Transparent things: cell fates and cell movements during early embryogenesis of zebrafish

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Summary

Development of an animal embryo involves the coordination of cell divisions, a variety of inductive interactions and extensive cellular rearrangements. One of the biggest challenges in developmental biology is to explain the relationships between these processes and the mechanisms that regulate them. Teleost embryos provide an ideal subject for the study of these issues. Their optical lucidity combined with modern techniques for the marking and observation of individual living cells allow high resolution investigations of specific morphogenetic movements and the construction of detailed fate maps. In this review we describe the patterns of cell divisions, cellular movements and other morphogenetic events during zebrafish early development and discuss how these events relate to the formation of restricted lineages.

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Introduction

Development of a zebrafish begins with the fertilization of an egg by a spermatozoon by way of a single sperm entry site located at the future animal pole of the zygote[1,2]. The animal-vegetal axis becomes visible within a few minutes as vigorous cytoplasmic streaming segregates the zygote into two distinct cellular regions, a clear cytoplasmic blastodisc situated atop a large sphere of translucent yolk. The first nine cleavages occur synchronously at 15 minute intervals. The first five cleavage planes are usually vertical in orientation and alternate at right angles to one another (Fig. 1). The sixth cleavage plane is horizontal, thus producing two tiers of blastomeres. These early cleavages are meroblastic and leave large cytoplasmic connections between the marginal blastomeres and the yolk cell (Fig. 1). By the 7th cleavage (2.3 hours after fertilization) a mound of blastomeres is centered on a large yolk cell and exhibits no obvious dorsal-ventral asymmetry (Fig. 2)[3-5].

Separation of lineages prior to gastrulation

Formation of the first distinct cellular lineage begins at about the 9th or 10th cleavage, when the marginal blastomeres collapse into the yolk cell to form the yolk syncytial layer (YSL) (Fig. 2). Whether a blastomere will contribute to the YSL appears to depend upon its marginal position within the embryo rather than on its lineage[6]. Once a cell becomes part of the syncytium it is restricted from contributing to the overlying blastoderm.

The second restricted lineage forms within the blastodermal cap. This lineage comprises the superficial blastomeres, which form an epithelial monolayer, called the enveloping layer (EVL). The EVL covers the remaining internal blastomeres, called the deep cell layer. Initially, EVL cell divisions occur both within the plane of the monolayer and perpendicular to it. Planar divisions produce daughters that remain within the enveloping layer, while perpendicular divisions leave one daughter in the EVL and introduce the other in the deep cell layer[7]. After 3 hours of development, however, the frequency of divisions contributing exclusively to the EVL increases. The EVL becomes a separate cellular lineage, when after 4 hours of development only planar divisions occur (Fig. 2)[7].

One striking feature of the formation of these first two embryonic lineages is that they are each accompanied by a dramatic morphogenetic change (Fig. 2). Separation of the YSL correlates with and may indeed be the consequence of the complete fusion of marginal blastomeres with the yolk cell. Lack of cytokinesis in the syncytium probably prevents the syncytial nuclei from contributing to the overlying blastoderm[8]. The gradual separation of the EVL lineage correlates with the flattening of the blastoderm on the yolk cell and is accompanied by both an increase in tension and cell shape changes within the EVL. It has been suggested that the separation of the EVL as a lineage might be the conse-

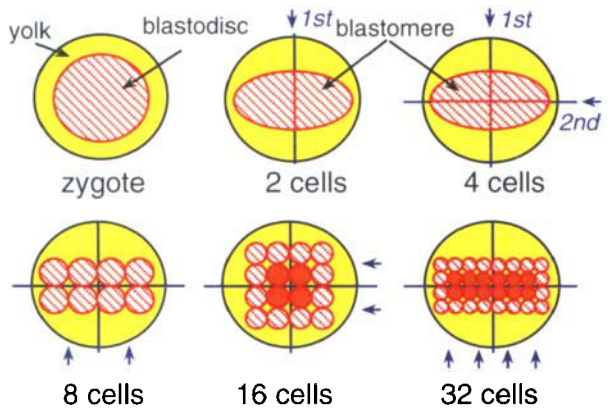

Fig. 1. Orientation of early cleavages in a zebrafish embryo. Drawings show animal views. In the zygote, cytoplasm forms a blastodisc (hatched red) positioned on top of a large yolk sphere (yellow). The first divisions (blue) are vertical, cleaving the blastodisc along the animal-vegetal axis, and alternate at right angles to one another. The cleavages are meroblastic so that initially all blastomeres (2-8-cell stages) and later the marginal blastomeres (16-512-cell stage) maintain cytoplasmic connections with the yolk cell (marked as hatched blastomeres). From the 16-cell stage on the central blastomeres (solid) are completely separated from the yolk. Note that at the 8-cell stage the embryo has bilateral symmetry with the longer axis of the blastodisc correlated with the second division plane. (Based on ref. 8, modified).

quence of increased tension that causes EVL cell divisions to occur preferentially within the plane of the EVL[8].

Separation of the YSL and the EVL from the deep cell layer roughly coincides with mid-blastula transition (MBT)[9]. MBT period in zebrafish and other teleosts begins at cycle 10 and, as in amphibia, involves the lengthening of the cell cycle, loss of cell division synchrony, activation of transcription and initiation of cell motility[9,10]. The yolk syncytial nuclei undergo a series of three to five rapid metachronous divisions eventually to cease mitosis at about 4 hours of development. The deep layer and EVL cells divide asynchronously. Cell cycle lengths of EVL cells are, however, markedly longer than those of the deep cell layer[11]. Thus, at the end of MBT period, that is at about cycle 13[9], three morphologically discrete lineages, the deep cell, enveloping and yolk syncytial layers, emerge as distinct mitotic domains exhibiting different cell cycle lengths and division synchrony[11]. This three layer blastodisc structure is a feature common to all teleosts and stands in marked contrast to the arrangement of cells in amphibia at an equivalent stage of development[12].

Epiboly

During epiboly, the deep cells, the EVL and the YSL lineages expand vegetally eventually to cover the yolk sphere completely. The mechanisms involved in epibolic movements of the three cell types are, however, quite different (Fig. 3). Studies of another teleost, *Fundulus heteroclitus*, suggest that epiboly of the blastoderm is largely passive and dependent on epiboly of the YSL. Epiboly of the blastoderm will not take place without concurrent epiboly of the underlying YSL. In contrast, epiboly of the syncytial layer can be completed even after the entire blastoderm has been removed[13].

At the beginning of epiboly, the embryo is spherical with a mound of blastoderm cells atop a flattened yolk cell (Fig. 3). The cortex of the yolk cell comprises two distinct regions. The YSL, a thickened cytoplasmic layer populated by the yolk nuclei, is located in the animal part of the yolk cell and is partially covered by the blastoderm. The remainder of the yolk cortex is a thin anuclear layer of cytoplasm called the yolk cytoplasmic layer. The beginning of epiboly is marked by a major morphological change in the yolk cell, which bulges toward the animal pole in a process called doming[14]. Subsequently, the YSL expands toward the vegetal pole. In contrast, the thin anuclear cytoplasmic layer of the yolk cell progressively disappears (Fig. 3)[15,16]. In both *Fundulus* and zebrafish, the surface of the yolk cytoplasmic layer decreases, probably due to a process of endocytosis occurring just vegetal to the YSL (Fig. 3)[16,17]. Concomitant with the decrease in the surface of the yolk cytoplasmic layer is an expansion of the surface of the YSL, which is probably mediated by the release of the excess of membrane stored in a form of membranous microvilli[15,18].

Epiboly of the YSL is at least partially driven by microtubule-dependent forces[16,19]. In zebrafish, the yolk cell is equipped with two distinct microtubule arrays that change their organization during epiboly (Fig. 3). One array, an extensive network of intercrossing microtubules, is part of the YSL and expands as the epiboly of the YSL proceeds. By contrast, another array of microtubules, oriented along the animal-vegetal axis within the yolk cytoplasmic layer, becomes shorter as this layer diminishes[16]. If microtubules are completely depolymerized, by treatment with high doses of nocodazole, the movements of the syncytial nuclei are blocked; however, endocytosis of the yolk cell and epiboly of the blastoderm are only partially inhibited. Thus, the yolk cell microtubules may be involved in the vegetal movements of the YSN and may also contribute to epibolic expansion of the EVL and deep cells.

As the yolk cell begins to dome, the mound of deep cells becomes thinner while simultaneously expanding vegetally. This is accomplished by the radial intercalation of cells from deeper to more superficial positions (Fig. 3)[14,20]. It has been proposed that such radial intercalation movements are driven by the doming of the yolk cell[21]. These movements thoroughly scatter deep blastomeres, dispersing clonally related cells[6,14]. There are regional differences in the degree of cell mixing. Cells in the center of the blastula undergo more extensive scattering and mixing than cells located near the blastoderm margin[22,23]. It has been suggested that these reproducible patterns of cell mixing during epiboly result from the passive response of deep cells to the doming of the underlying yolk cell[21,22].

In contrast to deep layer cells, when the EVL undergoes epiboly, cell rearrangements are observed to occur only

within the plane of the monolayer (Fig. 3)[14,24]. The expansion of the EVL in *Fundulus* appears to be driven by the epiboly of the surface of the YSL to which the monolayer is tightly linked by its margin (Fig. 3). Epiboly of the EVL is accompanied by an extension of the intercellular tight junctions and the increase in the tension within the monolayer[25].

Gastrulation movements of deep cells

(1) Formation of hypoblast – involution or ingression?

When the margins of the deep cells, the EVL and the YSL reach the equator of the yolk sphere, that is 50% epiboly, a second phase of morphogenetic movements begins. While the EVL, deep cells and YSL continue their vegetalward expansion, the deep cells initiate two new types of movement, involution/ingression and convergent extension. These gastrulation movements transform the late blastula, a thin hemisphere of deep cells, into a narrow, extended gastrula with three classically defined germ layers organized into the vertebrate body plan.

The involution/ingression movement brings the future mesoderm and endoderm cells to underlie the prospective ectoderm. This creates a circumferential thickening at the blastoderm margin called the germ ring, within which one can distinguish two distinct layers of deep cells, the hypoblast located next to the yolk and the epiblast located just below the EVL (Fig. 4)[14]. In amphibia the mesendodermal layer forms by the process of involution. This process is initiated by invagination of bottle cells, a structure that teleosts do not possess[12]. Subsequently, prospective mesoderm and superficial endoderm move as a continuous layer of cells around the blastopore lip to come to underlie the future ectoderm[26].

There is some controversy concerning the exact nature of the movement in teleost. Some reports describe the movement to be involution, wherein cells arrive at the margin, then move inward toward the yolk cell away from the EVL[14,27], and begin migrating toward the animal pole between the deep cell epiblast and the surface of the yolk cell (Fig. 4)[14]. This view is well supported by studies in a teleost with a similarly small yolk cell, *Barbus conchonius*[28]. For each of these studies, in teleosts, involution was observed to occur as a single cell phenomenon, rather than as in amphibia, where the movement is of layers of cells[14,27,28].

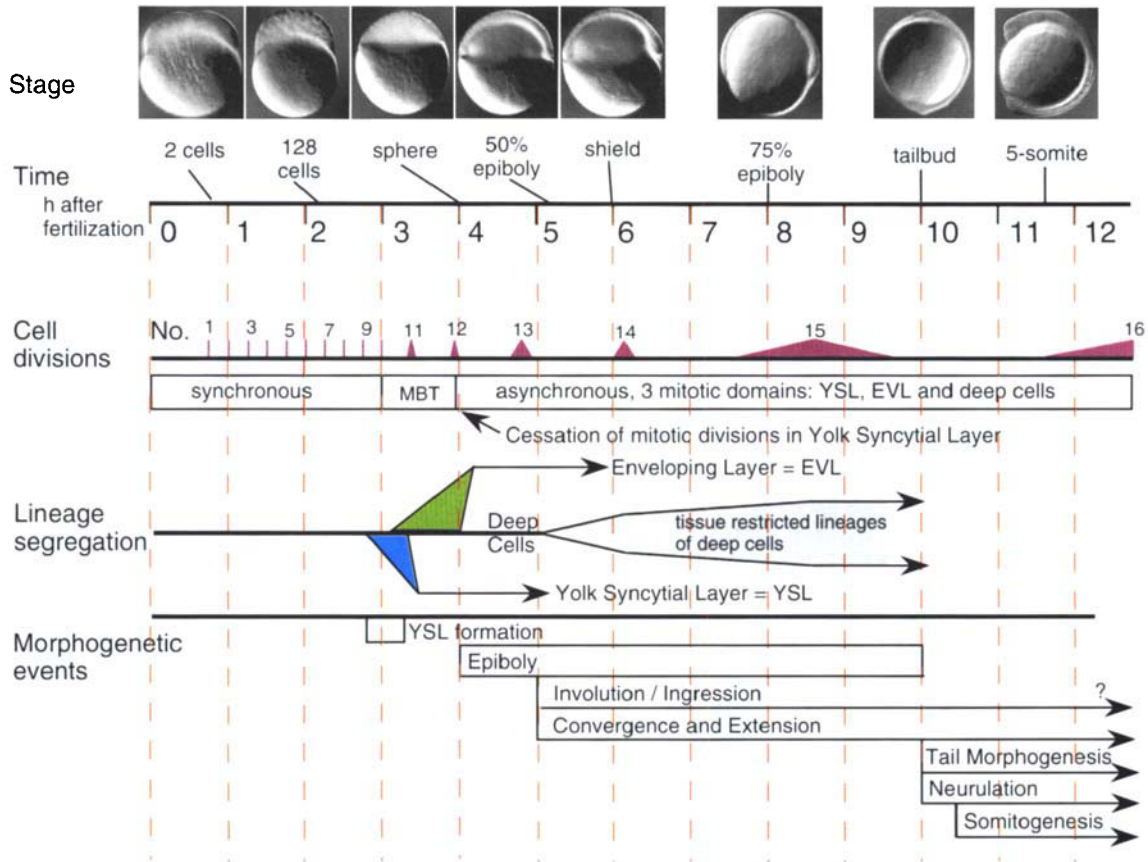

Fig. 2. Relative timing of cell divisions, formation of restricted lineage and morphogenetic events during early zebrafish embryogenesis. Developmental times are given in hours (h) after fertilization at 28.5°C and developmental stages are as described in ref. 14. The timing of the first 12 cell divisions is indicated as an average for all cells[9,11]. For divisions 13 through 16, the timings, including variation, are for deep cells exclusively[36]. Time correlations between segregation of distinct lineages, cell divisions and morphogenetic events are shown[6,7,14]. See text for details.

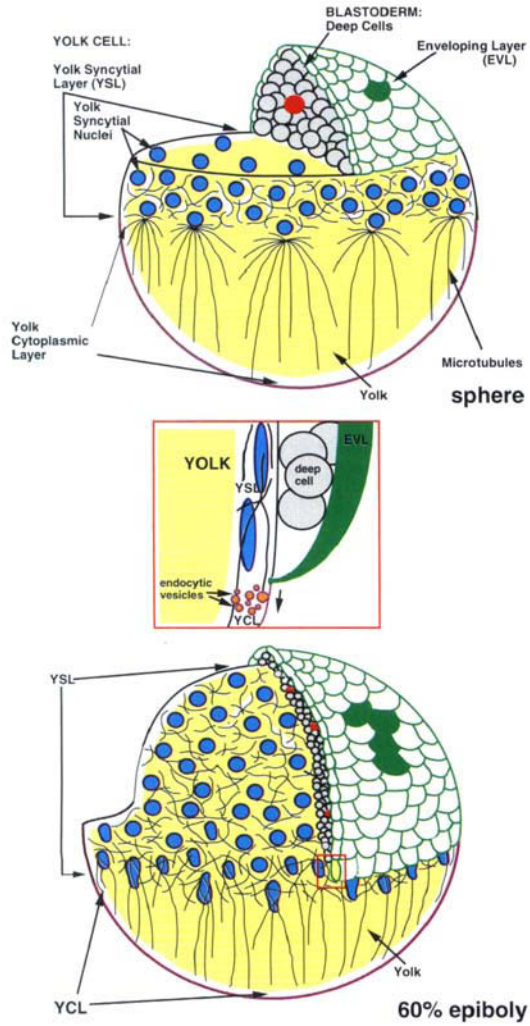

Fig. 3. Schematic illustration of changes in organization of the blastoderm and the yolk cell at the onset of epiboly (top) and at 60% epiboly (bottom) (based on ref. 16, modified). Just before the start of epiboly (sphere stage), the blastoderm is composed of the internal mass of deep cells and the superficial enveloping layer (EVL), and is positioned on top of the flattened syncytial yolk cell. Two compartments can be distinguished in the cortical layer of the yolk cell. A cortical yolk syncytial layer (YSL) is populated by the syncytial nuclei and covers the animal portion of the yolk sphere, underlying the blastoderm. A thinner anuclear yolk cytoplasmic layer surrounds the bulk of the yolk mass with the vegetal pole. The yolk cell is furnished with two types of microtubular arrays. A network of microtubules is present in the YSL while animal-vegetal-oriented microtubules that originate in the YSL span the yolk cytoplasmic layer. Epiboly starts with the yolk cell bulging towards an animal pole. This so-called doming is correlated with and might drive radial intercalations of deep cells that scatter clonaly related cells (illustrated by scattering of a red-marked clone of deep cells). Simultaneously the deep cell layer becomes thinner and spreads toward the vegetal pole. The YSL with the nuclei and intercrossed microtubule network spread vegetally, while the yolk cytoplasmic layer with animal-vegetal set of microtubules decreases in size. The surface of the yolk cytoplasmic layer (purple line) decreases due to process of membrane internalization (see inset). This is correlated with expansion of the YSL surface (black line). Epiboly of the EVL involves only limited rearrangements of clonally related cells (marked as solid green) that occurs exclusively in the plane of the monolayer. The EVL margin is tightly linked to the surface of the YSL (see inset).

Another mechanism for formation of the hypoblast had been held for many years, from pioneering work done in teleosts possessing much larger yolk cells, such as *Salmo gairdneri*[29]. Ballard concluded that the hypoblast must form by delamination of the deep cells, based on dye and chalk particle marking experiments. A delamination mechanism is one in which the layers of a multilayered embryo become uniquely specified as either epiblast or hypoblast without changing position.

Furthermore, using DIC optics and time-lapse video, Trinkaus found recently that cells translocate from the superficial to deeper positions as the germ ring forms in *Fundulus*, and described the movement as ingression (J. P. Trinkaus, personal communication). For delamination or ingression to occur cells do not necessarily need to be at the margin. Indeed, Trinkaus' observations indicate that most cells ingress within about 10 cell diameters of the margin. Many cells initially at the margin actually recede 1-3 cells from the margin before ingressing. From these studies it is not clear if cells that ingress contribute directly to the hypoblast or simply to a deeper layer of the epiblast. On the other hand, no egression of cells was noted. Recent observations of the behavior of dye-labeled cells in the embryonic shield of zebrafish indicated that at least some cells ingress at positions away from the margin and move toward the animal pole within the hypoblast without ever coming into contact with the margin[30].

Both involution and ingression mechanisms implicate movements of single cells in the formation of the hypoblast. To contrast this single cell movement observed in teleosts with the involution of layers of cells in amphibia, the term ingression might be more appropriate. The central difference is the position relative to the margin at which cells move to join the hypoblast. Involution (or marginal ingression) involves the translocation of cells from the epiblast to the hypoblast only at the margin (one cell diameter or less), whereas in sub-marginal ingression cells can move to join the hypoblast also up to several cell diameters away from the margin. One possibility is that these differences in the mode of hypoblast formation result from differences in either the developmental stage or position within the germ ring at which observations were made. To resolve these discrepancies, additional analysis is required in which the position of the cells relative to the dorsal side of the embryo and the stage of development are carefully noted. A recent report indicates that the formation of hypoblast in zebrafish is initiated on the dorsal side of the embryo[27], which is similar to *Fundulus* (J. P. Trinkaus, personal communication) and amphibia[26].

(2) Convergence and extension

Perhaps the most dramatic gastrulation movements are convergence and the consequent extension of embryonic tissues. As a result of these movements, deep cells of all germ layers become concentrated on the dorsal side of the embryo to form the embryonic shield, and eventually to form

the elongated embryo with distinct anterior-posterior, dorsal-ventral and medial-lateral axes.

The cellular basis of convergence and extension has been explored in great detail in the amphibian, *Xenopus laevis*. Involuting marginal zone, a region that will give rise to mesodermal and endodermal structures, was studied in explants. These experiments indicate that mediolateral intercalation of individual cells leads to convergence, seen as a narrowing of tissue at all circumferential points such that the dorsal side is brought closer to the ventral side. Consequently, this movement leads to the elongation of converging tissue in an anterior-posterior direction, called extension. Detailed analysis of the behavior of individual cells in the explants reveals that the cells exhibit protrusive activity, reaching in a mediolateral direction ultimately to intercalate between lateral neighbors, displacing them in an anterior-posterior direction[31-33].

The mechanisms of convergence have not been well studied in zebrafish. In *Fundulus*, however, several features of the convergent movements of deep cells in the germ ring were described (Fig. 4)[34]. Deep cells of the germ ring, with the exception of those most ventrally located, move toward the dorsal side of the embryo. The cells closest to the shield move the fastest. Cells meander in all directions, but the net result of their movement is dorsalward. Most cells move as part of a cluster. The great majority of moving cells exhibit protrusive activity, extending filolamellipodia (Fig. 4E). Cells moving by the filolamellipodial mechanism are contact inhibited. This means that the cluster as a whole is moved by cells at free edges of the cluster undergoing the filolamellipodial movement. A minority, about 10%, of the moving cells utilize blebs for their movement (Fig. 4F). Cells that move by blebbing are not contact inhibited and are therefore able to undergo intercalation. These observations indicate

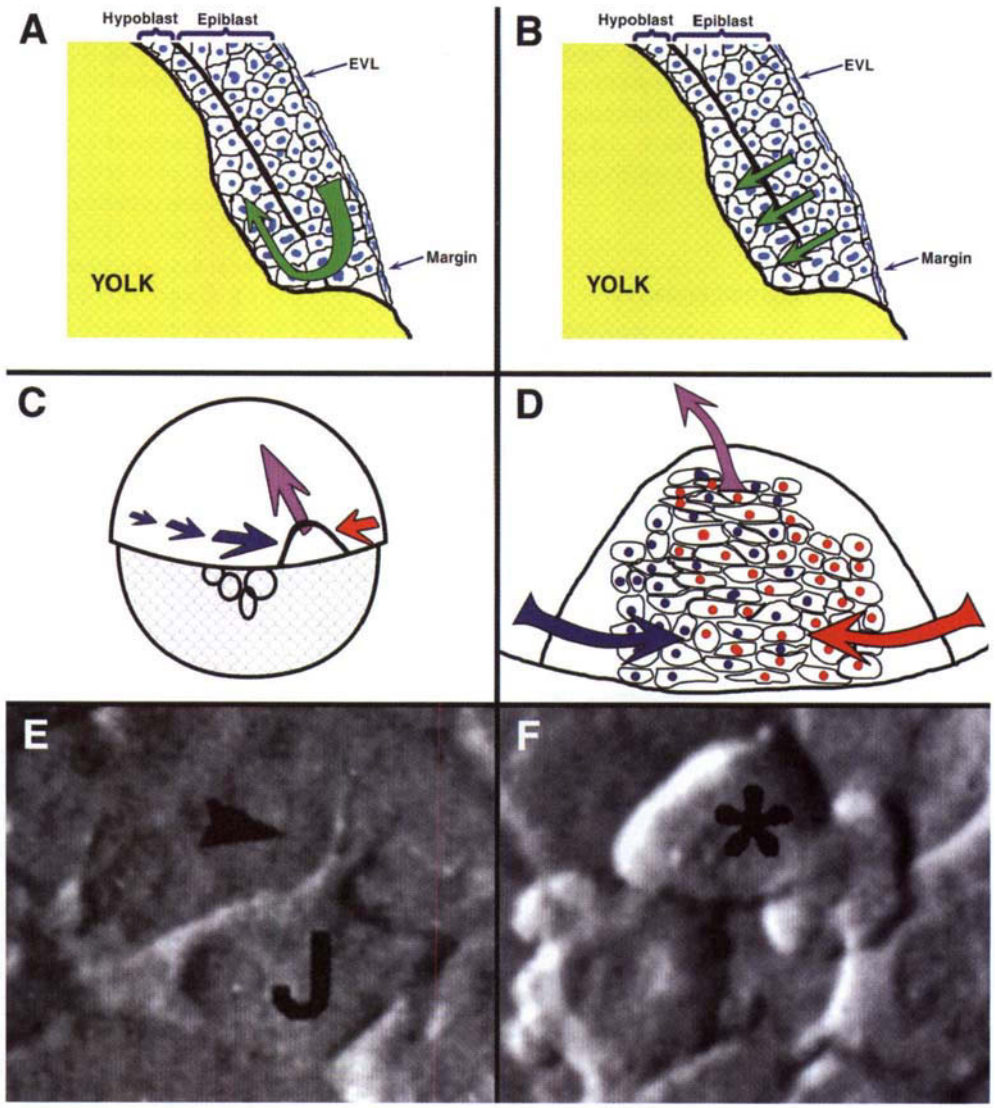

Fig. 4. Different types of tissue rearrangements could give rise to the multi-layered gastrula. In (A) involution movements of deep cells are described by the large green arrow. In involution, the more superficial cells of the deep layer must first move to the margin before they can contribute to the hypoblast. In (B) ingression movements of deep cells are described by the three green arrows. During ingression cells move to deeper layers to contribute to the hypoblast without first moving to the margin. In *Fundulus* embryos, convergence movements occur around the germ ring. In (C) the magnitude of the velocity of convergence movements is represented by the size of the red or blue arrows. Velocities are greatest near the shield. Cells of the shield are seen to move toward the animal pole (purple arrow), presumably by extension, which may be the direct result of mediolateral intercalation movements. In (D) mediolateral intercalation in the shield is depicted. Cells from the left side (blue nuclei) intercalate between cells from the right side (red nuclei), resulting in the extension of tissue toward the animal pole (purple arrow). In the germ ring of *Fundulus* embryos, cells undergo two characteristic types of movement. A cell (J) moving by extension of filolamellipodia is shown in (E); the black arrowhead indicates the filolamellipodium. A cell moving by blebbing is shown in (F) (asterisk). (E and F are modified from ref. 34).

that in *Fundulus*, the dorsalward convergence of lateral tissues is achieved by directional migration of small groups and individual cells. Mediolateral intercalation is not primarily responsible for the dorsalward movement. Dorsalward migration without mediolateral intercalation leads necessarily to the accumulation of cells in the embryonic shield, but not necessarily to extension.

For the zebrafish, it has been proposed that cells of ventrolateral hypoblast move by directional migration. Cells of the dorsal hypoblast and epiblast undergo mediolateral intercalation, which is inferred by the dispersion of clonally related cells along the anterior-posterior axis[27,35,36].

The following picture of the convergence and extension in teleosts emerges. Directional dorsalward migration of deep hypoblast cells leads to the thinning of the ventral and lateral parts of the embryo and to the thickening of the shield. Once cells are within the shield, mediolateral intercalation results in the narrowing and extension of the dorsally accumulated tissues. Continuing epibolic movements also contribute to extension of the axis. Clearly, what is required is a study of individual cell behaviors in the zebrafish epiblast and hypoblast, both in the ventral and lateral regions, and within the embryonic shield. Using fluorescent dye marking and high resolution time-lapse video, the spatial and temporal distribution of distinct types of cell movements will be readily apparent.

Emergence of deep cell lineages

The EVL and YSL cell lineages separate from the deep cells before the onset of epiboly and probably do not contribute to any adult structure of the zebrafish[7]. The deep cell layer, sandwiched between the EVL and YSL, will give rise to all the adult structures (Fig. 2). Two important questions are: when do tissue restrictions first appear in deep layer cells; and when does the relationship between the position of a cell in the embryo and its fate first become apparent?

Construction of a fate map is only possible in embryos that possess some intrinsic quality that will allow the assignment of unique cellular positions with respect to future embryonic axes. In addition, it is important that movements occurring subsequent to the marking of cells be stereotyped with respect to the original position of the marked cell. Otherwise, no consistent relationship between the original position of the cell and its fate can be described. Zebrafish embryos exhibit an animal-vegetal asymmetry at the beginning of development. Further, both the first and second cleavage planes can be unambiguously identified at the 8-cell stage (Fig. 1). Early studies, however, have reported either no correlation[37], or only a slight correlation[38] between the second division plane and the future dorsal-ventral embryonic axis. This lack of correlation between the cleavage planes and the future dorsal-ventral axis, and cell mixing due to the radial intercalation of the deep cells during epiboly, has prevented construction of the fate map for the early embryo[38].

The apparent lack of correlation between the cleavage planes and the future dorsal-ventral axis was later challenged by investigators using fluorescent lineage tracer dyes to map the fates of early blastomeres[39,40]. The distribution of the progeny of blastomeres from 4-16-cell stage embryos was analyzed at the end of embryogenesis, i.e. after 26 hours of development. The central conclusion from these experiments was that the position of an early blastomere will reliably predict the range of fates available to its progeny. Moreover, the invariant early cleavage planes were suggested to predict the principal body axes, with the second cleavage plane anticipating the position of the dorsal-ventral axis[39].

Three independent lines of evidence have recently demonstrated a lack of correlation between the second division plane and the future dorsal-ventral axis, confirming the original reports. In one type of experiment, two blastomeres of 8-cell stage embryos were injected with two distinguishable lineage tracers such that the position of the second plane of division was marked. The position of the second cleavage plane was then compared with the position of the dorsal-ventral axis at the beginning of gastrulation, as indicated by morphological[41] or molecular markers[23]. No correlation[23], or at best a slight correlation, was found[41]. Additional support for this view has come from the analysis of the maternal-effect mutation *janus*[41]. In *janus* mutant embryos the blastoderm becomes divided along the first cleavage plane into two detached half blastoderms. Consistent with the lack of correlation between the planes of early divisions and dorsal-ventral axis, the first division plane bisecting the blastoderm of the *janus* mutant embryos is positioned randomly with respect to the single dorsal organizer region recognized by the expression of *goosecoid* mRNA[41].

The unpredictability of the dorsal-ventral axis position in the early embryo precludes the prediction of fates of the early blastomeres. Recent observations, however, suggest that portions of zebrafish embryo retain their relative positions from blastula to gastrula, and consequently that rudiments of a fate map can be observed prior to the gastrula stage[23]. Analysis of the fates of injected cells located at distinct latitudes of blastula stage embryos has demonstrated that central blastomeres undergo more extensive mixing during epiboly than do marginal blastomeres[22,23]. Consequently, the fates of central blastomeres are less predictable. This observation explains why consistent differences are observed between contributions of central and marginal blastomeres to different tissues; why the progeny of marginal blastomeres give rise only to a limited set of fates; and, further, why progenitors of some common fates are clustered in certain regions of the blastula[6,40,42]. For example, single cell injections and tracking demonstrated that cardiac progenitors are located at the margin of the blastula stage embryo. Additionally, retrospective assessment of the position of injected cells at the shield stage showed that heart progenitors map to ventral and lateral regions of the blastula[43,44]. Therefore, there are rudiments of the fate map in the early zebrafish

embryo. This rudimentary fate map is cryptic because it exists prior to the earliest time the dorsal-ventral axis can be observed in live embryos. In fixed embryos, however, the dorsal-ventral axis is revealed by the expression patterns of a number of genes, such as *goosecoid*[45]. Furthermore, a recent report indicates that the blastoderm of a 30%-epiboly embryo is thinner on the dorsal side[27]. Using this feature as a dorsal marker it should be possible to construct a fate map at this early stage.

Fate mapping and cell lineage restrictions in the late blastula

The gastrulation movements of deep cells lead to the formation of the germ ring and embryonic shield. The shield is the earliest reliable marker of the dorsal side in living embryos, and as such provides a means for assigning unique cellular positions within the embryo. In addition, the movements of cells during this and later stages of development are stereotyped[14,36]. Together these facts have made possible the construction of a fate map for the late blastula to early gastrula[7]. The first important result to issue from these investigations is that for the deep cells in the 50% epiboly embryo, the first tissue-restricted lineages emerge. Injections with marker dyes reveal that in the great majority of cases, single cells generate clones that contribute to only single tissues[38,46]. The fate map of the zebrafish late blastula possesses an overall organization reminiscent of early gastrula fate maps of other vertebrates, with progenitors of a given tissue clustered in one region of the mapped embryo[7,47-50]. The three classically defined germ layers are arranged with ectoderm arising from near the animal pole, and the mesoderm and endoderm arising from marginal cells, with prospective endoderm holding the most marginal postions of the blastoderm (Fig. 5). Clustered on the dorsal side, near the margin, are the progenitors of the notochord and the hatching gland, prechordal mesoderm-derived tissue. The dorsal side of the late blastula will become the embryonic shield of the early gastrula. The shield is homologous to the dorsal blastopore lip of amphibians in its ability to organize the formation of an embryo when grafted into naïve host tissue[51-53] and because it also gives rise to the notochord and prechordal plate. Additionally, the shield and dorsal lip share the expression pattern of an array of molecular markers, indicating their shared functional significance[54]. One striking difference, however, between the zebrafish fate map and the *Xenopus* fate map is that the degree of overlap between prospective tissues appears to be more extensive in zebrafish (Fig. 5). On the other hand, the apparent overlap may simply reflect the low resolution of the current zebrafish map[7].

Fate map of the zebrafish embryonic shield

The late blastula /early gastrula fate map shows the distribution of tissue progenitors just before and at the onset of gastrulation movements of deep cells[7]. Recently, a high resolution fate map of the zebrafish embryonic shield (20-30 minutes later than the late blastula map) has been constructed. A combination of single cell labeling by dye injection and transplantation, and a method for assigning unique cellular positions that allows the consistent measurement of the fate of a given region of the shield in three dimensions from embryo to embryo, were used. The investigators obtained several important results[30]. Firstly, as expected from previous reports[7], single cells labeled in the early gastrula give rise to tissue-restricted lineages. Secondly, within the studied region, progenitors of neural, notochordal, somitic and endodermal lineages were present as an intermingled population. The third major result of this fate map is that there appears to be a cell-layer bias of cell fates within 5-cell-body diameters of the mid-line of the shield. In this region, the most probable fate of a cell in the superficial layer of deep cells is endodermal. For the second cell layer, mesodermal fates predominate. In the third cell layer the majority of cells have a neural fate. It is important to note that all three of these cell layers are within the epiblast. These results have several important implications which affect the interpretation of data concerning the specification of cell type and the movements of cells in the shield during gastrulation. There are at least three plausible explanations for the data.

One possibility is that cells within the shield are specified by the time the shield forms and that they subsequently sort themselves to appropriate locations during the ensuing gastrulation movements. One can imagine that cells with similar fates are specified and express the machinery necessary to come together in the same target tissue. Alternatively, it may be that cells of the shield are actually unspecified and that they move randomly within the shield becoming specified when they assume their final positions in either the epiblast

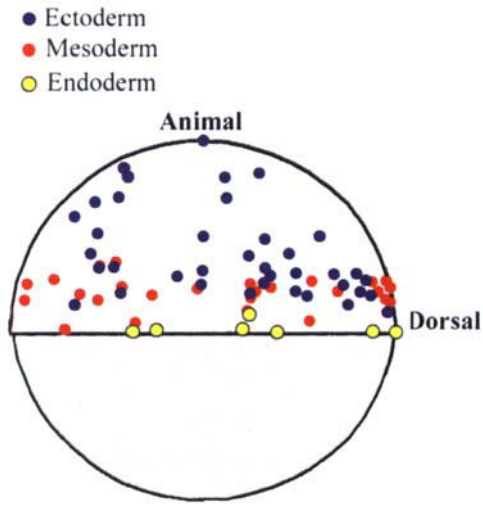

Fig. 5. Fate-map of the late-blastula zebrafish embryo, modified from ref. 7. Blue circles represent injected cells that produced ectoderm; red circles correspond to mesoderm and yellow represents endoderm fates. The animal pole is at the top and the prospective dorsal side of the embryo is to the right.

or hypoblast. Lastly, it is possible that movements within the shield are not random and that specific interactions at that time are an integral part of the specification process.

In this context it is important to consider that as much as 40 minutes earlier, cells were restricted to form only single tissues. Hence, the rate of cell divisions becomes an issue. If the cell cycle is short, such that one or more cell divisions on average occur in that interval, then it is more likely that the cells were specified prior to shield formation. If, however, the cell cycle is long, of the order of the length of the interval between the beginning of gastrulation and the formation of the shield, then all three possibilities are plausible. Indeed, the majority of deep cells become lineage-restricted when they are in cycle 14 or 15, which on average last 78 and 151 minutes, respectively (Fig. 2)[36], making it possible that the cells may be initially unspecified and become restricted during migration or after attaining their ultimate position in the gastrula[35].

Commitment to cell fates

The shield fate map raises several questions concerning the specification of cells during gastrulation. Distinguishing between the possible specification mechanisms will require one to determine when during development the cells become committed to a particular fate. It is not possible from lineage tracing experiments to ascertain whether a cell is 'committed' (or 'determined', as defined by Slack[55]) to a given fate; that is if the cell is challenged to a different environment, would it take on another fate? For zebrafish the issue of commitment has been addressed in one study[56], during the period of development spanning the transition from late blastula (5 hours), when tissue restrictions arise[38], to mid-gastrula (8 hours) (Fig. 2). Cells transplanted from the 5-hour-old marginal zone into the animal pole were found to contribute significantly to epiblast fates and not at all to hypoblast fates, i.e. they behaved as normal animal pole cells do[56]. When the same type of transplantation was done at 8 hours of development, using hypoblast cells from the marginal zone transplanted into the animal pole region, the transplanted cells contributed to hypoblast-derived lineages in 85% of the cases and to epiblast fates in only 5% of the cases. It was concluded that between 5 hours and 8 hours of development marginal zone cells, which had moved to the hypoblast, became committed to hypoblast fates. While the experiments seem to suggest that between 5 hours and 8 hours cells in the hypoblast become restricted from contributing epiblast fates, they do not address the question of whether those cells have been irreversibly assigned to a specific hypoblast fate, for example somite *versus* notochord. These experiments do indicate that even though tissue-specific lineage restrictions are observed for deep cells injected at the 5 hour time point, those cells can be respecified when they are transplanted to a new environment. That is, tissue-specific lineage restrictions do not necessarily reflect the state of commitment of the cell.

Future directions

A key point in zebrafish development is the late blastula stage when tissue-restricted deep cell lineages arise in a position-specific manner. A challenge to zebrafish researchers is to determine how this positional information is established, and how it relates to the gastrulation movements which in turn create a multilayered embryo with properly organized rudiments of organs and tissues.

The late blastula fate map of zebrafish exhibits overall organization similar to fate maps of other vertebrates. It predicts the normal fates of cells residing in a certain position of the blastula, as well as the types of movements they will undergo[7]. Although cells of the late blastula give rise to progeny with tissue-restricted fates, they are not committed to them at this stage[56]. Important questions are, to what degree are cellular fates specified in the late blastula before the cells embark on morphogenetic movements that generate germ layers; and to what degree is fate specification influenced by signals received while cells migrate in the process of germ layers formation, or when cells arrive at their final destinations? What molecules are involved in fate specifications?

Answering these questions will require high-resolution fate maps at the beginning, and at different stages during gastrulation, combined with analyses of cellular migration pathways, motile behaviors, and the commitments of cells to specific fates. The changes in position and behavior of cells need to be understood in the context of dynamic patterns of gene expression. Further, a variety of mutations affecting gastrulation have been identified[57-59]. Such mutations identify genes involved in the process, and the mutant phenotypes indicate their functions. Importantly, mutant embryos in which gastrulation is affected also provide a means to study the consequences of changes in specification of the late blastula fate map, abnormal cell movements and defects in formation of certain cell types on the other aspects of gastrulation. Combination of genetic and embryological methods applied to live zebrafish embryos will continue to complement other approaches to the problem of specification of cell fates and cell movements during vertebrate embryogenesis.

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