postimplantation mouse development: whole embryo culture and micro-manipulation

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ABSTRACT Methods for growing whole mouse embryos *in vitro* have been greatly improved in the last two decades. The present technology enables embryos to develop remarkably close to what can be achieved *in vivo* from the pre-gastrula to the early organogenesis stages. The ability to grow whole embryos for a substantial period outside the uterine environment offers a unique opportunity to observe the progression of development, and permits the performance of direct manipulation on the embryo. Experiments combining whole embryo culture and micro-manipulation have led to the discovery of new information on lineage differentiation, tissue interaction and morphogenetic mechanisms that are associated with the establishment of the fetal body plan.

KEY WORDS: *whole embryo culture, micro-manipulation, fate-mapping, lineage analysis*

Introduction

In vivo development of the mouse embryo beyond the blastocyst stage is dependent on the trophic and mechanical support provided by the uterine environment. The first embryo-maternal association is established by the implantation of the blastocyst on the uterine wall. Thereafter the differentiation of the trophoblast, the extraembryonic membranes and the uterine decidual cells culminate in the formation of a functional placenta. Concomitant with the formation of the placenta, the embryo establishes a vascular connection first with the visceral yolk sac by the vitelline vessels and later with the placenta via the allantoic derivatives (Downs and Gardner, 1995).

The reliance of the conceptus on the intrauterine environment for normal development places numerous constraints on direct experimentation on postimplantation embryos. *In vitro* culture of postimplantation embryos provides an opportunity for such manipulations. Three pre-requisites must be fulfilled to successfully establish postimplantation embryo culture. First, the mouse conceptus must be explanted from the uterus to allow direct observation and access to the embryo proper. Second, the explanted mouse embryo has to be maintained under *in vitro* conditions that support the growth and morphogenesis at a rate that is comparable to that *in utero* over the same period of time. Third, experimental techniques should be designed to enable continual monitoring of embryonic development and direct manipulation of embryonic cells and tissues for studying specific developmental process.

This review is an appraisal of the status of both *in vitro* culture and micro-manipulation as experimental techniques for studying the development of postimplantation mouse embryos. The application of these techniques to the elucidation of the process of gastrulation and body patterning are illustrated by several experiments performed in my laboratory. Lastly, I will draw attention to the potential value of these techniques to the analysis of mutant mouse embryos.

The culture of pre-gastrulation embryo

Although mouse embryos are routinely and efficiently cultured through all stages of pre-implantation development (see Biggers, 1998 this volume), previous attempts to culture mouse blastocysts to early organogenesis stages have met with only limited success (McLaren and Hensleigh, 1975; Hsu, 1978; Wiley *et al.,* 1978). This was achieved by employing complex culture media (CMRL 1066) supplemented with several types of sera (fetal calf, human cord and rat) and increasing oxygen levels (20% to 40%) in the gas phase (Chen and Hsu, 1982). It is also critical to maintain the morphology of the peri-implantation embryo by providing a collagen matrix for the attachment and differentiation of the extraembryonic tissues (Hsu, 1978). However, even with "optimal" conditions, at best only about 40-50% of blastocysts will develop through gastrulation and reach the early-somite-stage

Abbreviations used in this paper: Dil, 1,1' -dioctadccyl-3,3,3'3' -tetramethyl indocarbocyanine perchlorate; DiO, 3,3' -dioctadecyl oxacarbocyanine perchlorate; CFSE, 5 -(and -6) -carboxyfluoreseein diacetate succinimidyl ester.

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(Libbus and Hsu, 1980). Further development to the limb bud stage takes up to 10 days *in vitro* which is longer than the 6 days *in utero* and only 10 of 86 blastocysts have ever progressed to this stage. A success rate such as this hardly warrants using these culture techniques for experimentation. These results may have discouraged any serious efforts to improve the culture of mouse blastocysts through the peri-implantation period of development in the last 16 years. The lack of interest may also be partly due to the shift in the focus of research from peri-implantation to later stages of development such as gastrulation and early organogenesis.

The need to devise an efficient culture system for peri-implantation embryos has been rekindled by some recent observations into the development of embryonic axes prior to gastrulation. The consistent localization of the second polar body to one specific position in the pre-implantation mouse embryo has revealed the existence of an animal-vegetal axis that is related orthogonally to the embryonic-abembryonic axis of the blastocyst (Gardner, 1997). This observation has yet to be confirmed in mice other than the PO strain nor has it been correlated with other polarized features of the embryo (see Johnson *et al.*, 1986). Nevertheless, the findings that the asymmetrical characteristics of the mouse blastocyst could be traced back to the formation of the polar body at fertilization suggest that we should not ignore the possibility that the information for axis is derived from zygotic determinants. It is not known if this animal-vegetal polarity may be related to anterior-posterior embryonic axis of the pre-gastrulation embryo. This may be resolved if the development of peri-implantation embryo could be studied directly so that the emergence of the body axes could be traced prospectively and that the critical factors or conditions that promote the formation of the axis could be tested experimentally.

Two embryological experiments may illustrate the application of *in vitro* culture techniques of pre-gastrulation embryos to the elucidation of germ layer interaction and morphogenetic movement of the primitive endoderm in the pre-gastrula embryo. In an experiment which aimed to follow the movement of the endodermal cells during gastrulation, the primitive endodermal cells localized along the prospective anterior-posterior axis of the pre-primitive-streak-stage embryos were labeled by the iontophoretic injection of horseradish peroxidase. When the embryos were examined after they have been cultured for 22-44 h, the labeled cells were found mostly in the yolk sac, suggesting that the primitive endoderm has an extraembryonic tissue fate (Lawson and Pedersen, 1987). In another experiment, a specific group of primitive endodermal cells in the anterior region of the pre-gastrulation embryos has been surgically removed and the embryos were cultured until they reached the neurulation stage. It was found that the absence of these endodermal cells led to a weaker expression of the *Hesx1* gene in the forebrain (Thomas and Beddington, 1996). This result therefore shows that there may be some interaction between the prospective neural tissue and the primitive endoderm. The starting point of both studies has been restricted to the stage that is immediately before the onset of gastrulation because of the technical limitation of the culture system. Clearly, an improved technique that enables normal *in vitro* development of the pre-gastrulation embryo would be immensely helpful for studying axis determination and cell movement during the immediate postimplantation period.

Culturing embryos at gastrulation and early organogenesis

Initial attempts to culture postimplantation rodent embryos employed methods that are derived from the avian culture system or mammalian cell culture. Rat embryos at the neurulation stages were cultured on plasma clot supplemented with embryo extracts, on tissue culture substrates and in circulating medium (Nicholas and Rudnick 1934,1938; Deuchar, 1971,1976). Although development of the embryos was limited under these conditions, some useful insight into morphogenesis such as axis rotation, axis reconstitution and limb bud regeneration was obtained.

Significant improvement of whole embryo culture has been made primarily due to the extensive and meticulous analyses of the effects of different culture conditions and media formulation on the development of rat embryos carried out by Dennis New and his collaborators at the Physiological Laboratory in University of Cambridge (reviewed by New, 1978). For culturing rat embryos, the most critical ingredient of the culture medium was found to be the serum prepared by immediate separation from the blood cells and used either at full strength or diluted with a buffered Tyrode saline solution (Steele and New, 1974). Various mixtures of sera from both rats and humans have been tested with comparable results (Sanyal and Naftolin, 1983; Van Maele-Fabry *et al.*, 1991). Other critical factors for successful culture include the circulation of the culture medium, the adequate supply of essential energy substrates and the composition of the gaseous phase (New, 1978; Sanyal and Wiebke, 1979; Sanyal and Naftolin, 1983). With these methods, postimplantation rat embryos can be cultured efficiently from gastrulation to early organogenesis, with growth and development generally indistinguishable from that *in vivo*.

Methods for culturing postimplantation mouse embryos are adapted with only minor modifications from that used for the rat embryos (Cockroft, 1990). Similar to the rat system is the use of culture medium containing a high content of serum ranging from about 50% to 100% by volume (Sadler, 1979; Sadler and New, 1981). In addition, the use of human fetal cord serum and mouse serum enhances the growth of the gastrulating embryo, whereas the use of rat serum alone is sufficient for culturing embryos during neurulation and early organogenesis. Extended culture of mouse embryos for more than 48 h requires regular medium changes at 24 h intervals and boosting the level of oxygen in the gaseous phase (New, 1978; Morriss and New, 1979; Sanyal and Wiebke, 1979; Sturm and Tam, 1993). Sera from heterologous species such as the rat and human (adult or fetal cord) have been shown to contain embryotrophic activity comparable to that of the mouse, provided that the serum has been heat-inactivated before use (Tam and Snow, 1980; Tam *et al.*, 1987; Sturm and Tam, 1993). It is also known that variations in the embryotrophic quality of mouse serum is not strain-dependent or pregnancy-related (Tam and Snow, 1980) and that several types of tissue culture media can be used to dilute the serum for culture (Sadler, 1979; Sadler and New, 1981). For embryos that develop through gastrulation to early neurulation, a roller and a simpler static culture system has both been found to be adequate (Tam and Snow, 1980; Lawson *et al.*, 1986,1991; Lawson and Pedersen, 1987). The development of more advanced embryos, particularly of the organogenesis stages, is evidently enhanced if the culture medium is in constant motion or circulation (Sadler and New, 1981; Trainor *et al.*, 1994; Downs

and Gardner, 1995). These culture conditions have been reasonably successful in culturing postimplantation embryos to the stages that are equivalent to intrauterine development at about 10.5-11.0 days post coitum. Details of the protocols currently used for culturing post-implantation embryos can be found in Cockroft (1990), Sturm and Tam (1993) and Hogan *et al.* (1994). In our hands, about 70-80% of the early-primitive-streak-stage embryos develop to the early-somite-stage and about 85-90% of early-somite-stage embryos reach the hindlimb-bud (30-34 somite) stage after 48 h in culture (Trainor *et al.*, 1994; Quinlan *et al.*, 1995). This level of efficiency is adequate for studying the early events of gastrulation, neurulation, cardiovascular development, craniofacial morphogenesis and somitogenesis.

Future development of the culture method

The inability to extend embryonic development *in vitro* beyond the organogenesis stages (New, 1978) hampers the *in vitro* analysis of more advanced processes such as limb formation, skeletal development and secondary neurulation. The major hurdle is the failure to establish a feto-placental exchange to meet the nutritional demands of the conceptus under culture conditions. Historically, the development of embryo culture technology has taken an empirical approach. Very little is known about the metabolic and morphogenetic effects of the different nutritional components. There has been no reported success of the formation of a functional placenta in whole embryo culture. The culture media currently in use that favours the growth of embryonic tissues and fetal extraembryonic membranes might be inadequate for trophoblastic differentiation (New and Coppola, 1977; Sanyal and Nafotlin, 1983). The deliberate and necessary removal of most of the ectoplacental tissues when embryos are explanted for culture (Cockroft, 1990; Sturm and Tam, 1993) might also contribute to the lack of placental development *in vitro*.

The key to developing an effective method to support both embryonic and placental development will be a better definition of both the composition of the culture medium and the biological effects of the components of the culture system. The supplementation of the medium with chemically undefined serum makes it nearly impossible to achieve any uniformity in the culture medium used for different experiments. The protein components of rat serum have been analyzed by fractionation. It was found that a protein of two subunits (62 and 116 kDa) is sufficient to support embryonic growth when it is added to rabbit serum that normally lacks embryotrophic activity (Usami *et al.*, 1992; Usami and Ohno, 1996). More precise information about this protein is currently unavailable. Other serum components such as inositol and glucose also significantly affect embryonic growth (Cockroft, 1988; Chiu and Tam, 1992). The identification of key trophic factors will be critical for developing a defined culture medium that supports *in vitro* growth of both the embryonic and extraembryonic tissues.

Micro-manipulation *in vitro*

The ability to grow mouse embryos *in vitro* provides an unique opportunity to study development through direct experimental manipulation. This may involve the modification of culture conditions such as the addition of exogenous factors that may promote, inhibit or alter tissue differentiation and morphogenesis. Such studies constitute the majority of experiments seeking to elucidate the effects of metabolic compounds and teratogenic agents on mouse development (New, 1978; Eto and Osumi-Yamashita, 1995; Bavik *et al.*, 1996). The other venue of experimentation is the direct micro-manipulation of the embryo. This includes the micro-surgical ablation of groups of cells or parts of the conceptus, the labeling of single embryonic cells, groups of cells or the entire germ layer, the transplantation of cells or embryonic fragments and the retroviral transfection of embryonic cells (Beddington and Lawson, 1990). To perform such manipulative experiments, it is critical that the cells or tissues under test should be readily accessible for labeling, transfection or isolation and the technique of labeling, extirpation and transplantation can be performed with acceptable precision. Table 1 summarizes some micro-manipulative experiments that have been performed on the post-implantation mouse embryo in culture. Following manipulation, the embryos have to develop to a stage when the embryonic tissues or structures are of sufficient complexity to assess the outcome. Since the development of the embryo *in vitro* is limited, the end point of experiments must match with the stages of postimplantation development that can normally be sustained by the culture system. With the present culture system, the experimental embryos are usually assessed at stages between the completion of gastrulation (equivalent to 7.5 days post coitum) and the hindlimb bud/early tail bud stage (30-36 somites, 10.5 days post coitum).

Elucidation of mouse gastrulation through the application of micro-manipulation

Techniques for the analysis of cell fate

The most important contribution of the manipulative experiments of postimplantation embryos is the construction of fate maps that depict the prospective fate of cells in the germ layers of the mouse gastrulae. The fate of the cells is assessed by studying the types of embryonic tissues that are colonized by the descendants of cells that are marked by exogenous labels or express a genetic marker.

Cells in specific regions of the germ layer can be labeled *in situ* with enzymes or fluorescent dyes. Labeling of individual cells is achieved by the iontophoretic injection of horseradish peroxidase or lysinated rhodamine dextran (Lawson *et al.*, 1986,1991; Lawson and Pedersen, 1987; Lawson and Hage, 1994). Alternatively, groups of cells can be labeled by pressure injection of fluorescent dyes (e.g., lipophilic dye-DiI, DiO; cytoplasmic dye-CFSE) into the tissue layer with uptake into the cell membrane or the cytoplasm (Beddington, 1994; Smith *et al.*, 1994; Sulik *et al.*, 1994; Quinlan *et al.*, 1995,1997; Thomas and Beddington, 1996; Darnell and Schoenwolf, 1997; Tam *et al.*, 1997a). A blanket labeling of the epiblast or the ectoderm of the mouse gastrula can be accomplished by introducing the label into the proamniotic or amniotic cavity. The labels that have been successfully used include the lipophilic fluorescent dyes that were previously mentioned, and colloidal gold particles conjugated with wheat germ agglutinin, which are ingested via endocytosis mediated by cell surface lectin-binding glycoprotein (Tam and Beddington, 1987,1992). Labeling of cells can also be achieved by transfecting the cells by micro-injecting into the amniotic cavity a suspension of retroviruses carrying a genetic marker (Carey *et al.*, 1995).

Cell fate is also analyzed by following the distribution of the progeny of a transplanted cell population. Cells are isolated from defined regions of the donor embryo that express a genetic marker

TABLE 1

EMBRYOLOGICAL STUDIES INVOLVING MICRO-MANIPULATION OF WHOLE MOUSE EMBRYO IN CULTURE

Manipulation	Stage of embryo	Culture period	Culture conditions	Aim/topic of the study	References
Transplantation of cells	Early-somite	24 h	DMEM:RS (1:1); 5% CO_2/air; roller culture	Patterning effects of BMP-7 expressing cells on the hindbrain	(1)
	Early- to Late-streak	24-48 h	DMEM:RS (1:1); 5% CO_2/air; roller culture or DMEM:RS:HCS (1:2:1); 5% CO_2/air; static culture or DMEEM:RS (1:3); 5%CO_2/5% O_2/90% N_2; roller culture	Fate-mapping of the epiblast, the ectoderm, the mesoderm and the primitive streak Testing the lineage potency of epiblast and mesodermal cells	(2-9)
	Early-somite or forelimb-bud	24-48 h	DMEM:RS (1:3); 5% CO_2/5% O_2/90% N_2 → 5% CO_2/20% O_2/75% N_2 → 5% CO_2/40% O_2/55% N_2; roller culture	Mapping the migratory pattern of cranial neural crest cells Testing the somitogenetic potency of primitive treak and tail bud cells	(10-12)
Transplantation of fragments	Late-head-fold to early-somite	8-20 h	DMEM:RS (1:1); 6.2% CO_2/ air; roller culture	Development of the chorioallantoic connection	(13)
	Late-streak	24 h	DMMEM:RS (1:1) or DMMEM:RS:HCS (1:2:1); 5% CO_2/ air or; 5% CO_2/ 5% O_2/90% N_2; roller culture	Testing the organizing activity of the early gastrula organiser and node	(14,15)
	Earlly-head-fold	23 h	DMEM:RS (1:1); 6.2%CO_2/ air; roller culture	Lineage potency of the allantoic mesoderm	(16)
Labeling of single cells	Pre-to mid-streak	22-44 h	DMEM:RS (1:1); 5 or 6% CO_2/ air; roller culture	Fate-mapping of the epiblast and the primitive endoderm	(17-19)
Labeling of groups of cells	Early- to late-streak	24-48 h	RS (100%); 5% CO_2/ 5% O_2/ air; roller culture or DMEM:RS (1:3) 5% CO_2/ 5% O_2/90% N_2; roller culture or DMMEM:RS:HCS (1:2:1) 5% CO_2/ air; static culture	Fate-mapping of the primitive streak	(20, 21)
	Late-streak	24 h	DMEM:RS (1:1) or Tyrode saline: RS (1:1); 5% CO_2/ air; roller culture	Fate-mapping of the node	(22, 23)
	Early-Somite	24 h	DMEM:FCS:RS (2:1:1), 5% CO_2/ air; static culture or RS (100%); 5% CO_2/ 5% O_2/ air; roller culture	Segmental migratory pattern of cranial neural crest cells and cranial somitomeres	(11, 12, 24, 25)
Labeling of whole germ layer	Early-streak	6-48 h	DMEM:HCS:RS (1:1:2), 5% CO_2/ air; static culture	Recruitment of definitive endoderm from the epiblast	(26)
	Late-streak	24 h	DMEM:RS (1:1) 5% CO_2/ 5% O_2/ air; roller culture	Recruitment of mesoderm from the ectoderm and primitive streak	(27)
	Early-somite	24 h	DMEM:RS (1:1); 5% CO_2/ 5% O_2/ air; roller culture	Formation and migration of the cephalic neural crest cells	(10)
Transfection of embryonic cells	Late-streak	24-48 h	DMEM:RS (1:1); 6% CO_2/ air, roller culture	Restriction of clonal allocation of ectodermal cells marked by retrovirally introduced lacZ reporter	(28)
Ablation of tissues	Early- to late-streak	24-46 h	DMEM:HCS:RS (1:1:2), 5% CO_2/ air; static culture	Testing the re-constitution of the organizer	(29)
	Late-streak	24 h	DMEM:RS (1:1); 5% CO_2/ air; static culture	Morphogenetic regulation and autonomous development of embryonic fragments	(30)
	Forelimb bud	24 h	DMEM:MS:RS (1:2:1); 5% CO_2 40% O_2/55% N_2; roller culture	Regeneration of the forelimb bud	(31)

Abbreviations: DMEM, Dulbecco's modified Eagles Medium; FCS, fetal calf serum; RS, rat serum; MS, mouse serum; HCS, human cord serum.

References: (1) Arkell and Beddington, 1997; (2) Beddington, 1981, 1982; (3) Tam and Beddington, 1987; (4) Tam, 1989; (5) Tam and Tan, 1992; (6) Quinlan *et al.*, 1995; (7) Parameswaran and Tam, 1995; (8) Tam and Zhou, 1996; (9) Tam *et al.*, 1997a; (10) Chan and Tam, 1988; (11) Trainor *et al.*, 1994; (12) Trainor and Tam, 1995; (13)Downs and Gardner, 1994; (14)Beddington, 1994; (15) Tam *et al.*, 1997b; (16) Downs and Harmann, 1997; (17) Lawson *et al.*, 1986, 1991; (18) Lawson and Pedersen, 1987; (19) Lawson and Hage, 1994; (20) Smith *et al.*, 1994; (21) Tam and Quinlan, unpublished; (22) Sulik *et al.*, 1994; (23) Beddington, 1994; (24) Serbedzija *et al.*, 1992; (25) Osumi-Yamashita *et al.*, 1994, 1996; (26) Tam and Beddington, 1982 ; (27) Tam and Beddington, 1987; (28)Carey *et al.*, 1995; (29) Davidson, Schoenwolf, Behringer and Tam, unpublished; (30) Snow, 1981; (31) Chan *et al.*, 1991.

such as the *lacZ* gene (Beddington *et al.*, 1989; Parameswaran and Tam, 1995; Quinlan *et al.*, 1995; Trainor and Tam, 1995; Downs and Harmann, 1997; Tam *et al.*, 1997a, b) or from embryos that have been labeled with radioactive thymidine (Beddington, 1981,1982; Copp *et al.*, 1986; Tam and Beddington, 1987). These cells are then transplanted to a similar site (i.e., orthotopic transplantation) in the recipient embryo at the same developmental stage. The graft-derived cell population is identified by the expression of the genetic marker or label. Cell differentiation can be determined by histological characteristics or the expression of lineage-specific molecular markers (Tam and Zhou, 1996; Tam *et al.*, 1997a). Despite the technical differences of in situ marking, single-cell clonal analysis and cell transplantation, it has been reassuring to discover that there is a remarkable consensus of results obtained using these different techniques.

Cell fate, morphogenetic movement and developmental plasticity

Fate-mapping studies performed on mouse embryos *in vitro* have led to the construction of fate maps of the epiblast and the primitive endoderm of the early-primitive-streak-stage embryo (Lawson *et al.*, 1986,1991; Lawson and Pedersen, 1987; Parameswaran and Tam, 1995; Quinlan *et al.*, 1995), the mesoderm of the early-, mid- and late-primitive-streak-stage embryos (Parameswaran and Tam, 1995) and the ectoderm of the late-primitive-streak-stage embryos (Beddington, 1981,1982; Tam, 1989). These fate maps provide a description of the geographical distribution of the precursor cells of specific lineages in the germ layers at successive stages of gastrulation. From such cell localization data, it is possible to delineate the components (types of precursor cells) and the organization (e.g., cranio-caudal and dorso-ventral polarity of mesodermal precursors and the neural primordium) of the blueprint for subsequent patterning of the fetal body (Tam and Behringer, 1997).

The findings that cells localized in different regions of the germ layer have acquired divergent fates suggest that some degree of lineage specification has taken place. The results of clonal analysis of epiblast cells unequivocally show that individual cells can contribute to more than one lineage (Lawson *et al.*, 1991), suggesting that epiblast cells are pluripotent. However, it does not exclude the possibility that cells in different regions of the epiblast are differentially restricted in their potency to differentiate into specific tissue types (i.e., they are pluripotent but not of equivalent developmental potency). Experimentally, this can be tested by assaying the ability of the cells to differentiate differently from their presumptive fate after they have been transplanted to heterotopic sites in the embryo. Results of such studies show that epiblast cells display similar tissue potency, even including the ability to produce germline cells (Parameswaran and Tam, 1995; Tam and Zhou, 1996; Tam *et al.*, 1997a). Epiblast cells still retain their wide range of developmental potency if they are transplanted to mesoderm. Cells that have ingressed through the primitive streak, however, may lose the ability to differentiate into some mesodermal tissues if they are returned to the epiblast for another round of cell ingression (Tam *et al.*, 1997a). Cells in the ectoderm of the late-primitive-streak stage embryo are still able to switch between neural and mesodermal fates, but a bias for neural differentiation can be detected in the prospective neuroectodermal cells (Beddington, 1982). These observations of developmental plasticity raise the possibility that lineage specification takes place progressively during germ layer formation. To date, the experiments for testing potency have been performed only for specific cell populations and further insight into lineage potency at the level of individual cells awaits the refinement of techniques of single cell transplantation and the co-analysis of the molecular and histological phenotypes of clonal descendants.

A correlative study of the fate maps of successive stages of gastrulation and the distribution of clonal descendants of labeled or transplanted cells has permitted the reconstruction of the pattern of cell movement in the epiblast, the mesoderm and the endoderm (Lawson *et al.*, 1986,1991; Lawson and Pedersen, 1987; Tam and Beddington, 1992; Parameswaran and Tam, 1995). Epiblast cells are displaced posteriorly towards the primitive streak as more cells are recruited for ingression. Ingressed cells are distributed to both sides of the body and are sorted out into mesoderm and endoderm. Mesodermal and endodermal cells are displaced from the primitive streak towards the anterior and proximal regions of the gastrulating embryo. Cells that are recruited to the mesoderm and the endoderm at the same time during gastrulation may move together during gastrulation. Some of these cell movements, deduced from the results of fate-mapping, have been confirmed by tracking the migration of cells labeled *in situ* by carbocyanine dyes (Smith *et al.*, 1994; Tam *et al.*, 1997a; Quinlan and Tam, unpublished) or by short-term cinemicrographic tracking of cell movement (Nakatsuji *et al.*, 1986). Future application of whole embryo culture to the direct study of cell movement will be influenced by the efficacy of vital markers such as the green fluorescent protein (Zernicka-Goetz *et al.*, 1997). The potential value of such vital markers depends on first, the efficiency of in situ delivery of the markers, possibly by transfection of cells in situ using retroviral vectors or the transplantation of GFP-transgenic cells to the embryo, and second the ability to detect the signal in a small number of cells (Amsterdam *et al.*, 1996).

Establishment of the body plan

Whole embryo culture and micro-manipulation have been applied to the study of the functional role of the primitive streak and the organizer in the establishment of the body plan.

The composition of cell populations in the primitive streak during gastrulation has been analyzed by mapping the fate of cells that ingress through different segments of the primitive streak during gastrulation (Tam and Beddington, 1987; Lawson *et al.*, 1991; Smith *et al.*, 1994; Quinlan and Tam, unpublished). Essentially, the results show that cells destined for different mesodermal and endodermal tissues ingress through the primitive streak at different times. Cells destined for more cranial structures ingress earlier than those for caudal structures. Cells for more medial and dorsal mesoderm ingress near the anterior segment of the primitive streak whereas those for more lateral and ventral mesoderm do so near the middle segment of the primitive streak. Cells ingressing in the posterior segment colonize the extraembryonic mesoderm. Posterior primitive streak cells that ingress earlier during gastrulation occupy sites in the yolk sac mesoderm and the allantois further away from the embryo proper, whereas those that ingress later are found nearer to the amnion side of the yolk sac and the base of the allantois (Downs and Harmann, 1997; Quinlan and Tam, unpublished).

By following the fate of cells marked *in situ* with carbocyanine dyes, cells at the anterior end of the primitive streak (the node) of the late gastrula have been shown to contribute to the formation of

notochord and the floor plate (Beddington, 1994; Sulik *et al.*, 1994). Transplantation of the embryonic fragment that contains the node to another embryo results in the formation of an additional axis consisting of tissues derived from both the graft and the host tissues (Beddington, 1994). The cell fate and the patterning activity of the node of the late-gastrula are therefore consistent with the criteria for assigning this population as the equivalent of the organizer in other vertebrate gastrulae. Recently, we have identified a population of about 40 cells in the posterior epiblast of the early-primitive-streak-stage embryo which will contribute to the node of the late-gastrula and to the mesodermal tissues that will normally be derived from the node. By ectopic transplantation to another embryo, this population is found also to possess organizer activity (Tam *et al.*, 1997b). This epiblast population may therefore act as the early-gastrula organizer (EGO) for the mouse embryo. Some descendants of the EGO are later incorporated into the anterior end of the elongating primitive streak of the mid-primitive-streak-stage embryo and they can also induce a new axis in host embryos (Tam and Steiner, unpublished). The EGO alone, like the node, could not induce a complete neural axis in the host embryo. However, if the EGO is combined with the anterior epiblast and the anterior primitive endoderm that expresses the *Hesx1, Lim1, Hnf3β* and *Cerberus*-related genes (Shawlot and Behringer, 1995; Thomas and Beddington, 1996; Filosa *et al.*, 1997; Tsang and Tam, unpublished), a new axis that contains *Otx2* expressing tissues at its anterior end can be induced, suggesting this combination of tissues from the early gastrula comprises the full head and trunk organizer activity (Tam and Steiner, unpublished). The impact of the loss of organizer activity has been examined by surgical ablation of the node and EGO of the mouse gastrula. Axis formation continues unaffected in most node-ablated embryos. Apparently the germ layer tissues have the capacity to compensate for the loss of organizer activity and this is accompanied by the up-regulation of some organizer-specific genes (Davidson, Schoenwolf, Behringer and Tam, unpublished).

Analysis of gastrulation mutants

A number of spontaneous and targeted mutations that have a significant impact on gastrulation and body patterning have now been identified (Tam and Behringer, 1997). The current strategy for analyzing these mutants is to examine the phenotypes of the mutant embryos at different gestational ages, followed by the analysis of gene activity, usually by *in situ* hybridization, that may reveal the consequence of the mutation on genes involved with either upstream or downstream regulation. Alternatively, the mutant embryos are analyzed for tissue-specific gene activity that may provide some molecular insight into the nature of the morphological defects. In addition, the impact of the mutation on the lineage potency of the cells and the tissues affected by the mutation are assessed by studying cell differentiation and morphogenesis in chimaeras. For studying the functional interaction of the mutation with other genes, the compound phenotypes of mice carrying multiple mutations may be examined. Lastly, homologous genes are introduced into the genome of the mutant to attempt a functional rescue which if successful will ascertain whether the effect of mutation is specific to the gene in question.

The combination of whole embryo culture and micro-manipulation adds another dimension to the analysis of the mutant embryos particularly those that display defective gastrulation and early organogenesis. First, the embryo can be cultured for a direct study of the progression the mutant phenotype. Second, fate mapping studies can be performed on the mutant embryos and this will reveal if a normal body plan has been established and if not, whether this is due to the failure to specify particular tissue lineages or to the aberrant morphogenetic movement of cells. Cells from mutant and wild type embryos can be transplanted reciprocally to orthotopic and heterotopic sites to reveal if the mutation has any cell-autonomous impact on lineage potency. To overcome the problem of the paucity of mutant materials (especially when studying a recessive mutation), mutant embryos for experimentation may be generated as mutant embryonic stem cell <-> tetraploid chimeras. The analysis of tissue lineages may help to pinpoint the cell population that is most affected by the mutation. A critical test for the identification of the tissues that have been targeted by the mutation is the replacement of the wild type counterpart of the target tissues in the mutant embryo, which should theoretically ameliorate the mutant phenotype. If the mutant phenotype indicates the breakdown of tissue interactions then the reciprocal grafting of embryonic fragments (such as the organizer or the primitive streak) between wild type and mutant embryos should reveal which components of the interaction may be faulty. Another potentially useful experimental strategy to analyze the functional role of a specific gene on cell differentiation is to target cDNA, mRNA(sense or antisense) or protein encoded by the gene to selected cells. These molecules can be directly introduced, by intracellular injection, to the cells of the postimplantation embryo and the effect of altered gene activity may be analyzed by examining the development of the embryo *in vitro*. For mutations that interfere with organizer activity, ablation experiments may be performed to determine if the ability to re-constitute the organizer activity and to sustain axis development may require the activity of the normal allele of the mutant gene. This may yield novel insights into the gene activity that may be required to establish or maintain the organizer during normal embryogenesis.

The analysis of mutations that affect ligand-receptor signaling pathways involving growth factors is presently hampered by the obligatory use of serum-supplemented culture medium. The presence of a plethora of growth promoting factors precludes any meaningful assessment of the specific effects of these factors on embryonic development. Although it would be possible to test the effects of a high dose of growth factors delivered topically by bead implants (Davidson and Tam, unpublished) or grafting of ligand-producing cells (Arkell and Beddington, 1997), the results would have to be interpreted cautiously. Any effects of the depletion, substitution or addition of growth factors to the culture might either be masked or have elicited a non-physiological response from the embryo. It is therefore imperative to develop a chemically defined serum-free medium that can be re-constituted selectively with embryotrophic factors for specific experiments. An acceptable interim option would be to modify the composition of the serum either by inactivating or depleting the growth factors that may interfere with the experiment.

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