Dye-coupling and the formation and fate of the hypoblast in the teleost fish embryo, *Barbus conchonius*

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**Summary**

The present report describes Lucifer Yellow (LY) transfer between the syncytial layer of the yolk cell (YSL) and blastodermal cells during epiboly in the teleost fish *Barbus conchonius*. The fate of a group of labeled cells is described until germ layer formation.

At the onset of epiboly, LY seems to be transferred from the YSL to all blastodermal cells. Between 10 % and 40 % epiboly, dye-coupling appears to be restricted to the marginal region. Within 60 min individually labeled cells are distributed among unlabeled cells within the blastoderm. Between 40 % and 60 % epiboly, we observed a ring-shaped group of labeled cells, which probably have involuted during early gastrulation. Consequently, this cell group may correlate with the leading edge of the hypoblast layer within the germ ring.

At 60 % epiboly and later, the blastodermal cells are dye-uncoupled from the YSL.

A gradual translocation of the ring-shaped hypoblast towards a dorsally located bar-like structure is observed between 50 % and 100 % epiboly. At 100 % epiboly, fluorescent cells were located in contact with the YSL within the embryo proper, with the brightest fluorescence in the future head region. The translocation is due to dorsalwards convergent cell movements during the gastrulation process. The appearance of the hypoblast as a dye-coupled cell layer may correlate with some restriction in cell fate since the hypoblast differs in fate from the epiblast.

Key words: fish development, dye-coupling, gastrulation, hypoblast.

**Introduction**

During development, groups of cells within an embryo become progressively restricted in their presumptive functions and undergo structural differentiation. The appearance of cell groups, following different developmental pathways, may parallel changes in communication between cell groups (Lo and Gilula, 1979; Warner and Lawrence, 1982; Weir and Lo, 1982; Kalimi and Lo, 1988; for review, see Wolpert, 1978; Guthrie and Gilula, 1989). Crucial developmental processes like pattern formation and the formation of morphogenetic gradients appear to be mediated by gap junctions (Fraser et al. 1987; Lee et al. 1987; for review see Wolpert, 1978; Saxen et al. 1980; Lo, 1985; Warner, 1985; Guthrie and Gilula, 1989). Strong evidence for the key role of gap junctions has been obtained from experiments in which embryos of different species were injected with anti-gap junction antibody (Warner et al. 1984; Fraser et al. 1987; Serras et al. 1989a; Lee et al. 1987; for review, Warner, 1985) or gap junction antisense RNA (Bevilacqua et al. 1989). These treatments have led to specific developmental aberrations. The existence and size of a group of junctionally communicating cells can be demonstrated, for instance, by the transfer of low relative molecular mass dyes from one cell of a compartment to the others. Strict correlations between a dye-coupled compartment and a functional developmental compartment have been demonstrated in insects (Warner and Lawrence, 1982; Weir and Lo, 1982; Blennerhassett and Caveney, 1984). In some other species, such as Ascidia (Serras et al. 1989c), molluscs (Dorresteyn et al. 1983; Serras and Van den Biggelaar, 1987; Serras et al. 1989b) and mice (Lo and Gilula, 1979) the restriction of dye transfer at the segment border may not be complete, for that reason it is not a real compartment. In the mouse embryo, for example, a gradual limitation of dye spread from inner cell mass (ICM) to trophectoderm is observed from 36 h after implantation onwards.

Since the appearance of separate communication compartments may be correlated with cell fate restrictions, it is important to study the compartmentalization of other vertebrate embryos too. In the present study, the teleost fish embryo is chosen since very limited information is available concerning compartmentalization in embryos of this class of vertebrates.

Cell groups with a specific fate are the germ layers. In fish embryos, the germ layers are the hypoblast, which arises at gastrulation by involution within the germ ring.
(Wood and Timmermans, 1988), and the epiblast (non-involved cells). Directional cell movements within the hypoblast and epiblast are responsible for the formation of the embryo proper on the dorsal side of the yolk. As recently established by cell lineage experiments with Warga and Kimmel (1990) for the zebrafish, the epiblast has an ectodermal fate while the hypoblast has an endodermal/mesodermal fate. Although germ layers represent cell groups with specific fates, they do not have precise lineages and strict borders. This may indicate that they should not be considered as real compartments.

In this report, dye coupling between the syncytial layer of the yolk cell (YSL) and blastomeres during epiboly of the cyprinid fish Brachydanio rerio is described. In Fundulus heteroclitus (Kimmel et al. 1984) and Brachydanio rerio (Kimmel and Law, 1985), the YSL remains dye-coupled to the blastomeres during early epiboly but during early gastrulation the YSL and the blastomeres become dye-uncoupled. It is interesting that Kimmel and Warga (1986), Kimmel et al. (1990) and Warga and Kimmel (1990) concluded from cell lineage experiments in zebrafish that reproducible fates arise at the gastrula stage. Therefore, we have studied in Brachydanio rerio the correlation between the appearance of a dye-coupled group of cells, whether or not a real compartment, and cell fate.

Materials and methods

Embryos

Adult specimens of the Rosy Barb, Barbus conchonius (Cyprinidae, Teleostei) were kept in 201L tanks at 25°C, fed on Trouvit pellets and kept in a 12 h dark/12 h light cycle. Spawning and fertilization occurred in small tanks after the onset of the light period. Embryos were collected from the bottom and allowed to develop in modified Steinberg’s medium (60 mm NaCl, 0.7 mm KCl, 0.8 mm MgSO₄, 0.3 mm Ca(NO₃)₂) at 25°C. The embryos were dechorionated by incubation in a 0.2% protease solution (type XIV, Sigma) until the outer layer of the chorion could partly be removed with a hairloop. The chorion was subsequently removed by rinsing two times in Steinberg’s medium.

Dye injections

To establish the presence of dye-coupled compartments connected to the YSL, the YSL was injected iontophoretically at the outer margin with the low relative molecular mass dye Lucifer Yellow CH (LY; Sigma; di-Li-salt; 545, dissolved in 0.3 mm LiCl (hyperpolarizing current, 50 nA, 5 Hz, 5 min). From the formation of the YSL onwards (0% epiboly; 4 h after fertilization, a.f.), every 30 min until 100% epiboly (9 h a.f.) 10 embryos were injected. As a control, similar experiments were carried out using the relatively high molecular mass dye LY-dextran (LY-D; Sigma; M_r=40000) to confirm that the LY label in the former experiments was not transferred by endo/exocytosis or cytoplasmic bridges. Since it might be possible that, after a certain time, LY aggregates with cytoplasmic determinants (due to its charge and/or chemical structure), which would prevent dye transfer, a second low molecular weight dye fluorescein-complexon (FC; Kodak; M_r=710; negatively charged) was injected into the YSL at the previously mentioned stages (5 embryos at each stage).

Furthermore, 30 embryos were injected with LY (same procedure as described before) into the YSL at the 40% epiboly stage to study the fate of groups of labeled cells, that were found in the above experiments.

Electrodes were pulled from Clark Electromedical Instrument electrodes (GC 150F-15) with a DKI electrode puller. Injections were visualized with a 6.3 times epifluorescence objective on a Zeiss microscope, supplied with a 50 W mercury epilight source. To prevent light damage to the embryo, the lightpath was blocked during injection. Embryos were photographed with a 400 ASA Kodak T-max Black/White film.

Embryos injected with LY or LY-D at several stages of epiboly

The 10 embryos per stage per injected dye were fixed in groups of 5 in 2% formaldehyde in Steinberg’s medium (16 h, 4°C) either 10 min or 60 min after injection. Afterwards, the embryos were rinsed in Steinberg’s medium, orientated in 1% agarose (type VII, Sigma), dehydrated in a graded series of ethanol and embedded in Technovit. 5 μm sections were made with an LKB Histoprime microtome, perpendicular as well as parallel to the direction of epiboly. According to Stewart (1978) and our own observations in vivo, this method does not alter the staining pattern of LY substantially. However, it is almost impossible to prove definitively that there are no fixation artifacts. Fluorescent cells were visualized with a standard fluorescence microscope and photographed with a 400 ASA Kodak T-max Black/White film.

Embryos injected with LY at the 40% epiboly stage in order to follow the labeled cell group

The 30 injected embryos were fixed in groups of 5 at the 50%, 60%, 70%, 80%, 90% and 100% epiboly stage in 2% formaldehyde in Steinberg’s medium (16 h, 4°C), rinsed in Steinberg’s medium (60 min) and in methanol (30 min) and cleared in a 1:2 solution of benzylalcohol/benzylbenzoate, as also described by Dent et al. (1989). The presence and location of labeled cells were subsequently studied by confocal scanning laser microscopy (CSLM; Biorad) and photographed with a 125 ASA Kodak PX Black/White film.

Embryos injected with FC

The embryos, injected with FC, were fixed 60 min after injection in 2% formaldehyde (16 h; 4°C), rinsed in Steinberg’s solution (60 min) and methanol (30 min), placed in clearing solution and observed in toto by fluorescence microscopy. They were photographed using a 400 ASA Kodak T-max Black/White film.

Results

General development

The meroblastic cleavage pattern of fish embryos results in a cell mass, the blastoderm set, at the animal pole, and a yolk cell at the vegetal pole. At 4 h a.f. a yolk syncytial layer (YSL) is formed below the blastoderm (Fig. 1A) and an enveloping layer (EVL) can be observed at the outer side of the blastoderm. The cells between YSL and EVL are the deep cells (DC), which will form the embryo proper. After YSL formation epiboly starts, during which the yolk cell will be surrounded by the cell mass (Fig. 1B, C). At about
Fig. 1. Schematic drawing of median sections of three different stages of development. (A) The 4 h stage, shortly before the onset of epiboly, just after the formation of the YSL. (B) The 60% epiboly stage. (C) The 90% epiboly stage. DC, deep cells; EVL, enveloping layer; YSL, yolk syncytial layer; Y, yolk; A, animal side; V, vegetal side.

50% epiboly (6 h a.f.), gastrulation starts: at the blastoderm margin a germ ring arises, in which involution is responsible for the formation of the hypoblast below the epiblast cell layer (see also Wood and Timmermans, 1988). At 9 h a.f. epiboly is completed.

Injections into the YSL at several stages of epiboly
The EVL cells were shown to be LY-coupled to the YSL, as long as deep cells were coupled to the YSL. However, the label did not spread evenly to all EVL cells, but showed a gradient-like distribution from brightly labeled cells near the YSL to undetectable labeling towards the animal pole of the embryo (see also Fig. 2D). In the remaining part of the results, we will only consider the labeling of the deep cells as only these cells contribute to the embryo proper.

Embryos, injected at the onset of epiboly, showed a gradient-like distribution of the label with the brightest cells near the YSL. Even 1 h after injection, the label was not able to reach all cells (Fig. 2A). Embryos, injected at 10% epiboly and fixed 10 min later, showed similar labeling as the above group but if they were allowed to develop for 60 min, many individually labeled cells had spread within the blastoderm, with a relatively high density of labeled cells in the marginal region (Fig. 2B). The embryos injected at 40% epiboly were the first to show a clear ring-shaped unit, which probably represents the hypoblast. At 10 min after injection this labeled cell group had a height (indicated by A in Fig. 6b) of only a few cells (Fig. 2C), but after 1 h (60% epiboly) the fluorescent cell group had increased in height (Fig. 2D). Furthermore, it had a sharp border at the animal side (hypoblast/epiblast boundary; Fig. 2D, E) while near the margin (germ ring) the border was more diffuse. The height of the fluorescent hypoblast was broadly the same along the whole circumferential margin of the blastodisc. Injections, carried out at 60% epiboly and thereafter, showed that all blastodermal cells were dye-uncoupled from the YSL (Fig. 2F).

In the embryos, that were injected with LY-D, no labeled blastoderm cells could be detected (Fig. 2G). In embryos injected with FC at the onset of epiboly, the dye spread to a number of layers of blastodermal cells and showed a decreasing gradient in fluorescence, as is also observed after LY injection. Embryos, injected with FC at 40% epiboly and fixed at 60% epiboly showed the ring-shaped hypoblast (Fig. 3), but less sharply delineated than after LY injections. A three-dimensional image of the ring-shaped labeled cell group is shown in Fig. 4.

The fate of the ring-shaped labeled cell group
The change in shape of the LY-labeled cell group (see Fig. 4) was studied by CSLM during the subsequent progress of epiboly. In embryos injected at 40% epiboly and fixed at 50% epiboly, a ring-shaped labeled cell group was present (Fig. 5A, B). At the side of the future rostrocaudal embryonic axis (dorsal side), the height of the labeled cell group gradually increased during the progress of epiboly (Fig. 5C, D), whereas at the opposite side (ventral side) labeled cells disappeared (Fig. 5C). During epiboly the brightness of the fluorescent cell group gradually decreased towards the germ ring, due to the diffuse nature of the label. The weakly labeled cells may reflect the fact that connections between labeled and unlabeled cells were present. The weakly labeled cells may correspond to cells involuted after dye-uncoupling, or to cells, involuted previously but having made contacts with unlabeled cells. Finally, at 100% epiboly, the brightest labeled cells were located at the future head side in a rostrocaudal direction, closely adhering to the YSL (Fig. 5E, F). A graphical model of the translocation of the labeled compartment is given in Fig. 6.

Discussion
In the present paper, we described the LY dye transfer from the YSL to the blastodermal cells during epiboly of Barbus conchonius embryos. Around the onset of
gastrulation, we observed the emergence of a ring-shaped group of labeled cells, which probably represents involuted cells, forming the hypoblast (Wood and Timmermans, 1988). Furthermore, we studied the fate of the labeled hypoblast after dye-uncoupling between YSL and blastomeres: it changed into a dorsally located bar-like structure along the rostrocaudal axis. Since the injections with LY-D into the YSL did not result in labeled blastomeres, it can be concluded that dyes are not transferred by endo/exocytosis or cytoplasmic bridges from the YSL to the blastomeres. Consequently, LY injections presumably demonstrate cells linked by gap junctions. Since we did not study electrical coupling between cells, we do not know if complete uncoupling occurs between the labeled and unlabeled cell layer. However, because these two layers (e.g., germ layers) are physically separate layers, uncoupling may be complete.

Embryos injected with either LY or FC into the YSL just after the onset of epiboly showed a gradient-like distribution of the label in the blastomeres, even when fixation followed 1 h after injection. Since FC injections showed exactly the same result as LY injections, this phenomenon may be simply due to fixation before
Fig. 2. Sections of embryos that were injected with LY into the YSL at several stages of epiboly. The time after fertilization (a.f.) is given in brackets. When not otherwise indicated, embryos are vertically sectioned, parallel to the direction of epiboly. Small arrows indicate the EVL.

(A) Injection at the onset of epiboly (4 h a.f.) and fixation at 20% epiboly (60 min after injection). This figure shows distinct labelling of deep cells adjacent to the YSL. A gradual reduction in fluorescence can be seen in the deep cells in a direction perpendicular to the YSL (arrow). Abbreviations as described in Fig. 1. Scale bar: 100 μm.

(B) Injection at 10% epiboly (4.5 h a.f.) and fixation at 30% epiboly (60 min after injection). This figure shows many individually labeled cells, distributed among unlabeled cells. A high density of labeled cells can be seen at the blastoderm margin (arrow). Scale bar: 100 μm.

(C) Injection at 40% epiboly (6 h a.f.) and fixation 10 min later. A few deep cells at a certain distance from the margin are labeled (thick arrow). Note that a labeled EVL cell can also be seen at the blastoderm margin (arrowhead). Scale bar: 100 μm.

(D) Injection at 40% epiboly (6 h a.f.) and fixation at 60% epiboly (60 min after injection). A group of deep cells (hypoblast) is labeled. It has the highest fluorescence at the animal side of the blastoderm, and shows a gradient towards the margin (germ ring). Note also that the EVL cells show a gradient-like distribution of the label (thick arrow). A number of individually labeled cells outside the bright fluorescent cell group (epiblast) can be seen. This is probably due to injection at a time when cell mixing is not yet completely finished. Scale bar: 100 μm.

(E) Injection at 40% epiboly (6 h a.f.) and fixation at 60% epiboly (60 min after injection). The section is made in a horizontal plane, perpendicular to the direction of epiboly. All cells are dye-uncoupled from the YSL. Scale bar: 100 μm.

(F) Injection with LY-D at 40% epiboly (6 h a.f.) and fixation at 60% epiboly (60 min after injection). The section is made in a vertical plane, parallel to the direction of epiboly. We did not find any labeled cells. If the injected dye was actively transported between the cells, one would expect a picture as shown in (D). The thick arrow indicates where labeled cells might be found. Scale bar: 100 μm.

Diffusion processes have been completed. Consequently it is not clear whether during this stage another dye-coupled cell group is present.

The embryos injected with LY between 10% and 30% epiboly and fixed 60 min later showed many individually labeled cells, distributed among unlabeled cells. This result correlates with the existence of 'cell mixing' at the same developmental stage as shown for Barbus conchonius (Gevers et al. unpublished data) and the zebrafish (Kimmel and Warga, 1987, 1988). The contrast between labeled and unlabeled neighboring cells suggests that during this stage no LY communication is present between the deep cells, although they may be electrically coupled. The higher density of labeled cells at the blastoderm margin indicates that dye-coupling occurs at the blastoderm margin at these stages.

In the embryos injected with LY at 40% epiboly (fixation at 60% epiboly), we observed the appearance of a labeled group of cells at the same location as found after LY injections. The high background is due to the fact that this photograph is a standard fluorescence picture and not a CSLM picture. Scale bar: 25 μm.

Fig. 3. Embryo of B. conchonius in toto, injected with FC at the 40% epiboly stage and fixed at the 60% epiboly stage. The arrow indicates the presence of a labeled group of cells, at the same location as found after LY injections. The high background is due to the fact that this photograph is a standard fluorescence picture and not a CSLM picture. Scale bar: 25 μm.

Fig. 4. Three-dimensional drawing of a Barbus conchonius embryo at 60% epiboly, shortly after the formation of the ring-shaped cell group, the hypoblast (H). It is clear that at the animal side (A) of the fluorescent cell group a sharp boundary is present, while the vegetal side (V) shows a more diffuse boundary. The arrow indicates the involution movement of the cells within the germ ring. Y, yolk, YSL, yolk syncytial layer.
of a dye-coupled ring-shaped cell group, apparently representing the hypoblast cell layer. Since the height of the labeled cell group is almost equal around the entire circumferential margin, the rate of involution may also be nearly equal at every point along the circumference. The same suggestion was made by Warga and Kimmel (1990) with respect to the formation of the germ ring in zebrafish. Since dye transfer is relatively slow in the Rosy Barb, it can not be excluded that dye-coupling occurs just before the onset of gastrulation. Consequently, it is not clear whether dye-coupling is a result or a preceding event of hypoblast formation. From 60% epiboly onwards, shortly after the onset of gastrulation, the YSL and the blastomeres are dye-uncoupled.

In conclusion, in B. conchonius, a dye-coupled cell group, the hypoblast, is formed within the blastoderm during early gastrulation. It is interesting in this respect that Kimmel and Warga (1986), Kimmel et al. (1990) and Warga and Kimmel (1990) concluded that in zebrafish cell lineage restrictions occur at the gastrula stage. Although the hypoblast cells of B. conchonius appear to form a dye-coupled unit, we cannot consider it as a developmental compartment, because these compartments have a precise lineage. Furthermore, because we did not find a strict border at the margin of

Fig. 5. Translocation of the labeled hypoblast at successive stages of development as observed by means of confocal scanning laser microscopy (CSLM). All embryos are injected with LY into the YSL at 40% epiboly (6 h a.f.). Scale bar: 100 μm (A, C, E) or 25 μm (B, D, E). (A) Embryo fixed at 50% epiboly. The presence of a labeled cell group can be seen on both sides of the blastoderm at a certain distance from the margin (arrows). Also the gradual decrease of fluorescence in the direction of the margin is clear on both sides. Arrowhead indicates YSL. Y, yolk. (B) A detail of the labeled cells can be seen on the right (arrow). (C) Embryo fixed at 80% epiboly. The labeled cells can be seen only at one side of the blastoderm (arrow). Despite the reduction in fluorescence, the labeled cells can be followed to a short distance from the margin (arrowhead). At the opposite side (right), the cells belonging to the labeled hypoblast have already migrated dorsalwards. (D) Detail of the 'head' of the hypoblast at 80% epiboly (arrow). In this picture, it is very clear that the hypoblast is a separate layer of cells, located between the YSL and the unlabeled part of the blastoderm (epiblast). (E) Embryo fixed at 100% epiboly. At this stage, the label is diluted among a large number of cells. Labeled cells can be observed in the future head region (arrow) as a separate layer against the YSL. (F) Detail of the future head region of (E). Arrow indicates the presence of a layer of labeled cells.
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Fig. 6. Model of the change in morphology of the LY-containing cell group (hypoblast) at successive stages of epiboly (a). We measured the relative height of the hypoblast as indicated in (b). This is the height from the 'head' to the margin (A) related to the height of the embryo (B). The relative height was measured at the highest point along the circumference of the embryo (the future rostrocaudal embryonic axis, the dorsal side) and at the lowest point (the ventral side). In this figure, the ring-shaped structure at 50% epiboly is represented as a solid line (most left and right points of the lines close the ring) with the same height at every distance from the embryonic axis. The lines represent the hypothetical boundaries of epiblast/hypoblast around the circumference of the embryo as we did not measure exactly the slope of the labeled cell group.

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