CELL FATE ESTABLISHMENT DURING EARLY DEVELOPMENT OF CYPRINID FISHES, WITH SPECIAL EMPHASIS ON THE FORMATION OF THE PRIMORDIAL GERM CELLS

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CELL FATE ESTABLISHMENT DURING EARLY DEVELOPMENT OF CYPRINID FISHES, WITH SPECIAL EMPHASIS ON THE FORMATION OF THE PRIMORDIAL GERM CELLS

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
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in het openbaar te verdedigen
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STELLINGEN

1. De oorsprong van PGCs van cyprinide vissen suggereert dat de "fate map" van deze vissen beter vergeleken kan worden met die van urodelen dan met die van anuren.
(Delarue et al., Development 114, 135-146, 1992; Kimmel et al., Development 108, 581-594, 1990; dit proefschrift).

2. De YSL speelt met grote waarschijnlijkheid een belangrijke (determinerende) rol tijdens de gastrulatie van vissen.
(Dit proefschrift).

3. In tegenstelling tot de visie van Ballard (1966; 1973) vindt ook tijdens de gastrulatie van vissen involutie van cellen plaats.
(Ballard, J. Exp. Zool 161, 201-220, 1966; Ballard, J. Exp. Zool. 184, 27-48, 1973; Wood en Timmermans, Development 102, 575-585, 1988; dit proefschrift).

4. Vis-mutanten voor het *Cdx [Zf-cad1]* homeobox-gen zouden een belangrijke bijdrage kunnen leveren aan het ontrafelen van het mechanisme van de gastrulatie.
(Joly et al., Differentiation 50, 75-87, 1992).

5. De term hypoblast, zoals gebruikt bij verschillende groepen vertebraten, suggereert ten onrechte dat het gaat om ontwikkelingsbiologisch homologe strukturen.

6. De ontwikkelingsbiologische basisbegrippen, in het verleden gedefinieerd vanuit een klassieke achtergrond, kunnen bij gebruik in een moderne context leiden tot onnodige diskussie en spraakverwarring.

7. Het beeld van de evolutie als "een rivier die tegen de berg op stroomt" kent behalve "draaikolken" en "stroomversnellingen" mogelijk ook "watervallen".
("De rivier die tegen de berg op stroomt" door W. Calvin, 1990).

8. Het is onterecht dat de vis als proefdier een betere wettelijke bescherming geniet dan wanneer deze dient als consumptievis.
("Inventarisatie-onderzoek naar de methodes waarop palingen, meervallen en forellen in de praktijk worden gedood" door ir. P. Lievense, 1992, Dierenbescherming).

9. Het patenteren van DNA fragmenten is een vorm van plagiaat.

10. De trend van tijdelijke aanstellingen, gekombineerd met een beoordeling op basis van het aantal publikaties, leidt tot een kwaliteitsvermindering van de individuele publikatie.

11. Aftekeningen aan de benen van het moderne rijpaard zouden mogelijk het gevolg kunnen zijn van een ontwikkelingsbiologisch interessante mutatie.

12. De waarde van de officiele erkenning van het beroep "dierfysiotherapeut" (Wet op de Uitoefening van de Diergeneeskunde) zal aanmerkelijk verhoogd worden wanneer dierenartsen goed voorgelicht worden over de (on)mogelijkheden van de fysiotherapie.

13. Bij het vestigen van internationale rekords in de sport speelt het gebruik van de meest geperfektioneerde uitrusting een relatief (te) grote rol ten opzichte van daadwerkelijke verhoging van het biologisch prestatievermogen.

14. Probiotica kunnen werken als anti-biotica.

P. Gevers
Cell fate establishment during early development of cyprinid fishes, with special emphasis on the formation of the primordial germ cells
Wageningen, 14 oktober 1992

Aan mijn ouders

CONTENTS

ABBREVIATIONS

h:	hours
a.f.:	after fertilization
PGC:	primordial germ cell
EVL:	enveloping layer cells
YSL:	yolk syncytial layer
DC:	deep cells
ULC:	upper layer cell
LLC:	lower layer cell
LLM:	lower layer marginal cell
LLN:	lower layer non-marginal cell
LY:	lucifer yellow
LY-D:	lucifer yellow - dextran
R-D:	rhodamin B isothiocyanate - dextran
FC:	fluorescein complexon
ECM:	extracellular matrix
FN:	fibronectin
EM:	electron microscope
XTC-MIF:	*Xenopus* tissue culture - mesoderm inducing factor
FGF:	fibroblast growth factor
TGF ß:	transforming growth factor ß

DEFINITIONS OF DEVELOPMENTAL BIOLOGICAL TERMS

Fate: Prospective feature(s) of the progeny of a cell or cell group.

Commitment: Restriction of cell fates, either reversible or irreversible.

Determination: Sequence of events, resulting in an irreversible restriction of cell fates.

Induction: Transfer of signal(s) from one cell or cell group to another, resulting in determination.

Epigenesis: Development of great diversification of structures or cell types, from eggs with homogeneously distributed cytoplasmic determinants.

Preformation: Cell fate establishment by segregation of ooplasmic determinants before and during early cleavage stages.

Differentiation: Sequence of events, following determination, resulting in cells with a specialized phenotype.

Chapter 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

In this thesis it is studied whether cells, especially the primordial germ cells (PGCs), are already committed to their fate during early cleavage stages of fish embryos. As no evidence for early commitment could be obtained, it was studied during which later stage commitment occurs and which processes may be involved in the establishment of cell fate. This chapter will deal with the following subjects: animals, embryonic development, cell fate, morphogenetic movements, cell interactions, primordial germ cells and the aim of the present investigations.

Animals

The present study is carried out mainly on the rosy barb (*Barbus conchonius*) and partly on the common carp (*Cyprinus carpio*, Chapter 5). Both are fresh water fishes, belonging to the family of Cyprinidae (order Cypriniformes), in which also the zebrafish (*Brachydanio rerio*) is classified. Cyprinids have a single dorsal fin and cycloid scales. Their jaws are toothless. Instead, under the gill cover, behind the gill arches, pharyngeal grinding teeth are present. Cyprinids can be found in nearly every part of the world, being absent only from Madagascar, Australia, New Zealand, South America, southern central America, northern Canada and Alaska, Greenland and Iceland. About 1450 species are known, which vary enormously in their outward appearance. Most species of the genus *Barbus* are non-migratory and confined to one watershed. The original undomesticated rosy barb specifically lives in the fresh waters of about 22°C of Northern India and Bangladesh (see Sterba, 1973).

It is only recently that the teleost fish has attracted attention of developmental biologists and geneticists as a useful model for vertebrate development, due to a number of distinct advantages. Among fishes, much research is performed on the zebrafish. This fish, and the narrowly related *Barbus conchonius*, used in the present study, are inexpensive, relatively easy to rear and breed in laboratory aquaria, and, under optimal conditions, will produce rather large numbers of transparent eggs. Spawning can occur all year round on the morning following reintroduction of the male after a one month separation. Furthermore, the transparency of the embryos and the usually strictly alternating orientation of the cleavage planes form attractive characteristics for cell lineage studies. Another advantage for embryological studies is that the

developmental period until hatching is relatively short (i.e. for *B. conchonius* only 27 hours at 25°C). Thus, developmental processes can be studied within a relatively short period.

Embryonic development

The following descriptions will refer to early development of cyprinids, especially reported by Hisaoka and Battle (zebrafish; 1958), Neudecker (carp; 1976) and Timmermans (rosy barb, 1987). Fig. 1 and 2 are schematical representations of early cleavage stages and several developmental stages until hatching, respectively. Immediately after spawning the outer chorionic membrane will be formed and after fertilization cytoplasm streams towards one side (the animal pole), forming a yolk-poor cytoplasm on top of the yolk. Cell divisions are restricted to the yolk poor cytoplasm, leaving the yolk uncleaved (meroblastic cleavage). Early cleavages are regular in time and occur about every 20 minutes. The first five cleavages are all in a vertical plane, but each one at right angles to the previous one. As demonstrated in zebrafish, all cells form a kind of syncytium until this stage since a membrane between yolk and cleavage cells is absent (Kimmel and Law, 1985a). The 6th cleavage, leading to the 64 cell stage, is the first horizontal division, and, thus, results in two cell layers (Kimmel and Law, 1985a). After this stage cleavage is not perfectly synchronized anymore, but still cleavages follow each other within a relatively short time. Concerning the relationship between the early planes of first cleavage and the plane of bilateral symmetry of the embryo, inconsistency is

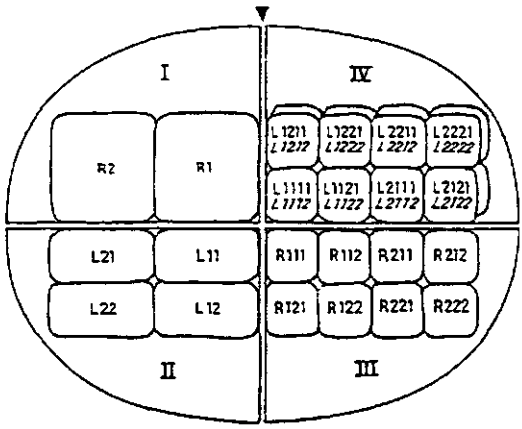

Fig. 1. Schematical drawings of quadrants of cleavage stage embryos of ***Barbus conchonius*** *(upper view). They represent the 8 cell (I), the 16 cell (II), the 32 cell (III) and the 64 cell (IV) stage. The arrowhead points to the plane of first cleavage. The blastomeres are numbered according to their lineage; diagonally opposed quadrant pairs are labeled R and L, respectively. The 64 cell stage embryo consists of two layers of 32 cells, indicated with the standard letter type (upper layer cells) and the italic letter type (lower layer cells). See also Chapter 3.*

reported for the zebrafish (Kimmel and Law, 1985c). This is different in *Xenopus*, where the first cleavage furrow demarcates the dorsal-ventral axis (Klein, 1987).

As described by Kimmel and Law (1985b), after the 9th/10th cleavage a yolk syncytial layer (YSL) is formed by collapse of a subset of descendants of the early marginal blastomeres into the yolk cell. Before formation of the YSL marginal blastomeres appear to be connected with the yolk by cytoplasmic bridges. It is observed in zebrafish that, thereafter, gap junctions represent the connection between marginal blastomeres and yolk via the YSL, which can from now on be considered as the cytoplasmic part of the yolk cell. Kimmel (1989) compared this stage with the midblastula transition of the *Xenopus* embryo since from this developmental stage onwards the cleavage time of cells is prolonged, cells begin to migrate and a number of other morphogenetic processes start (see below).

In addition to the formation of the YSL during the early blastula stage, just before the onset of epiboly, peripheral blastomeres become organized as a one cell thick epithelium, covering the mass of the deeper blastomeres. The space between this enveloping layer (EVL) and the YSL is occupied by the deep cells (DCs), which exclusively will form the embryo proper. According to Bouvet (1976), early EVL cells of *Salmo trutta fario* are capable of delamination by spindle orientation at right angles to the blastoderm surface for a certain period. This results in addition of one daughter cell to the subjacent mass of DCs and a superficial daughter cell to the EVL. Since between the EVL cells of *Salmo gairdneri* junctions of the zonula adherens type have been observed (Lentz and Trinkaus, 1971; Betchaku and Trinkaus, 1978), these outer cells may have a protective function.

In the following period cellular movements start in the embryo and the YSL and overlying EVL undergo epiboly (spreading) to surround the uncleaved yolk mass (Betchaku and Trinkaus, 1978). These authors suggest that epibolic spread is passive for EVL and DCs, occurring in response to pull exerted by the independently expanding YSL. During the first part of epiboly (0%-50% epiboly) DCs are present between YSL and EVL all around the circumference of the embryo. The second part of epiboly starts with gastrulation (50% epiboly). Three directions of cell movement (see Fig. 3) finally result during this second part of epiboly in the presence of germ layers and an embryonic axis (Warga and Kimmel, 1990; see Morphogenetic movements).

When epiboly is completed, gastrulation movements cease and all DCs have migrated dorsally towards the position of the embryo proper

(Warga and Kimmel, 1990). Also the notochord has been formed and somitogenesis will start shortly afterwards. The head will be formed at the side of the animal pole, and the tail at the side of the vegetal pole, at the location of the yolk plug. In *Barbus conchonius* at 15 h a.f. the optic cups and the neural tube are clearly visible. During the next hours the tail becomes gradually separated from the yolk sac. At 27 h a.f. the *Barbus conchonius* embryos leave the chorion (hatching) and arrive at the larval stage. At this stage the eye lens, otic vesicles, mesonephric tubules, heart, blood vessels and myotomes are present.

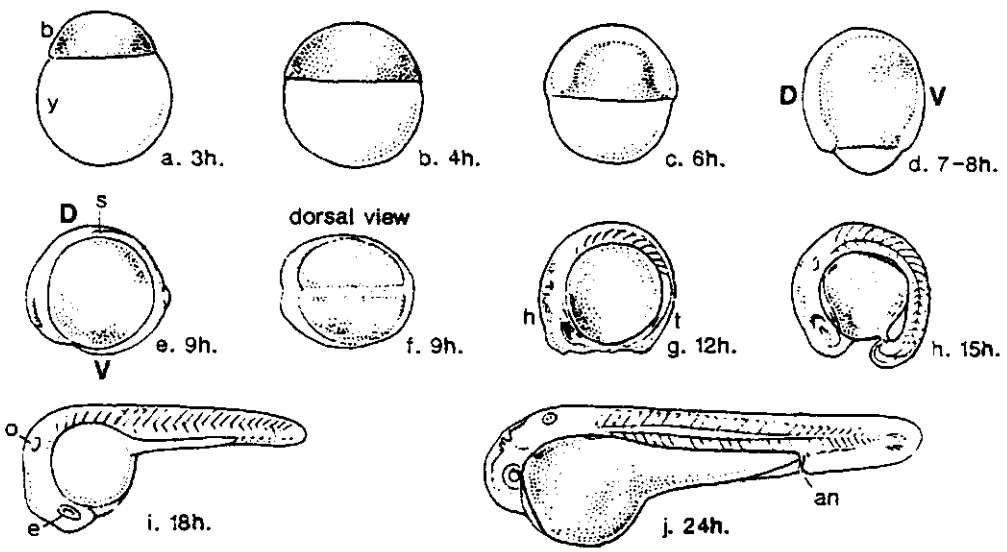

Fig. 2. Embryonic development of ***Barbus conchonius*** *during the first day. (a)-(e), (g)-(j): lateral views. (f): dorsal view. Note that from (e) onwards the dorsal and ventral sides are changed. an, anus, b, blastoderm; e, eye; h, head; o, otic vesicle; t, tail; s, somite; y, yolk (After Timmermans and Taverne, 1989).*

Cell fate

During development of a number of invertebrate groups (nematodes, ascidians) early patterns of cell divisions generate cell lineages that make invariant contributions to the differentiated tissues of the body (Sulston *et al.* 1983; Jeffery, 1985). An isolated blastomere from these types of embryos will generate progeny, following the same developmental pathway as it should have done in the intact embryo. Ablation of blastomeres of such embryos results in individuals, lacking

the tissue which should have been derived from the removed cell. These phenomena are the result of a coordinated and ordered segregation of ooplasmic determinants among different blastomeres prior to or during early cleavage. A striking example of cytoplasmic segregation is the localization of the germ granules of *Caenorhabditis elegans* in the posterior pole of the zygote, from which part the germ cell line originates (Schierenberg, 1988). Because cell lineage is important in specifying cell fate, consistent lineage relationships must occur among the cell types. Thus, if cell interactions occur, they are highly reproducible. This type of development is determinative.

In vertebrates cell determination occurs predominantly according to cell position within the embryo. In the mouse embryo, for instance, inner cells will form the ICM and outer cells form the trophoblast, whereas the inner or outer position is not neccessarily related to a cell's history. Isolated blastomeres from an early cleavage stage can generate a complete new individual (Rossant and Pederson, 1986). Cell lineage apparently is not the primary factor to establish the fates of blastomeres, arising during early cleavage. Instead, interactions between cells play a key role in cell determination, in addition to the history of the cell. This type of development is regulative. Cells are involved in those interactions (inductions) at different developmental stages and at different locations, which makes it necessary to study the migration paths of cells.

Cell lineages must be studied in order to distinguish between a determinative or a regulative type of development. In embryos, exhibiting a regulative type of development, also some determinative elements may be present, and vice versa. Cell lineage studies in vertebrates, using fluorescent probes as markers of individual cells, have been performed especially on *Xenopus* embryos (Dale and Slack, 1987; Moody, 1987) and mouse embryos (Winkel and Pederson, 1988). With respect to teleosts, zebrafish cell lineages have been extensively studied by Kimmel and co-workers (1985a, b, c, 1986, 1987a,b, 1988, 1989, 1990) and Warga and Kimmel (1990).

In general, it is possible that cleavage yields cells that are indistinguishable from each other with respect to potency, and not determined to prospective fate, which may be related to an unpredictable migration path of individual cells. In mouse embryos cell lineage does not restrict the fate of the progeny of cells of the inner cell mass, due to extensive cell mixing (Winkel and Pederson, 1988). Genetic marking of single embryonic mouse cells has demonstrated that such cells frequently contribute to a number of somatic tissues. In *Xenopus* too, cell lineage is not the sole determining factor in the establishment of a cell's fate.

Nevertheless, experiments with these embryos revealed that prospective fates of blastomeres could be roughly mapped at early cleavage stages, generally based on the position within the embryo. However, regional subdivisions can not be mapped to unique non-overlapping regions in the early gastrula, indicating the indeterminate fate of the progeny of at least part of the embryonic cells. As Moody (1987) and Wetts and Fraser (1989) described, this may be due to the limited cell mixing in the *Xenopus* embryo (producing a partly consistent fate map), if compared with mammalian embryos. According to Klein and Jacobson (1990), a relatively low rate of cell mixing in *Xenopus* seemed to be correlated with a reduction in the rate of cell division. If this is true for the mouse embryo, it may be hypothesized that in mouse embryos the rate of cell division is relatively high during the specific stage of cell mixing.

Kimmel and Warga (1987a) described for zebrafish embryos an extensive cell mixing, starting at the onset of epiboly. During the course of embryonic development, cells become allocated in separate lineages that express a stereotyped set of cell fates. It seems likely that in fish embryos clonal restrictions are not present before the late blastula/early gastrula stage because the movements that take place in the late blastula occur without respect for clonal boundaries (Kimmel and Law, 1985a; Kimmel and Warga, 1986, 1987a,b, 1988; Kimmel *et al.* 1988, 1990). Although regular trends in cell lineages of zebrafish exist, single blastomeres can generate quite different cell types, populating various types of tissues at diverse regions of the developing embryo. Thus, blastomeres, identical in cell lineage, can generate progeny of different tissue, located among different positions along the body axis.

Vertebrates apparently exhibit a regulative type of development: their cells have unpredictable fates, until at a specific developmental stage commitment occurs, according to the position of a cell. This conclusion, based on cell tracer experiments, was previously drawn with respect to early fish development by experiments with vital dye marking or movement of chalk particles (Pasteels, 1936; Oppenheimer, 1938; Ballard, 1966a,b, 1973a,b). Since cell lineage is not the sole determining factor, cell interactions must play an important role. Thus, cells must be able to communicate with other cells, both to registrate their own position within an embryonic field and to be involved in induction processes. Therefore, communication mechanisms need to be present.

Morphogenetic movements

Directional mass cell migration, morphogenetic movements, play a key role during early embryogenesis. The migration paths establish the position of a cell, and, especially during the regulative type of development, the cells require position dependent cell interactions, giving them a commitment. There are a number of poorly understood aspects of directional cell movements, such as what starts them, what keeps them going, what gives them directionality, and finally, what let the cells stop their migration movement and establish the definitive position within the differentiating tissue. The mechanism of these morphogenetic movements and the nature of the processes that control and coordinate them, is one of the key problems associated with the establishment of cell fate. According to Kimmel and Law (1985c) morphogenetic movements in the zebrafish start at the onset of epiboly. For the *Xenopus* embryo Newport and Kirschner (1982) reported that morphogenetic cell movements appear to start as the cells have reached a critical ratio of nucleus/cytoplasm.

At some moment during morphogenesis cells must become gradually restricted in fate. In this respect, one of the most important and widely studied cell fate restricting morphogenetic movement is gastrulation, leading to the formation of germ layers. In fish embryos controversy has arisen about the origin of the hypoblast, and the migration path of individual cells during the formation of this cell layer. Pasteels (1936) and Oppenheimer (1938) already studied gastrulation in *Salmo gairdneri* by means of vital staining. Ballard (1966a, b, c, 1973a, b) conducted an extensive series of marking experiments with chalk particles using different teleost species. As a result of his studies Ballard concluded that no involution occurred at the rim of the fish blastodisc, and that the hypoblast arises by delamination and outward migration of centrally placed DCs. Furthermore, he proposed a three dimensional fate map of the fish blastula, accounting for boundary overlap. However, the use of time-lapse filming of living embryos during gastrulation of *Barbus conchonius* revealed that an involution movement (inward migration) of individual cells occurs at the germ ring from 50% epiboly onwards (Wood and Timmermans, 1988). This result is confirmed by Warga and Kimmel (1990) for the zebrafish by cell labelings experiments. They observed that, in addition to gastrulation, convergence movements occur, leading to the formation of the embryonic axis (see also Trinkaus *et al.* 1992). As summarized by Warga and Kimmel (1990) three different directions of cell movements cooperate in order to form the final, dorsally located,

embryo proper with the three different germ layers (Fig. 3): epiboly (vegetally directed mass cell migration), gastrulation (involution) and embryonic shield formation (convergence).

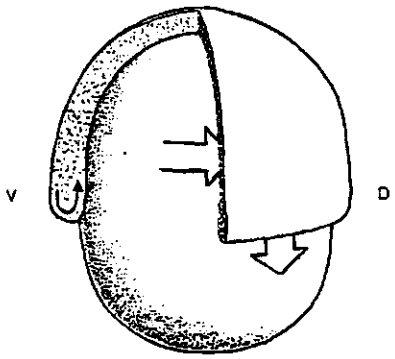

Fig. 3. Schematical drawing of a 60% epiboly stage of a ***Barbus conchonius*** *embryo, showing three different directions of cell movement: vegetally directed mass cell movement (epiboly), gastrulation (involution) and dorsally directed cell movement (convergence), finally resulting in the formation of the embryo proper on the dorsal side. V, ventral side; D, dorsal side.*

Cell interactions

Morphogenesis and differentiation are thought to be mediated by interactions between cells or cell groups during early regulative development. In order to reveal the developmental importance of such interactions, it is first necessary to establish their pattern and timing. Developmental information may be transferred via direct cell to cell communication pathways, such as gap junctions and cytoplasmic bridges, or through ligand-receptor mechanisms at the cell surface.

A specific type of direct cell to cell interaction is the gap junction, which might be involved in the transfer of small molecules (MW below 1200 D; Stewart, 1978). The appearance of these channels has been investigated using the transfer of low molecular weight fluorescent dyes. Gap junctionally coupled cell populations can be considered as partial syncytia (communication compartments) in that they share a common pool of ions, metabolites and small molecules. Junctional communication is thought to be involved in developmental signalling (see Bennett and Spray, 1985): cells require positional information, which may be provided by a gradient of molecules, possibly transferred through gap junctions. Indeed, it was found that in insects groups of gap junctionally communicating cells, communication compartments, were coincident with developmental compartments, identified in cell lineage studies (Weir and Lo, 1982; Blennerhassett and Caveney, 1984). Also in molluscs this appears to be the case (Serras *et al.* 1989, 1990). Thus, restriction in developmental potential may coincide with reduction in gap junctional

communication. In vertebrates the relationship between communication compartments and developmental compartments appears to be less strict (Lo and Gilula, 1979).

Morphogenesis and determination in vertebrates may also be mediated by receptor-ligand interactions, resulting in induction. Mesoderm formation of the *Xenopus* embryo, for instance, is a result of induction of the ectoderm by endodermal cells: XTC-MIF, FGF and TGF ß (activin ß) all appear to exhibit mesoderm inducing capacity (for review, see New *et al.* 1991; McClay, 1991). The mesodermal cells, on their turn, can induce the neural tissue, which results in activation of the phosphoinositol pathway in induced cells (Otte *et al.* 1990).

Another type of receptor-ligand interactions, involved in cell migration and morphogenesis, is mediated by the extracellular matrix (ECM). A large number of studies have demonstrated the importance of ECM in morphogenesis in embryos of several species (see for reviews Hay, 1981a, 1984; Bard, 1990; Trelstad, 1984). Its involvement in a variety of cell processes has been reported, i.e. cell to cell and cell to substrate adhesion, cell migration and cell positioning and modulation of cell shape. The matrix of the ECM is formed by collagens and proteoglycans, which contain a core protein with covalently bound glycosaminoglycan chains and oligosaccharides. Developmentally important molecules of the ECM are collagen, hyaluronic acid (glycosaminoglycan) and the glycoproteins fibronectin and laminin (see Hay, 1981). Some types of collagen are involved in the guidance of cell migration, cell shape and cell polarity (Hay, 1981b). The presence of hyaluronic acid also plays a role in cell migration: it causes a high degree of hydration at the time of onset of cell movement, providing opening of pathways for cell migration. Fibronectin (FN) has generally been found to promote cell-cell adhesion, cell-substrate adhesion, cell migration and cell morphology, and with respect to development, it may mediate cell differentiation processes (Yamada, 1981; Yamada *et al.* 1984). Laminin seems to exhibit a comparable but weaker function and is especially present in basement membranes.

An important number of studies on the developmental role of the ECM is concentrated on FN, which can be studied both *in vivo* and *in vitro*. FN is found in a wide variety of species throughout the animal kingdom (Yamada *et al.* 1984), possibly indicating an important role. FN consists of a series of functional domains, that are specific for particular biological activities: collagen binding domain, heparin binding domain, hyaluronic acid binding domain and a cell binding domain. The existence of separate binding sites suggests that FN may be a central molecule in

the organization and binding of a variety of extracellular or membrane-linked molecules. The cell binding domain of the FN molecule contains a tripeptide sequence Arg-Gly-Asp (RGD; Ruoslahti and Pierschbacher, 1986), which interacts via the integrin cell surface receptor (Buck and Horwitz, 1987; Hynes, 1987) with cytoskeletal elements as actin (Tamkun *et al.* 1986) within the cell.

With respect to vertebrates, in chick and amphibia FN is shown to be involved in the gastrulation process: immunolabeling studies demonstrated that the fibrils covering the inner surface of the blastocoel roof of amphibians and the basal surface of the epiblast of chick embryos contained FN (Boucaut and Darribère, 1983a, b; Harrisson *et al.* 1988). Furthermore, mesodermal cell migration is inhibited by using either anti-FN antibodies, anti-integrin antibodies or the synthetic peptide of the cell binding site of FN, which interferes with the binding of FN to the integrin receptor (Boucaut *et al.* 1984a, b; Boucaut *et al.* 1985; Brown and Sanders, 1991; Darribère *et al.* 1988; Smith *et al.* 1990).

Primordial germ cells

An interesting cell type for the study on cell lineages and determination is the primordial germ cell (PGC), since this type of cells may differ from somatic cells with respect to their way of determination. PGCs will be defined as all early developmental stages of germ cells until female and male gonads can be distinguished from each other. Weismann (1892) first proposed the idea that part of the embryo formed the reproductive material (preformation) and the rest formed the individual. Subsequently he stated that rather than continuity of the germ cells there was a continuity of a substance transferred from parent germ cells to the germ cells of the new individual. He termed this substance "Keimplasma" and proposed that it was responsible for heredity, being uninfluenced by the individual itself (see Eddy, 1975; Nieuwkoop and Sutasurya, 1979). Indeed, in insects and nematodes the unfertilized egg already possesses vegetal pole plasm, which will later be distributed over the pole cells or vegetative cells, giving rise to the germ cell lineage (Nieuwkoop and Sutasurya, 1979). Also in anuran amphibians germinal plasm is present, and will be distributed over four germ cell precursors (Dixon, 1981). Nieuwkoop first reported that Weismann's generalisation was not true: embryos of urodelan amphibians did not show a continuity between germ cells of succeeding generations (preformation), but PGCs arise epigenetically from the mesoderm via induction processes by the endoderm, relatively late during development (see Nieuwkoop and

Sutasurya, 1979). In birds and mammals PGCs also arise epigenetically, and until recently they were first recognized in the embryonic ectoderm (Eyal-Giladi *et al.* 1981; Copp *et al.* 1986). Ginsburg *et al* (1990), however, suggested a mesodermal origin of mouse PGCs. Thus, striking differences seem to exist between a number of species with respect to their germ cell origin and the moment of obtaining germ cell characteristics. Until now the debate is going on which are the characteristics of PGCs and at which developmental stage do they appear.

In all species studied so far the PGCs contain an electron dense material, nuage, from a specific stage onwards (Nieuwkoop and Sutasurya, 1979). Satoh (1974) and Eddy (1975) described nuage as electron dense bodies or granules, not bound by a membrane, often found in close association with the nuclear membrane or clusters of mitochondria. As reported for the *Oryzias latipes* embryo (Cyprinidontidae), the location and ultrastructure of the nuage may depend on the developmental stage of the PGCs (Hamaguchi, 1985). Furthermore, avian and mammalian PGCs are characterized by their high alkaline phosphatase activity or their high glycogen content, respectively.

In fish embryos the origin of the PGCs is as yet unclear because they can not be recognized by alkaline phosphatase activity or glycogen as avian and mammalian PGCs (Timmermans and Taverne, 1989). Although PGCs of carp can be selectively stained with monoclonal antibodies, raised against carp spermatozoa, this occurs only from hatching onwards and not during embryogenesis (Parmentier and Timmermans, 1985). However, according to morphological studies of Timmermans and Taverne (1989) PGCs of *Barbus conchonius* can be light-microscopically recognized at 12 h a.f. by their large size and their location between mesoderm and YSL. At the ultrastructural level nuage was observed by these authors in PGCs of 5 day old larvae. Following of specific cell lineages during embryonic development and identification of nuage-containing cells may give more clarity about the formation and determination of the germ cell line of fish embryos.

Aim of the present investigations

In the present study cell lineages of the teleost fish *Barbus conchonius* have been described, with special emphasis on the origin of the germ cell line.

Chapter 2 describes a morphogenetic study on the different locations of PGCs during early developmental stages. Evidence for their

PGC-identity is given by the presence of nuage, i.e. electron-dense material near the nuclear pores, which is generally known to be specific for germ cells. The location of nuage-containing cells was correlated with their contacts with surrounding somatic cells.

In Chapter 3 cell lineages, especially the germ cell line, are studied by means of microinjection of fluorescent tracers into blastomeres of early cleavage stages. The progeny of the injected cell was followed until 12 h a.f., at which stage the PGCs can be recognized with certainty. The results suggest that cell lineages remain undeterminate during a relatively long period. Since development always results in an ordered arrangement of cells and tissues, there must be a moment in its course at which indeterminate cells become committed to a specific fate.

In Chapter 4 we studied from what stage onwards general cell fate restrictions may occur, since this stage may also be important for the determination of the PGCs. Therefore, the formation of a compartment of a group of dye-coupled cells, indicating a group of gap junctionally communicating cells, was studied, since changes in communication properties between cells or cell groups may be correlated with differences in developmental pathways of cell groups. During gastrulation, developmental pathways between different cell groups may gradually disperse. Knowledge of the control on the uncoordinated migration of individual cells during blastula stages and the directional mass cell migration during gastrula stages may be important for the study on cell fate establishment.

In Chapter 5 we described the involvement of fibronectin in epiboly and gastrulation in another cyprinid fish, *Cyprinus carpio*. Fibronectin is a glycoprotein of the extracellular matrix, generally known to be involved in migration processes and differentiation. It may be involved in the guidance of the migratory hypoblast cells during gastrulation.

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Chapter 2

ORIGIN OF PRIMORDIAL GERM CELLS, AS CHARACTERIZED BY THE PRESENCE OF NUAGE, IN EMBRYOS OF THE TELEOST FISH, *BARBUS CONCHONIUS*

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ORIGIN OF PRIMORDIAL GERM CELLS, AS CHARACTERIZED BY THE PRESENCE OF NUAGE, IN EMBRYOS OF THE TELEOST FISH, *BARBUS CONCHONIUS*

Summary

The presence, location and morphology of cells containing nuage, an ultrastructural characteristic of primordial germ cells (PGCs), is described from the moment of first morphological recognition of PGC-like cells (around 100% epiboly) in embryos of the teleost fish *Barbus conchonius*. Thus characterized cells were studied in relation to their cellular contacts with somatic germ layer cells, possibly involved in the determination of PGCs.

The results show that from the very moment that cells, likely to be PGCs, can be lightmicroscopically identified with morphological and positional criteria (from 10 h after fertilization onwards), they contain nuage near the nuclear envelope, which is a strong indication of their PGC-identity. During the studied period (9 - 12 h and 24 h a.f.) nuage-containing cells seem to translocate from the mesoderm towards the yolk syncytial layer (YSL). These PGCs usually appear not to be directly connected with the YSL but to remain separated from the YSL by one or more endodermal extensions, at least up to 12 h a.f. Also at 24 h a.f. somatic cells separate the PGCs from the YSL.

Key words: embryonic development, primordial germ cell, nuage, ultrastructure, fish.

Introduction

The study on cell fates is of crucial importance for developmental biology. Cell fates can be established either by predetermination or by epigenesis. Induction processes are necessary for the epigenetic commitment of embryonic cells. In several phyla, mainly invertebrates, primordial germ cells (PGCs) become determined during very early development (for review, see Nieuwkoop and Sutasurya, 1979, 1981; McLaren and Wylie, 1983; Eddy, 1984). Due to the presence of germ plasm, an early germ cell determinant, the germ cell origin can even be traced back to the vegetal region of the egg. In vertebrates, however, germ plasm is only observed in eggs and embryos of anuran amphibians. In urodelan embryos the PGCs arise epigenetically from the ventral mesoderm (Nieuwkoop and Sutasurya, 1976; Dixon, 1981; Eddy, 1984; Nieuwkoop, 1991). In birds (Eyal-Giladi *et al.* 1981) and mammals (Copp *et al.* 1986) PGCs were long supposed to arise epigenetically from

embryonic ectoderm (for review, McLaren, 1983; Nieuwkoop, 1991). Ginsburg *et al.* (1990), however, recently suggested a mesodermal origin for mouse PGCs.

A number of studies have been performed on the origin of fish PGCs, but the results are not conclusive (for review, see Johnston, 1951). Both mesodermal and endodermal origins have been reported for fish PGCs. Recently, Hamaguchi (1982) suggested for PGCs of *Oryzias latipes* (Cyprinodontidae) an endodermal origin. The present report describes the origin of PGC-like cells of the cyprinid fish *Barbus conchonius*.

Recently, cell lineage experiments of Gevers *et al.* (1992) suggested an epigenetic origin of all *Barbus conchonius* cell types, including PGCs. Cell labeling experiments indicated that epigenetic commitment of cells may not occur before the onset of gastrulation (Gevers and Timmermans, 1991).

Avian and mammalian PGCs can be distinguished from somatic cells during early development by their high glycogen content and their high alcaline phosphatase activity, respectively (Eddy, 1984). Fish PGCs have never been shown to possess these characteristics during early development (Timmermans and Taverne, 1989). These authors reported for *B. conchonius* that at 12 h after fertilization (a.f.) 18-19 single cells, probably PGCs, have arrived at the mitotically resting stage until proliferation starts at the age of about three weeks. These cells were lightmicroscopically recognized by their size and location between the syncytial layer of the yolk cell (YSL) and the mesoderm, to the left and right of the notochord. Their ^{3}H-thymidine experiments suggested that the mitotically resting stage was probably already reached between mid-epiboly and the early somite stage. At 5 days a.f. a specific germ cell determinant, "nuage", was found to be present in PGCs of *Barbus conchonius*. In a number of species, ranging from Hydra to mammals, this electron dense material appears to be highly specific for germ line cells (Noda and Kanai, 1977; Spiegelman and Bennett, 1973; Eddy, 1975; for review, see Nieuwkoop and Sutasurya, 1979; Eddy, 1984). Hogan (1978) and Satoh (1974) described the presence of "germinal dense bodies" ("nuage") in fish PGCs. Hamaguchi (1982, 1985) reported for *Oryzias latipes* that nuage was present during early gonad formation.

The present report describes an attempt to obtain more information on the origin of PGCs of *Barbus conchonius* using the presence of nuage as a criterion in addition to lightmicroscopic morphological criteria (used in earlier studies), in order to identify PGCs with more certainty. The lightmicroscopical (location, size, staining

intensity of the cells) and ultrastructural (the presence of nuage) parameters were correlated to contacts of the possible PGCs with cells of different germ layers, possibly involved in the determination of the PGCs. The observed characteristics of PGC-like cells until 12 h a.f. were compared with those at the time of hatching (24 h a.f.).

Material and methods

Natural matings between adult specimens were used in order to obtain embryos. Embryos were collected and dechorionated in 0.2% protease (type XIV, Sigma) in Steinberg's medium (see Gevers and Timmermans 1991) and cultured in Steinberg's medium until mentioned stages. Fixation occurred in groups of five at 9 h, 10 h, 11 h, 12 h and 24 h a.f. in Karnovsky's fixative (4% paraformaldehyde, 5% glutaraldehyde in 0.2 M phosphate buffer; pH=7.2; 30 min, room temperature), followed by orientation in 1% agarose (type VII, Sigma), postfixation in 1% osmiumtetroxide (30 min, 0° C), dehydration in a graded series of ethanol and embedding in epon 812. Embryos were staged according to the time of development and the number of somites. Localization of possible PGCs occurred in toluidin blue stained semithin sections, made perpendicular to the rostrocaudal axis. Subsequently ultrathin sections were made using a Reichert Ultracut ultramicrotome. If possible, per stage at least 4 PGC-like cells in at least 2 embryos were ultrathin sectioned. In all stages also endodermal cells were ultrathin sectioned in order to confirm that nuage was not present in these cells. Photographs were made with a Nikon standard lightmicroscope, using a Kodak 100 ASA film or with a Philips EM 201 microscope, using an Eastman KS 1870 film.

Due to the difficulties in recognizing PGC-like cells in the early stages, embryos of 9 h a.f. were fixed in Bouin's fluid, orientated in agarose (type VII, Sigma), dehydrated in a graded series of ethanol, embedded in Paraplast, sectioned at 5 μm and stained with haemalum/eosin. In these sections the presence of PGC-like cells was studied too.

Results

Cells, which had a large size, stained dark with toluidin blue, and located between endodermal and/or mesodermal cells, were identified as PGC-like cells. As determined in the epon sections, the size of the PGCs of all studied stages varied between 16 μm and 24 μm, which is especially

in the later stages larger than most somatic cells. Furthermore, at the earliest stages the different staining quality between PGC-like cells and somatic cells was less clear. PGC-like cells could be distinguished from endodermal cells because the latter were always in close contact to the YSL, in contrast to the former. Nuage was only found in cells determined as PGC-like cells with these lightmicroscopical criteria. Except the presence of nuage, the ultrastructure of the PGCs did not differ from the somatic cells (see legend fig. 2B).

Since PGC-like cells are easier recognized in elder stages, we started our study at 24 h a.f. (28-34 somites). At that stage PGCs were located between the mesonephric tubules and the YSL, in close connection with surrounding somatic cells (Fig. 1A, B, D). Nuage was present near the nuclear envelope with mitochondria in close vicinity (Fig. 1C). In some cases the nucleus was extremely lobular.

At 12 h a.f. (7-10 somites) the PGCs were located in between the endodermal cells, while in three cases only very thin extensions of endodermal cells separated them from the YSL (Fig. 2A, B, D). In one case direct contact between the PGC and YSL was observed, while in another single case a PGC and the YSL were separated by a complete layer of endodermal cells. Dorsally, the PGCs contacted the mesoderm. The distance between the notochord and the PGC-like cells varies within one embryo and between the embryos. At the ultrastructural level nuage was observed around the nuclear envelope (Fig. 2C), indicating the identity of these cells.

At 11 h a.f. (2-4 somites) PGC-like cells were located alike 12 h a.f., although the endodermal extensions (one or two), separating the PGC from the YSL, were distinctly thicker (Fig. 3A, B, D, E). In only 1 embryo (4 somites) a small tip of a PGC was directly connected to the YSL. In all cases nuage was present near the nuclear envelope (Fig. 3C).

At 10 h a.f. (0 somites) only three recognizable PGC-like cells could be identified and ultrathin sectioned, although one embryo was semithin sectioned completely. These cells contained nuage and were located relatively laterally within the mesodermal cell layer, contacting endodermal cells only ventrally (Fig. 4A, B, C, D).

In all stages as a control endodermal cells, in close contact with the YSL, were studied ultrastructurally. Some of these cells, especially those at 10 h a.f., had only slight characteristics of PGC-like cells. In none of them nuage was observed (not shown).

We also sectioned at 11 h and 10 h a.f. three laterally located PGC-like cells (comparable staining, size and morphology as previously described cells) showing cell division. However, nuage could not be

recognized, possibly due to chromatin condensation and the absence of the nuclear envelope.

At 9 h a.f. lightmicroscopical recognition of PGC-like cells was not yet possible, and searching for nuage-containing cells without recognizing them by lightmicroscopy was not undertaken.

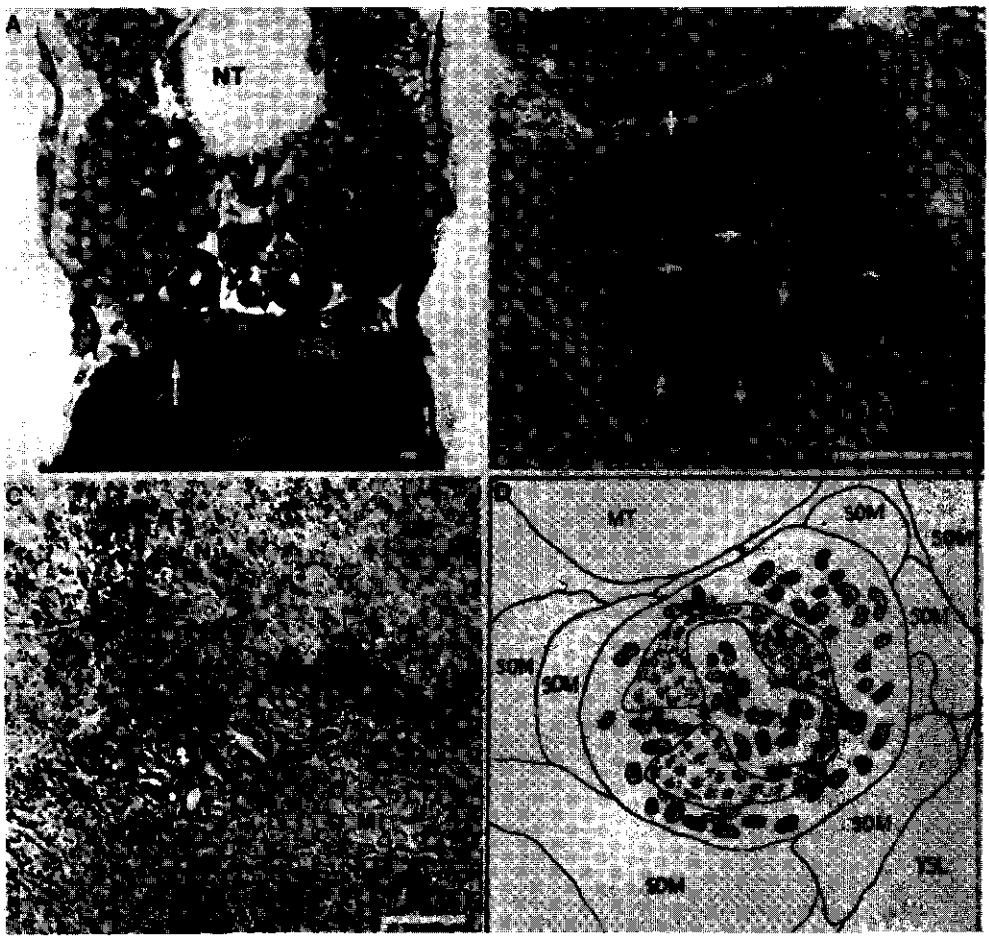


Fig. 1. A PGC at 24 h a.f. (A). Lightmicroscopical view. Arrow points to PGC. Scale bar: 10 μm. (B). EM view. In this case the nucleus was extremely lobular. Arrows indicate location of nuage. Brackets indicate the position of Fig. 1C. Scale bar: 10 μm. (C). Detail of Fig. 1B, containing nuage (arrows). Scale bar: 1 μm. (D). Drawing of Fig. 1B, showing the contacts between the PGC and the somatic cells. The drawing is composed by using large magnifications of the PGC-like cell. I, intestine; M, muscle tissue; MT, mesonephric tubule; NT, notochord; NU, nucleus; Y, yolk; YSL, yolk syncytial layer; SOM, somatic tissue; Mi, mitochondria.

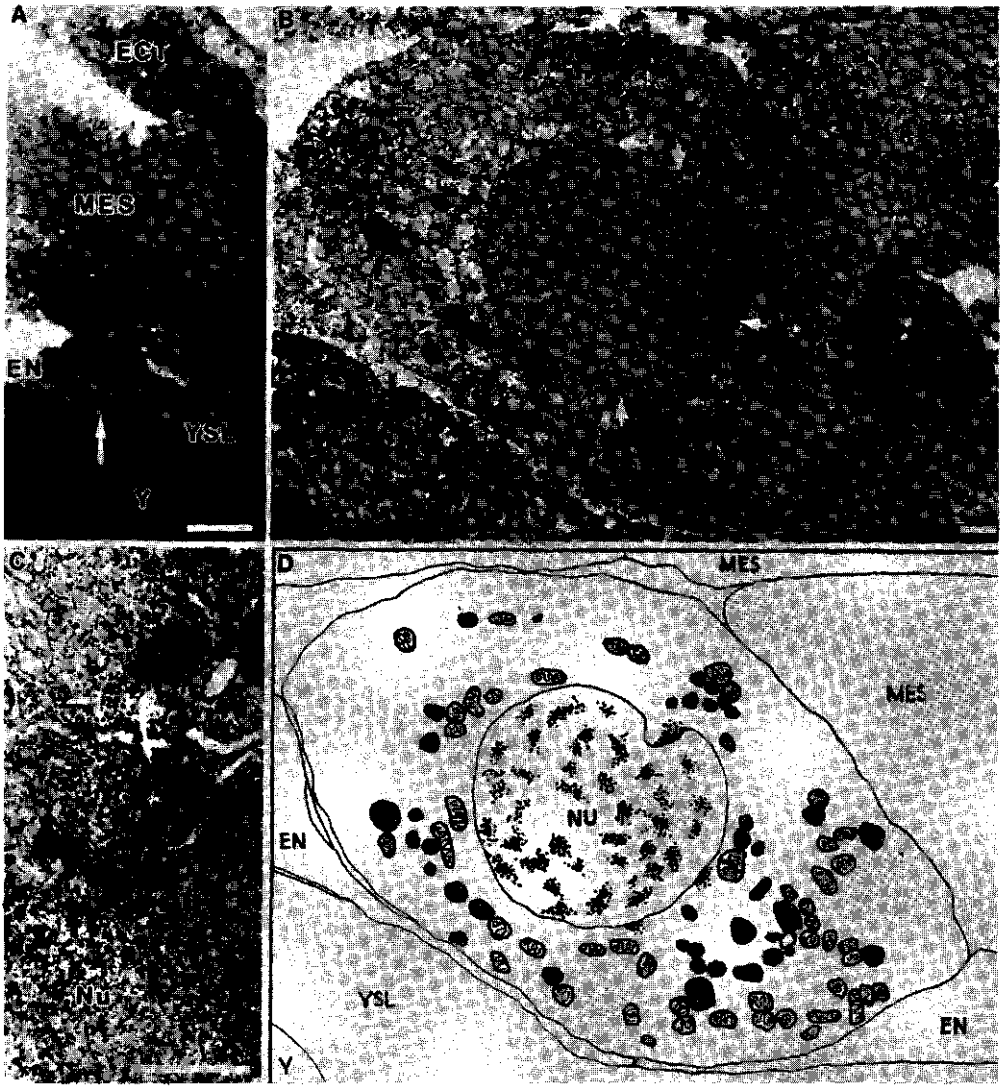
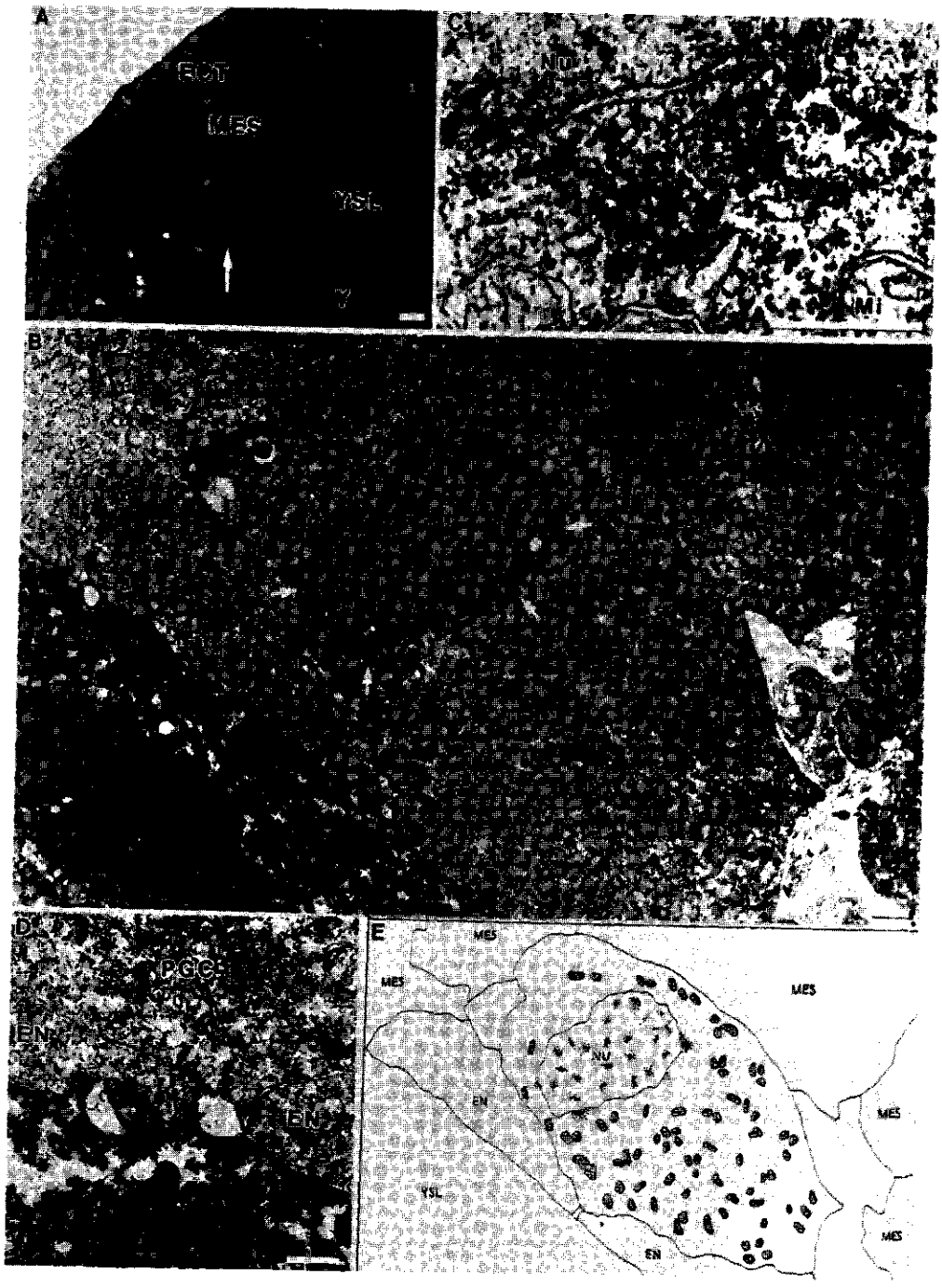


Fig. 2. A PGC at 12 h a.f. (A). Lightmicroscopical view. Arrow points to PGC. Scale bar: 10 μm. (B). EM view. Arrows indicate the location of nuage. Brackets indicate the position of Fig. 2C. Except the presence of nuage, PGCs do not differ from somatic cells with respect to their morphology. As in somatic cells many mitochondria, numerous ribosomes, some golgi areas and ER can easily be found. Yolk-like bodies (arrowheads) are found as well in some somatic cells as in some PGCs, independent on their developmental stage. Scale bar: 1 μm. (C). Detail of Fig. 2B, containing nuage (arrows). Scale bar: 0.5 μm. (D). Drawing of Fig. 2B, showing the endodermal extension between the PGC and the YSL. The drawing is composed by using large magnifications of the PGC-like cell. ECT, ectoderm; EN, endoderm; MES, mesoderm. Other abbreviations as described before.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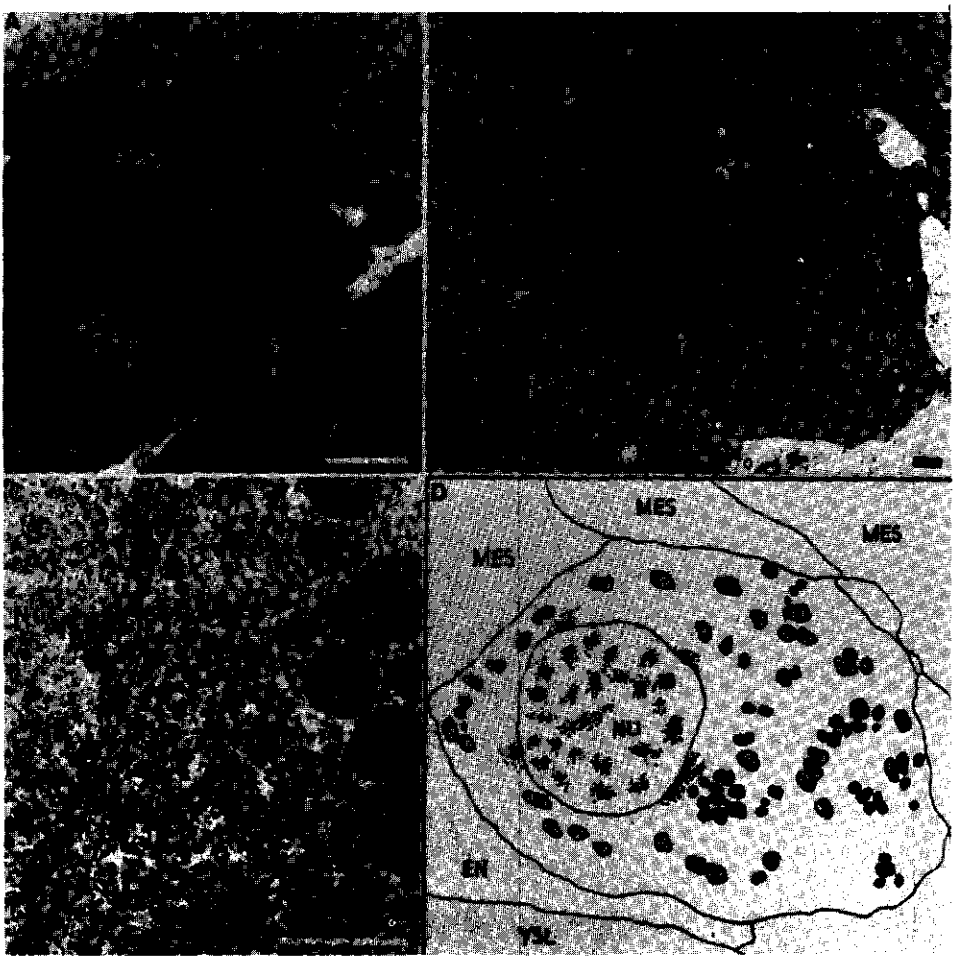

Fig. 4. A PGC at 10 h a.f. (A). Lightmicroscopical view, showing its location within mesoderm. Arrow points to PGC. Scale bar: 10 μm. (B). EM view. Arrows indicate the location of nuage. Brackets represent the position of Fig. 4C. Scale bar: 1 μm. (C). Detail of Fig. 4B, containing nuage (arrows). Scale bar: 0.5 μm. (D). Drawing of the PGC of Fig. 4B, showing the presence of endoderm between YSL and PGC-like cell. The drawing is composed by using large magnifications of the PGC. Abbreviations as described before.

Fig. 3. (pg 39) A PGC at 11 h a.f. (A). lightmicroscopical view. Arrow points to PGC. Scale bar: 10 μm. (B). EM view. Arrows represent the location of nuage. Brackets indicate the location of Fig. 3C and Fig. 3D, respectively. Scale bar: 1 μm. (C). Detail of Fig. 3B containing nuage (arrows). Scale bar: 0.5 μm. (D). Detail of Fig 3B, showing the separation between PGC and YSL by two endodermal cells on both sides. Scale bar: 0.5 μm. (E). Drawing of the PGC and its contacts with somatic cells, as photographed in Fig. 3B. The drawing is composed by using large magnifications of the PGC. Abbreviations as described before.

Discussion

The present report describes the identification of PGC-like cells by an electronmicroscopical criterion (presence of nuage) in addition to lightmicroscopical criteria (size, location, staining intensity). The latter data are in agreement with previous data (Timmermans and Taverne, 1989). However, whereas Timmermans and Taverne (1989) could distinguish the PGC-like cells from the 10 somite stage onward, in the present study they were distinguished already, and shown to contain nuage, around 100% epiboly. The somewhat smaller size of the PGCs observed by Timmermans and Taverne (1989) probably is due to shrinkage.

Nuage appears to be specific for germ cells (Eddy, 1984). In fish embryos nuage, also described as "electron opaque substance", "germinal dense bodies" or "mitochondria-associated granular material", is reported to be present in several stages of germ cells (Hogan, 1978; Satoh, 1974; Hamaguchi, 1985). Therefore, the presence of nuage was used to identify the PGCs with more certainty, which is necessary in order to study the germ cell origin. Conclusive proof, however, about the identity of PGC-like cells can only be obtained by transplantation and reproduction experiments, which in fishes was not possible hitherto. It is not clear whether nuage has a germ cell specific function. The granular materials of *Xenopus* are thought to be composed of nucleolar material extruded in the cytoplasm via the nuclear pores (Ikenishi and Kotani, 1975). Experiments of Clérot (1979) with carp embryos also indicated nuclear compounds to be present in nuage.

We selected PGC-like cells to be ultrathin sectioned by lightmicroscopical criteria. The slight difference with somatic cells at very early stages may result in insufficient recognition of PGCs to allow selection for ultrathin sectioning. At 10 h a.f. (no somites) only three nuage-containing cells could be identified. PGC-like cells which were more difficult to distinguish from the somatic cells, did not contain nuage. Possibly, the appearance of the lightmicroscopical characteristics are correlated with the presence of nuage. At 9 h a.f. we were not able to identify nuage-containing cells by lightmicroscopical criteria. Because it is not possible to screen all embryonic cells for the presence of nuage, PGCs may nevertheless be present at this early stage. Therefore, these studies make not clear whether nuage-containing cells, e.g. PGCs, arise epigenetically. Furthermore, because a high comparibility exists between nuage and nuclear material (Ikenishi and Kotani, 1975; Clérot, 1979), we were uncertain whether dividing cells, lacking the nuclear envelope,

contained nuage.

Between 10 h - 12 h the cellular contacts of PGCs change by a shift in location of the cells. First they are surrounded by mesodermal cells with endodermal connections at the ventral side (10 h); later they are located between endodermal cells with mesodermal connections at the dorsal side (12 h). At 24 h a.f. PGCs were found within the somatic tissue between the mesonephric tubules and the YSL.

We can conclude that as soon as PGC-like cells can be lightmicroscopically identified with morphological and positional criteria, they possess nuage. At least until 12 h a.f. the most important difference between nuage containing cells and endodermal cells was that all endodermal cells were directly connected with the YSL, while the PGCs usually were separated from the YSL by an endodermal extension.

The PGC migration path may indicate that they arise epigenetically from the mesoderm and translocate ventrally, remaining separated from the YSL by an endodermal extension. A number of contrasting reports about the origin of fish PGCs are present (reviewed by Johnston, 1951). However, due to the lack of early recognition of PGCs, it is still not clear whether there is only one type of origin in all fishes, or that the PGCs of different fish groups arise in different germ layers. In the amphibians the origin is different for the orders of urodeles and anurans. This led Nieuwkoop and Sutasurya (1976) to the proposal of a polyphyletic origin of the amphibians from different groups of the osteichtyan fishes. Since the osteichtyans form a rather diverse group, a different origin of PGCs for different orders of fishes might be expected too.

At least between 9 and 12 h a.f. the translocation of *Barbus conchonius* PGCs is exactly the reverse of that reported by Hamaguchi (1982) for *Oryzias latipes*. He reported for successive stages a translocation from the subendodermal space, where PGCs could be first recognized, towards the mesoderm. However, since Hamaguchi (1982) could recognize PGCs only from stages just before and during early gonad formation, his observations may involve later developmental stages than ours. Possibly, also in *Barbus conchonius* the PGCs translocate before and during early gonad formation from endodermal towards mesodermal tissue. Since it is not clear whether the somatic cells, surrounding the PGCs at 24 h a.f., have a mesodermal or an endodermal origin, we don't know whether between 12 and 24 h a.f. the direction of translocation of PGCs of *B. conchonius* is still the reverse of the direction described for PGCs of *O. latipes*. The transitional contact of PGCs to endodermal cells might be necessary for their normal development. Because in one case a PGC contacted the YSL, it needs to be elucidated

whether and at what moment between 12 h and 24 h a.f. the PGCs become directly connected with the YSL. In that case the YSL also might be involved in PGC differentiation.

Acknowledgements

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Chapter 3

A STUDY ON CELL LINEAGES, ESPECIALLY THE GERM CELL LINE, IN EMBRYOS OF THE TELEOST FISH, *BARBUS CONCHONIUS*

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Roux's Arch. Dev. Biol., *in press*

A STUDY ON CELL LINEAGES, ESPECIALLY THE GERM CELL LINE, ON EMBRYOS OF THE TELEOST FISH, *BARBUS CONCHONIUS*

Summary

Lucifer Yellow-Dextran labeling of lower layer cells (LLC), sometimes together with upper layer cells (ULC), of the 64 cell *Barbus conchonius* embryo resulted in labeled primordial germ cells (PGCs) at 12 h after fertilization (a.f.) in about 25% of the cases. The presence of labeled PGCs was independent on the location of the injected blastomere with respect to the later orientation of the embryonic axis. After injection of an ULC alone, however, labeled PGCs were never found. Also the distribution of labeled somatic cells differed between the ULC and LLC injected embryos. In the case we found fluorescent PGCs always only a few of them were labeled, suggesting that either a single predecessor exists earlier than the 64 cell stage or that the formation of germ cells is a polyclonal process.

Tracing the fluorescent cells at successive stages of development shows an extensive cell mixing with unlabeled cells during the epiboly stage, which might well be the cause of partly unpredictable cell lineages. The chance to be committed to a specific fate is different for the ULC and LLC descendants. This might be due to relatively limited cell mixing between these two cell populations.

Keywords: cell lineage, primordial germ cell, fish development

Introduction

The issue of cell fate has long been a topic of interest in developmental biology. Both cell lineage and cell interactions may be involved in the establishment of a cells fate (by predetermination and epigenesis, respectively). In a number of species (mainly invertebrates), primordial germ cells (PGCs) become determined during very early development, suggesting the relative importance of predetermination for these cells (for review, see Nieuwkoop and Sutasurya, 1979; McLaren and Wylie, 1983; Eddy, 1984). In vertebrates, however, only anuran amphibians seem to possess an early germ cell determinant. In urodelan, avian and mammalian embryos the PGCs appear to arise epigenetically from a region in the ventral mesoderm (urodeles; Nieuwkoop and Sutasurya, 1976, for review, see Nieuwkoop and Sutasurya, 1979; Dixon, 1981; Eddy, 1984) or from the epiblast (birds and mammals; Eyal-Giladi *et al.* 1981; Copp *et al.* 1986; for review, see McLaren, 1983).

Conflicting ideas have arisen about the origin of fish germ cells

because, in contrast to avian and mammalian PGCs, they can not be recognized by their high alkaline phosphatase and glycogen content (Eddy, 1984; Timmermans and Taverne, 1989). However, EM studies have shown that at 5 days after fertilization (a.f.) electron dense germ cell specific material or "nuage" (Nieuwkoop and Sutasurya, 1979; Eddy, 1984), is present in PGCs of *Barbus conchonius* (Timmermans and Taverne, 1989). At earlier stages PGCs (mean number: 18-19 per fish) can only be recognized by their large size and their location between mesoderm and YSL. Timmermans and Taverne (1989) could distinguish these cells from 12 h a.f. onwards, shortly after the onset of somitogenesis. Recently, Gevers *et al.* (in press) reported the presence of nuage in these cells from 10 h a.f. onwards, supporting evidence that these cells are PGCs indeed.

Kimmel and coworkers (1985a,b,c, 1986, 1987, 1988, 1990) and Warga and Kimmel (1990) studied cell lineages in zebrafish (Cyprinidae) in order to define fate maps of early developmental stages. They reported in 1986 and 1990 that predictable cell lineages arise at gastrula stage. In 1987 they found that detailed cell fates vary considerably before epiboly (for review, see also Kimmel and Warga 1988). The fact that cell fates were unpredictable was ascribed to uncoordinated movements of the undetermined blastomeres before gastrulation.

Since Kimmel and Warga (1988) only described the appearance of label in somatic cell types, it remains possible that, in contrast to somatic cells, germ cells are determined early in development. Moreover the variance in cell fate might be diminished by taking the orientation of the embryonic axis into account. Moody (1987) described for *Xenopus* embryos that the fates of identified 16 cell stage blastomeres were predictable if embryos were preselected for specific cleavage patterns.

In order to identify cell lineages, especially the germ cell line, we injected the cell lineage tracer Lucifer Yellow-Dextran (LY-D) into early cleavage blastomeres of *Barbus conchonius*, and followed the progeny until 12 h a.f., at which time the germ cells can be identified (Timmermans and Taverne, 1989; Gevers *et al.*, to be published). The presence of fluorescent PGCs at that developmental stage was correlated with the location and fate of labeled somatic cells and with the orientation of the embryonic axis (angle with first cleavage plane) that can be recognized by that time. For identification of the blastomeres to be injected, we used a numerical method, as previously proposed by Kimmel and Law (1985a), since the early cleavage pattern up to the 64 cell stage of *Barbus conchonius* is, to our vision, identical to that of the zebrafish.

Material and methods

Embryos

Freshly laid eggs were obtained from natural matings between adult specimens of the Rosy Barb, *Barbus conchonius* L. (Cyprinidae, Teleostei). The adult fishes were fed on Trouvit pellets (Trouw and Co, Putten, The Netherlands) and kept under a 12 h light - 12 h dark regime. Spawning and fertilization occurred at dawn in small tanks. Embryos were collected from the bottom and transported to Steinberg solution (60 mM NaCl, 0.7 mM KCl, 0.8 mM $MgSO_4$, 0.3 mM $Ca(NO_3)_2$; pH=7.4). Development occurred 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 be partly removed with a hairloop, followed by rinsing for two times in Steinberg medium, until the chorion is lost. Developmental capacity after dechorionation was compared to developmental capacity of untreated embryos, and appeared to be equal or nearly equal.

Dye injections

Dye was injected into one of the blastomeres after the completion of the 2nd, 3rd, 4th, 5th or 6th cleavage. At the 6th cleavage two layers of blastomeres are formed, the upper layer cells (ULC) and the lower layer cells (LLC), the latter consisting of marginal blastomeres (LLM) and non-marginal blastomeres (LLN). Because it is technically very difficult to label a LLN, these blastomeres were labeled by injection of their sister ULC before completion of the 6th cleavage. Furthermore, we injected dye into the YSL (just after its formation at 4 h a.f.) of 5 embryos, in order to find out whether labeled cells could arise from the YSL. We used pressure injections as well as iontophoretic injections (applied with a 40 nA current in 5 Hz pulses during 5 minutes). In order to prevent light damage, the illumination was interrupted during injection and kept as low as possible during subsequent development. Electrodes were pulled from Clark Electromedical Instruments capillaries (GC 150F-15) with a DKI electrode puller. Tip diameter, as measured by SEM, was between 1-5 μm. The tip was backfilled with 5% Lucifer Yellow - Dextran (LY-D; Molecular Probes; M_r =40,000), dissolved in either 0.3 M KCl (pressure injections) or in 0.3 M LiCl (iontophoretic injections). As a control, also Rhodamin B isothiocyanate - Dextran (R-D; Sigma; M_r=38,900), dissolved in 0.3 M KCl, was injected into blastomeres of the 64 cell stage. The electrode shank was filled with 0.3 M KCl or 0.3 M LiCl. Cell penetrations were visualized with a 6.3x epifluorescence objective on a Zeiss microscope, supplied with a 50W Mercury epilight source. In the case of pressure injections, injections

were ended before a visible volume expansion could be observed. Embryos were photographed with a 400 ASA Kodak Ectachrome film or a 400 ASA Kodak Black/White film.

Optical examinations

To identify each injected blastomere, and to determine its position and, at three different stages, that of its progeny relative to the future embryonic axis i.e. embryonic shield, embryos were immobolized during development in 1% agarose (type IX, Sigma) with their blastomeres orientated upwards (according to the method described by Wood and Timmermans, 1988). The agarose block was supplied with buffer and mounted between coverslips with a perspex spacer. We determined whether the embedding procedure has a retarding effect on development. Only a slight retardation was observed.

Fluorescence microscopy

In order to establish the location of the fluorescent cells exactly, LY-D injected embryos were fixed 12 h a.f. in 2% formaldehyde in Steinberg solution (12 h; 4°C), rinsed in Steinberg solution, embedded in 1% agarose type VII (Sigma; according to the method of van der Wal and Dohmen, 1978), dehydrated in a graded series of ethanol and embedded in Technovit. Blocks were serially sectioned at 5 μm with an LKB Historange microtome, perpendicular to the embryonic axis. Fluorescent cells were visualized with a Zeiss microscope equipped as described above. Moreover, the distribution of fluorescent somatic cells over head-, trunk- and tail region was studied in the sections and it was established for each part whether these cells were present in the enveloping layer, ectoderm, mesoderm or endoderm layer. Only the presence of fluorescent blastomeres was recorded for each of these parts and they were not quantified. Photographs were made using a 400 ASA Kodak Ectachrome film or 400 ASA Kodak Black/White film.

Results

General development

The first five cleavages of the *Barbus conchonius* embryo, following each other about every 20 minutes, are in a vertical plane, each one at right angles to the previous one. The sixth cleavage (64 cell stage; about 2.5 h a.f.) is the first horizontal cleavage and, thus, creates two layers of blastomeres, which will be named upper layer cells (ULC) and lower layer cells (LLC), respectively. A drawing of an upper view of early cleavage stages is given in Fig. 1., and blastomeres of one quadrant are numbered according to their lineage. Occasionally variant cleavage

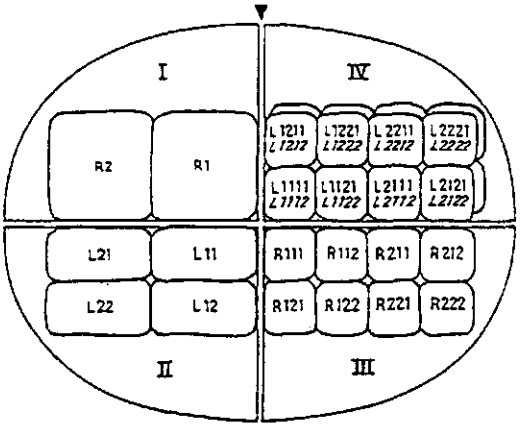


Fig. 1. Schematical drawings of quadrants of cleavage stage embryos of ***Barbus conchonius*** *(upper view). They represent the 8 cell (I), the 16 cell (II), the 32 cell (III) and the 64 cell (IV) stage. The arrowhead points to the plane of first cleavage. The blastomeres are numbered according to their lineage. Diagonally opposed quadrant pairs are labeled R and L, respectively. The 64 cell stage embryo consists of two layers of 32 cells, indicated with the standard letter type (upper layer cells) and the italic letter type (lower layer cells). See also Kimmel and Law (1985a).*

patterns were observed, but these embryos were excluded from the experiments. About 4 h a.f. (approximately 1000 cell stage) the yolk syncytial layer (YSL) arises below the blastomeres and the epiboly process starts. At 9 h a.f. epiboly is completed, three different germ layers are present and somitogenesis starts. At 12 h a.f. PGCs are present as single cells between the mesoderm and the YSL, in the region of the first somites, and to the left and right of the notochord (Timmermans and Taverne, 1989).

Developmental capacity of dechorionated and agarose embedded embryos

Twenty embryos were dechorionated in the 4-8 cell stage. Ten of them were embedded, orientated in agarose and placed in Steinberg solution while the other ten embryos were directly placed in Steinberg solution. Another ten embryos, used as controls, were placed in Steinberg medium with intact chorion. The development was checked at the appearance of the first somite at about 9.5 h a.f. No significant difference in developmental stage was observed between the dechorionated embryos and the control embryos. In 7 out of 10 and 8 out of 10 embryos, respectively, the formation of the first somite could already be observed. The development of embryos that were dechorionated as well as agarose embedded, however, was slightly retarded if compared with the control group: only in 1 embryo the formation of the first somite could be observed. However, in the other embryos epiboly was completed, and the first somite was formed shortly afterwards. All embryos appeared to be normal and all were alive at the swimming larvae stage. No morphologic abnormalities were observed.

Correlation between the first cleavage plane and the direction of the embryonic axis

Twenty embryos were dechorionated and orientated in agarose and the orientation of the first cleavage plane was established. Another 20 embryos, not dechorionated nor embedded, served as controls and developed normally. At 9 h a.f. the direction of the embryonic axis (i.e. embryonic shield) was established. In 11 embryos (55%) the direction of the embryonic axis was correlated with the orientation of the first cleavage plane. In 4 embryos (20%) the two orientations were at right angles to one another. The other 25% of the embryos showed relationships in between.

Injections with LY-D into early cleavage stages

We injected LY-D into the 2, 4, 8, 16, 32 and 64 cell stage. Until the 32 cell stage, LY-D injections resulted in labeling of a group of blastomeres due to spreading of the dye from the injected blastomere to adjacent blastomeres (Fig. 2A, B). From the 64 cell stage onwards, when the embryo consists of two layers of blastomeres, the label remained restricted to the injected blastomere (Fig. 2C, D; ULC), or spread to adjacent blastomeres (Fig. 2E, F; LLC). After injection of LLM blastomeres the spreading was observed to one or more adjacent LLM or LLN blastomeres, whereas in a few cases also an adjacent ULC received label. We used the 64 cell stage to start cell lineage studies.

Cell lineages from the 64 cell stage onwards

Injection of LY-D into one of the blastomeres of the 64 cell stage always resulted after a few cleavages in a compact cluster of labeled descendants (Fig. 3A, B). During epiboly the fluorescent descendants of the labeled blastomere(s) became extensively mixed with unlabeled cells (Fig. 3C, D). At 12 h irreproducible and widely disparate patterns were observed (Fig. 3E, F) concerning the location of the fluorescent cells, the cell types they represented, and their position along the rostrocaudal axis. At that stage the labeled cells were exactly localized in sectioned material. Since the LY-D might be damaging, we ascertained by interference microscopy that the labeled cells were still alive. A detail of LY-D containing blastomeres is shown in Fig. 4A, B. Furthermore, as a control we also photographed a detail of the progeny of R-D injected blastomeres (Fig. 4C, D). Although those labeled cells appear to be alive, we can never be sure that the behaviour of the fluorescent progeny was normal.

First of all we injected 31 embryos with LY-D into ULC (Table 1). These injections never resulted in labeled germ cells. Then we injected LLC, LLM as well as LLN. LLN were labeled by injection before the 6th

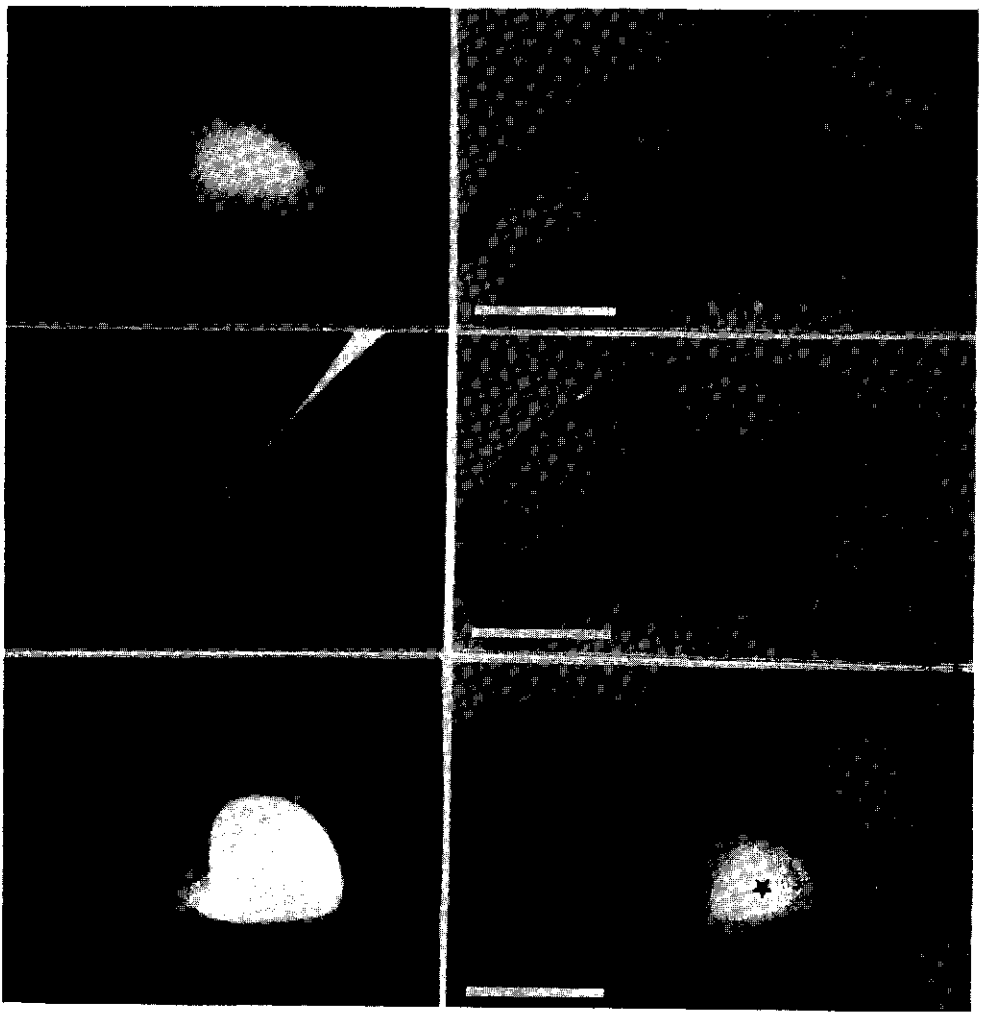
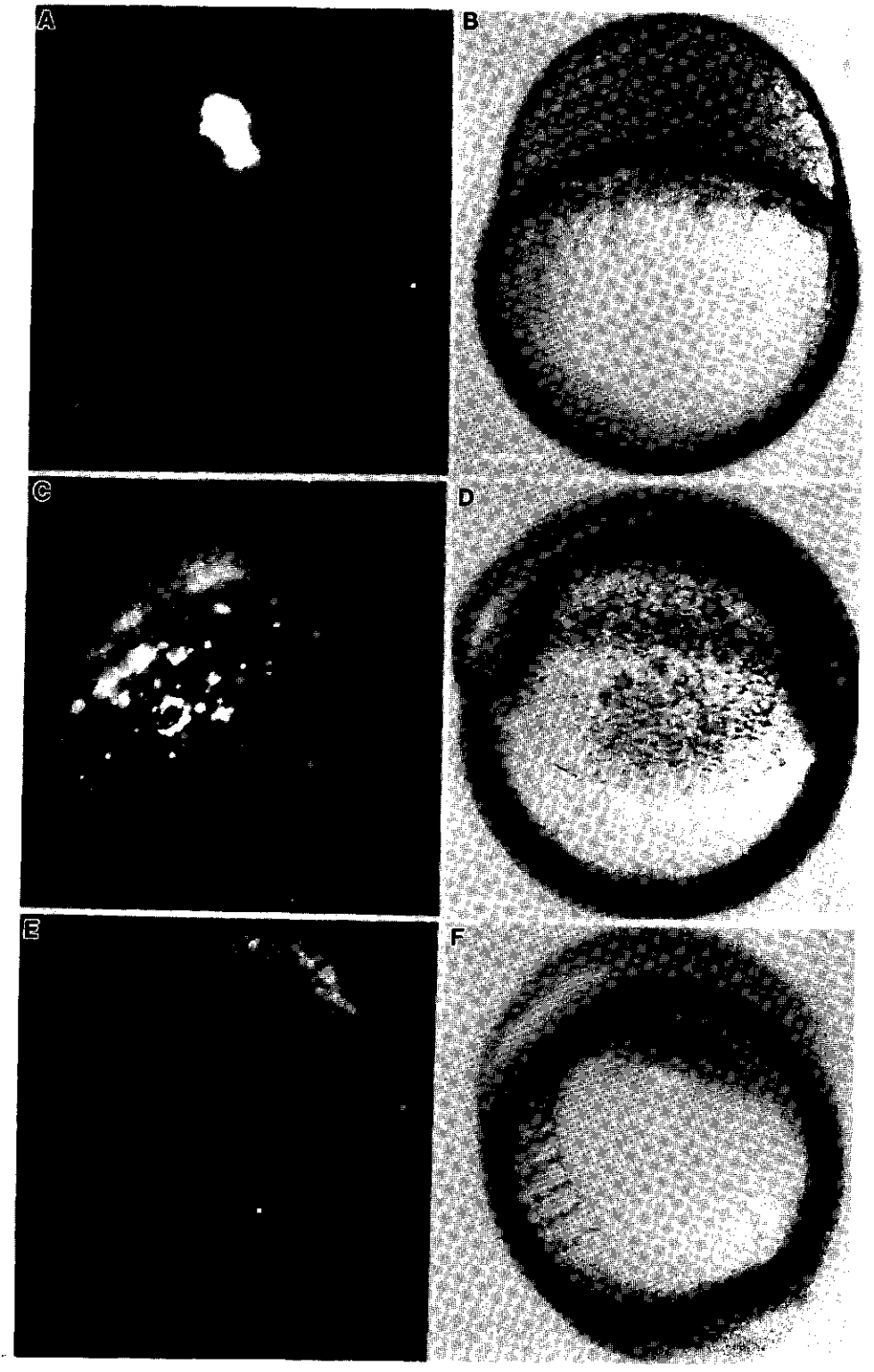


Fig. 2. (A). Fluorescence picture of a living 16 cell stage embryo, injected with LY-D into blastomere 21, (indicated with the asterisk in (B)), located near the margin of the one layered blastoderm. The label is transferred to adjacent blastomeres. (B). Bright field picture of the same embryo as (A). Scale bar: 100 μm. (C). Fluorescence picture of a living 64 cell stage embryo, injected with LY-D into an ULC, indicated with the asterisk in (D). The label remains restricted to the injected blastomere. (D). Bright field picture of the same embryo as (C). Scale bar: 100 μm. (E). Fluorescence picture of a living 64 cell embryo, just after injection of LY-D into a marginal blastomere, indicated with the asterisk in (F). The fluorescence is not restricted to the injected blastomere because at this developmental stage marginal blastomeres appear to have cytoplasmic connections with adjacent blastomeres. (F). Bright field picture of the same embryo as (E). Scale bar: 100 μm.

cleavage was completed, resulting in labeling of the LLN with its corresponding ULC. No dye-spread was observed to other blastomeres. The LLM injected embryos showed dye-spread from the injected blastomere to one or more adjacent blastomeres, so the labeled cells, found at later stages, could derive from a couple of blastomeres of the 64 cell stage. In all cases the LLM injection also resulted in a weakly fluorescent YSL. Injection with LY-D into the YSL itself (just after its formation at 4 h a.f.), however, never resulted in labeled blastomeres (not shown). Table 1 presents the dye-spread (due to cytoplasmic connections) to adjacent blastomeres, the number of labeled germ cells at 12 h a.f., and (in LLM injected embryos) the orientation of the embryonic axis and the relative position (left, right, head end, tail end) of the injected blastomere. Only in 6 out of 28 cases (LLM) and in 6 out of 15 cases (LLN) we found a part of the PGC population labeled (PGC-positive; Fig. 5A, B). Fig. 5C shows a haemalum/eosin stained section of a Bouin fixed embryo with a similarly located PGC.

In the LLM injected embryos the relative location of the injected cell could be established at 9-12 h a.f. when the rostrocaudal orientation of the embryonic axis was visible. Table 1 shows that the orientation of this axis in 3 of the 6 PGC-positive embryos was parallel to the plane of first cleavage. When viewing from dorsal, in one embryo the location of the injected blastomere was to the right side, in two embryos to the left side. Furthermore, Table 1 shows that in two of the PGC-positive embryos the embryonic axis was perpendicular to and in one embryo at an angle of 45° with the plane of first cleavage; in one of them the location of the injected blastomere was at the head end and in two embryos at the tail end. Thus, all directions of the embryonic axis are represented in the PGC-positive as well as in the PGC-negative LLM injected embryos (Table 1). Consequently, we were not able to predict the position of labeled cells at 12 h a.f., even not after identifying the relative position of the injected blastomere. Since this was so, we did

Fig. 3. (pg 55). (A). Fluorescence picture of a blastula stage of a living embryo (3.5 h a.f.), injected with LY-D at the 64 cell stage. The labeled blastomeres can be observed as a coherent cluster. (B). Bright field picture of the same embryo as (A). Scale bar: 100 μm. (C). Fluorescence picture of a 50% epiboly stage of a living embryo (6.5 h a.f.), injected with LY-D at the 64 cell stage. The fluorescent cells have started migration movements and become mixed with unlabeled cells. (D). Bright field picture of the same embryo as (C). Scale bar: 100 μm. (E). Fluorescence picture of a living embryo, 12 h a.f., injected with LY-D at the 64 cell stage. The labeled cells are extensively mixed with unlabeled cells. In this case most of the labeled cells are located in the future head. Because of the thickness of the embryo a lot of cells are out of focus. (F). Bright field picture of the same embryo as (E). Scale bar: 100 μm.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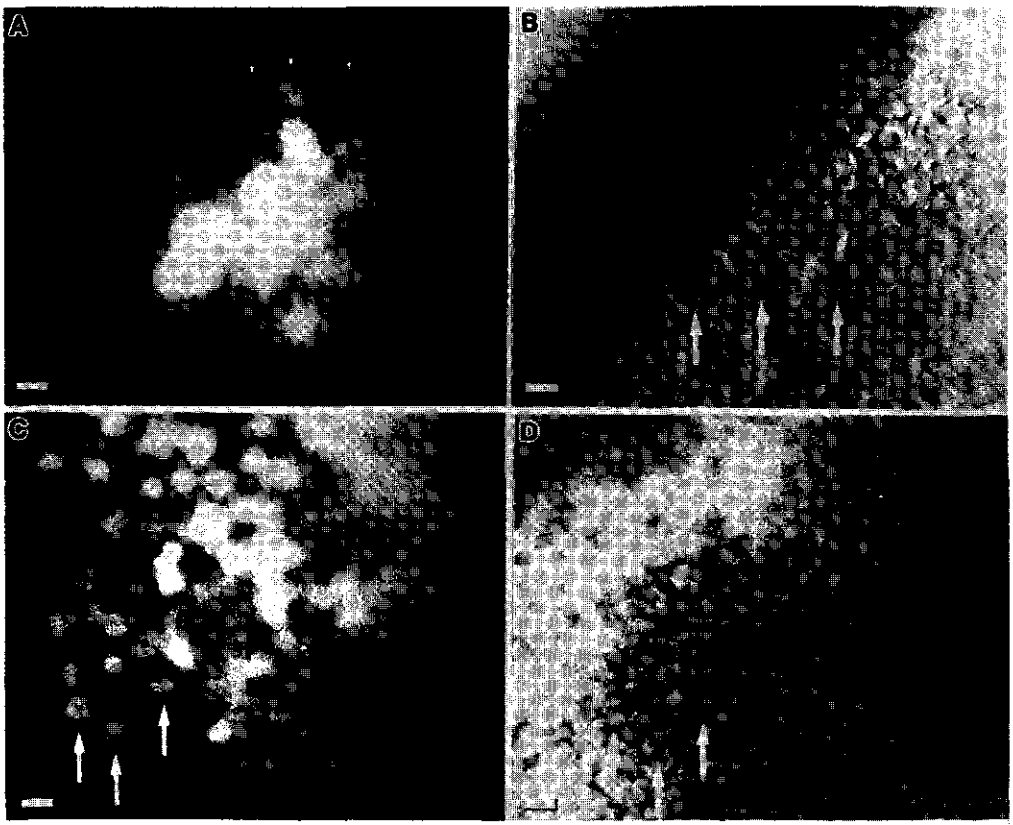
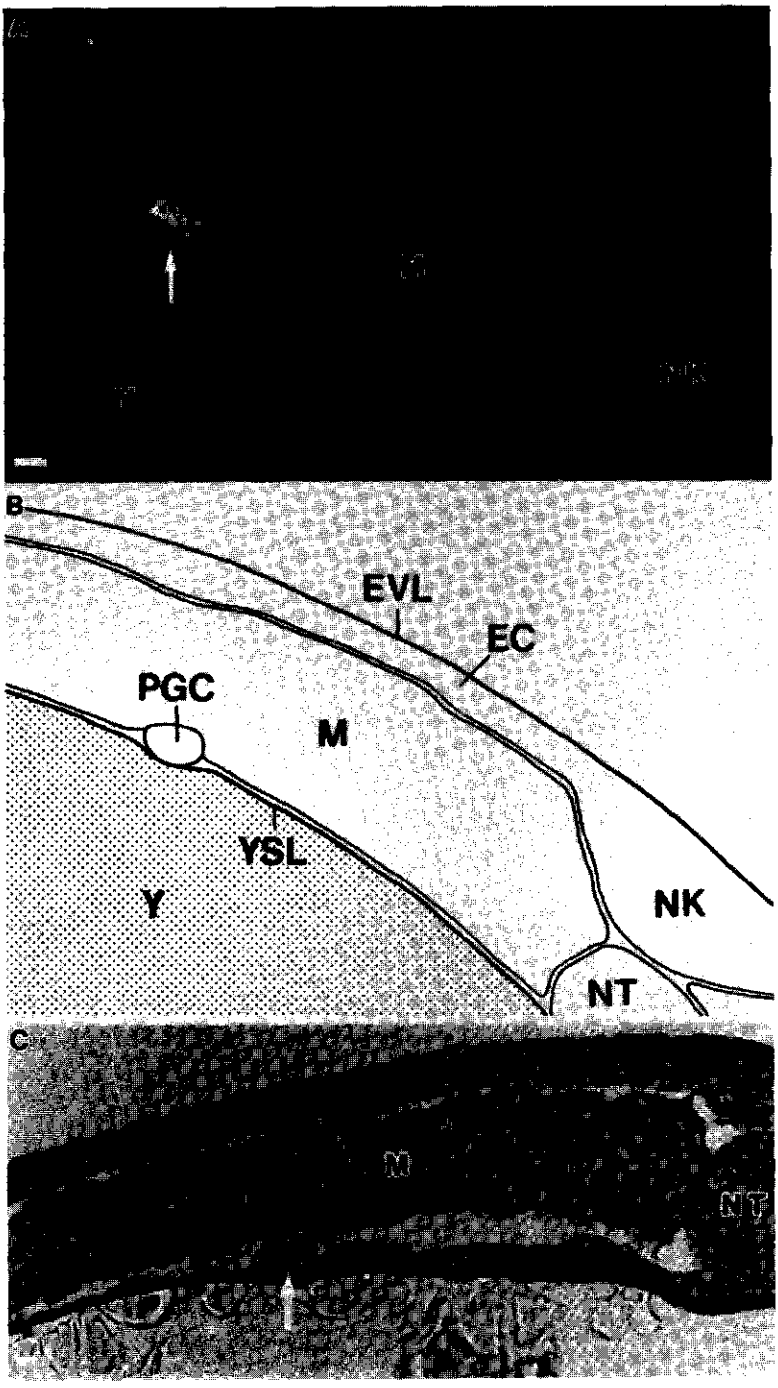


Fig. 4. (A). Fluorescence picture of a detail of LY-D containing cells at 20% epiboly. Three labeled cells are indicated with the arrows. Scale bar: 20 μm. (B). Nomarski picture of the same embryo but at higher magnification. The three arrows show the cells, marked in (A). Scale bar: 10 μm. (C). Fluorescence picture of R-D labeled cells at 50% epiboly. (D). Nomarski picture of the same embryo as in (C). The three arrows show the same cells as indicated in (C). Scale bars: 20 μm.

Fig. 5. (pg 57). (A). Cross section of a 12 h embryo, injected at the 64 cell stage with LY-D. A bright labeled PGC (arrow) can be observed between the mesodermal cells (M) and the yolk syncytial layer. NK, neural keel. Scale bar: 10 μm. (B). Drawing of the same section. Y, yolk, EVL, enveloping layer; EC, ectoderm; M, mesoderm; PGC, primordial germ cell; YSL, yolk syncytial layer. (C). Haemalum/eosin stained section of a Bouin fixed 12 h embryo, in which a PGC is present with a comparable location as in (A). Scale bar: 10 μm.


B
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not establish this correlation for the LLN injected embryos.

Furthermore, as evident from Table 2, we did not see a difference in the distribution of labeled somatic cells between the PGC-positive and the PGC-negative part of one injection group. However, there is a difference in the distribution of labeled somatic cells between the ULC, LLM and LLN injected groups (Table 2). ULC injected embryos more often showed ectodermal labeling and less often endodermal labeling if compared with the LLM injected embryos. The LLN injected embryos, in which always ULC were labeled (due to the injection method), showed characteristics of both the ULC and the LLM group. More LLN injected embryos (100%) were observed to possess labeled ectodermal cells, if compared with the LLM group (about 80%). However, if compared with the ULC group, more LLN embryos were observed with endodermal labeling (about 26% and 80% for the ULC and LLN group, respectively).

Discussion

The present results lead to the conclusion that the germ cell lineage of a 64 cell stage *Barbus conchonius* embryo is indeterminate. However, the germ cells appear to develop from the LLC, since injection of only ULC never resulted in labeled germ cells. For the LLM injected embryos we in fact studied the location of the progeny of a couple of blastomeres, because the dye spreads to adjacent blastomeres. The dye-spread of LLM injected embryos is probably caused by transport via the yolk cell because the formation of a membrane between the blastomeres and the yolk cell is not yet completed. This has been shown indeed to be the case in zebrafish (Kimmel and Law, 1985a). Injection into the LLN remained restricted to the injected blastomere and its upper neighbour as in LLN the membrane between the yolk cell and the blastomeres may be already present. In some cases the LLM showed dye spread to the LLN, while the reverse direction was never observed.

In this study LY-D was used as a cell lineage marker for its bright fluorescence and its good preservation in histological sections. As a

*Table 1. (pg 59). The table shows the numbers of the injected blastomeres and the dye-spread to adjacent blastomeres after injection. Also the numbers of labeled PGCs, the orientation of the embryonic axis (with respect to the plane of first cleavage) and the injection site, correlated with the embryonic axis, are shown. The different orientations of the embryonic axis are presented in both the embryos with labeled germ cells, as in those without. LLM, lower layer marginal blastomeres. LLN, lower layer non-marginal blastomeres. ULC, upper layer cells. HS: head end. TS: tail end. R: right. L: left. //: in the same plane as the first cleavage plane. *: perpendicular to the first cleavage plane. 45°: angle between the first cleavage plane and the embryonic axis. n.d.:not determined.*

EMBRYOS, INJECTED WITH LUCIFER YELLOW-DEXTRAN AT THE 64-CELL STAGE.

Number of embryos	Injected cell	Dye spread to adjacent cells ULC	LLM	LLN	Labeled PGCs (total number)	Embryonic axis related to 1° cleavage plane	Injection side correlated with embryonic axis
				ULC			
5	2121	-	-	-	-	n.d.	n.d.
8	2111	-	-	-	-	n.d.	n.d.
6	1121	-	-	-	-	n.d.	n.d.
8	1111	-	-	-	-	n.d.	n.d.
1	2221	-	-	-	-	n.d.	n.d.
2	2211	-	-	-	-	n.d.	n.d.
1	1221	-	-	-	-	n.d.	n.d.
				LLM			
1	2122	+	+	-	2(12)	//	R
1	2122	-	+	-	1(21)	*	TS
1	2122	-	+	+	5(16)	//	L
1	2122	-	+	-	4(17)	45°	TS
1	2122	-	+	-	3(26)	//	L
1	2122	-	+	+	2(14)	*	HS
2	2122	-	+	+	-	//	L,R
2	2122	-	+	+	-	45°	TS,TS
4	2122	-	+	-	-	//	R,L,L,R
4	2122	-	+	-	-	*	HS,HS,TS,HS
4	2122	-	+	-	-	45°	HS,HS,TS,TS
3	2122	+	+	-	-	//	L,R,L
1	2122	+	+	+	-	45°	HS
1	2122	+	+	+	-	//	R
1	2122	+	+	+	-	*	TS
				LLN			
6	LLN	+	-	-	2,2,3,1,1,3 (n.d.)	n.d.	n.d.
9	LLN	+	-	-	-	n.d.	n.d.

LUCIFER-YELLOW DEXTRAN LABELING OF SOMATIC CELLS *

Number of embryo	Injection at 64-cell stage	Distribution at 12h a.f. EVL	Ecto-derm	Meso-derm	Endo-derm
31	ULC, PGC$^-$	100%	100%	90%	26%
6	LLM, PGC$^+$	100%	83%	100%	83%
22	LLM, PGC$^-$	100%	82%	100%	83%
6	LLN, PGC$^+$	100%	100%	100%	83%
9	LLN, PGC$^-$	100%	100%	100%	66%

* Percentage of embryos with labeled cells in a specific germ layer.

Table 2. Percentage of embryos, in which the different locations of labeled somatic cells at 12 h a.f. are represented after LY-D injection into one of the blastomeres of the 64 cell stage. Only the presence of labeled cells within a germ layer is noted and not the number of labeled cells. So, 100% ULC/EVL means that in 100% of the ULC injected embryos labeled EVL cells were present. Independent on the injected blastomere in all cases some EVL cells were labeled, which is represented as 100% in every injection group. For the LLM and LLN injected embryos the location of somatic labeled cells can be compared between the labeled and unlabeled PGC subgroup. No significant difference can be detected. However, there is a difference between the ULC and the LLM group with respect to the ectodermal and endodermal labeling. The LLN injection group shows characteristics of both the ULC and the LLM injection group.

control R-D was used to present evidence that similar pictures were obtained, indicating that living cells were studied.

According to Timmermans and Taverne (1989) cells could be identified as PGCs at 12 h a.f. because of their position and size. Recently, an early germ cell constituent, nuage, could be observed during early somitogenesis at the ultrastructural level in cells with a comparable morphology, size and location (Gevers *et al.*, to be published), providing evidence for their PGC identity. In a number of species the presence of nuage in germ cells is reported (for review, see Nieuwkoop and Sutasurya, 1979; Eddy, 1984). However, definitive proof, showing nuage and a cell lineage marker in one and the same cell, still awaits elucidation.

We found labeled PGCs in about 25% of the LLC injected embryos. In these embryos only a part of the PGC population showed fluorescence. This implies that if there is only one predecessor, it arises

before the 64 cell stage. Alternatively, the ontogeny of germ cells might be a polyclonal process. By means of genetic experiments Walker and Streisinger (1983) calculated five pregonial cells to be present in the 1000 cell stage of the zebrafish.

Even after establishing the relative orientation of the embryonic axis, we were not able to identify a constant progenitor blastomere of the germ cells because the location of the injected blastomeres, giving rise to PGCs, varied in relation to the orientation of the embryonic axis. The results suggest that at the 64 cell stage the germ cells do not have a progenitor, committed to form the PGCs, but the PGCs later arise epigenetically. The chance to be committed to PGC or a somatic fate appears to differ for the ULC and LLC.

The impossibility of predicting cell fates of the 64 cell stage embryo is due to irregular patterns of cell movement during the epiboly stage. This uncoordinated blastomere motility, which impairs fate mapping, was previously reported for *Brachydanio rerio* (Kimmel and Warga, 1987; Kimmel *et al.* 1990), *Salmo* (Ballard, 1973), *Catostomus* (Ballard, 1982), *Fundulus* (Trinkhaus and Lentz, 1967), *Oryzias* (Kageyama, 1977) and *Notobranchius* (Lesseps *et al.* 1979).

However, the cell fate of the 64 cell stage embryo is not completely unpredictable since ULC injections never resulted in labeled germ cells in contrast to LLC injections. Furthermore, ULC injections showed more embryos with ectodermal labeling while LLM injections resulted in more embryos with endodermal labeling (Table 2). These results suggest that the mixing between the descendants of the ULC and LLC is limited if compared to the cell mixing between the descendants of only one blastomere layer. Since the LLN injections always resulted in a labeled LLC and corresponding ULC this group of embryos showed somatic labeling patterns intermediate between the ULC and the LLM injected group (Table 2). Cell fate correlated with cell depth was also proposed by Ballard (1973, 1982), suggesting that LLN indeed can give rise to endoderm. Kimmel and Warga (1987, 1990), however, reported for the zebrafish a completely indeterminate cell lineage at the 64 cell stage and LLM as possible endoderm precursor. Our results suggest that LLM as well as LLN can give rise to endoderm, but the chance to be committed to endoderm differs for the progeny of these cell groups.

Kimmel and Warga (1986), Kimmel *et al.* (1990) and Warga and Kimmel (1990) reported that specific cell lineages in zebrafish arise at gastrula stage (after cell mixing). Also Gevers and Timmermans (1991) suggested for *Barbus conchonius* reproducible cell fates from 50% epiboly (onset of gastrulation) onwards. Probably cells will be committed to their fate dependent on the location at the onset of gastrulation. Because this

location is apparently not completely unpredictable, the chance to be committed to a specific fate may be different for the ULC and LLC progeny.

Acknowledgements

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Chapter 4

DYE-COUPLING AND THE FORMATION AND FATE OF THE HYPOBLAST IN THE TELEOST FISH EMBRYO, *BARBUS CONCHONIUS*

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Development 112, 431-438 (1991)

DYE-COUPLING AND THE FORMATION AND FATE OF THE HYPOBLAST IN THE TELEOST FISH EMBRYO, *BARBUS CONCHONIUS*

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; Lee *et al.* 1987; Serras *et al.* 1989a; 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 molecular weight 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, 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-involuted 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 by 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 have not 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 *Barbus conchonius* 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 gastrula stage. Therefore, we have studied in *Barbus conchonius* the correlation between the appearance of a dye-coupled group of cells, whether or not a real compartment, and cell fate.

Material and methods

Embryos

Adult specimens of the Rosy Barb, *Barbus conchonius* (Cyprinidae, Teleostei) were kept in 200 liter tanks at 25 °C, fed on Trouvit pellets and kept at 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 solution (60 mM NaCl, 0.7 mM KCl, 0.8 mM $MgSO_4$, 0.3 mM $Ca(NO_3)_2$) 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 molecular weight dye Lucifer Yellow CH (LY; Sigma; di-Li-salt; M_r=457), dissolved in 0.3 M 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 weight dye LY-dextran (LY-D; Sigma; M_r=40,000) 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 50W 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 Steinbergs 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 Historange 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 artefacts. 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 Steinbergs 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 50% epiboly (6 1/2 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.

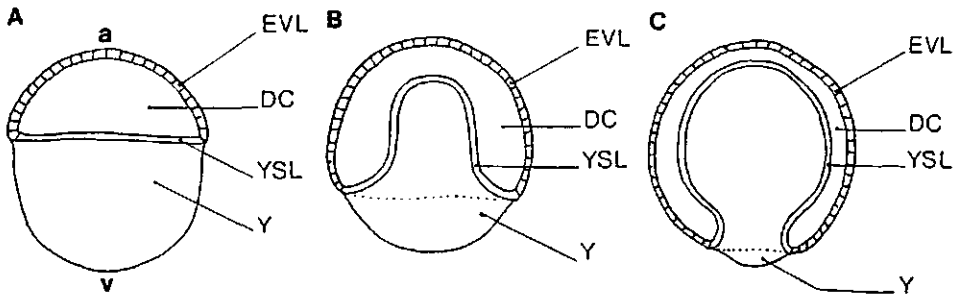
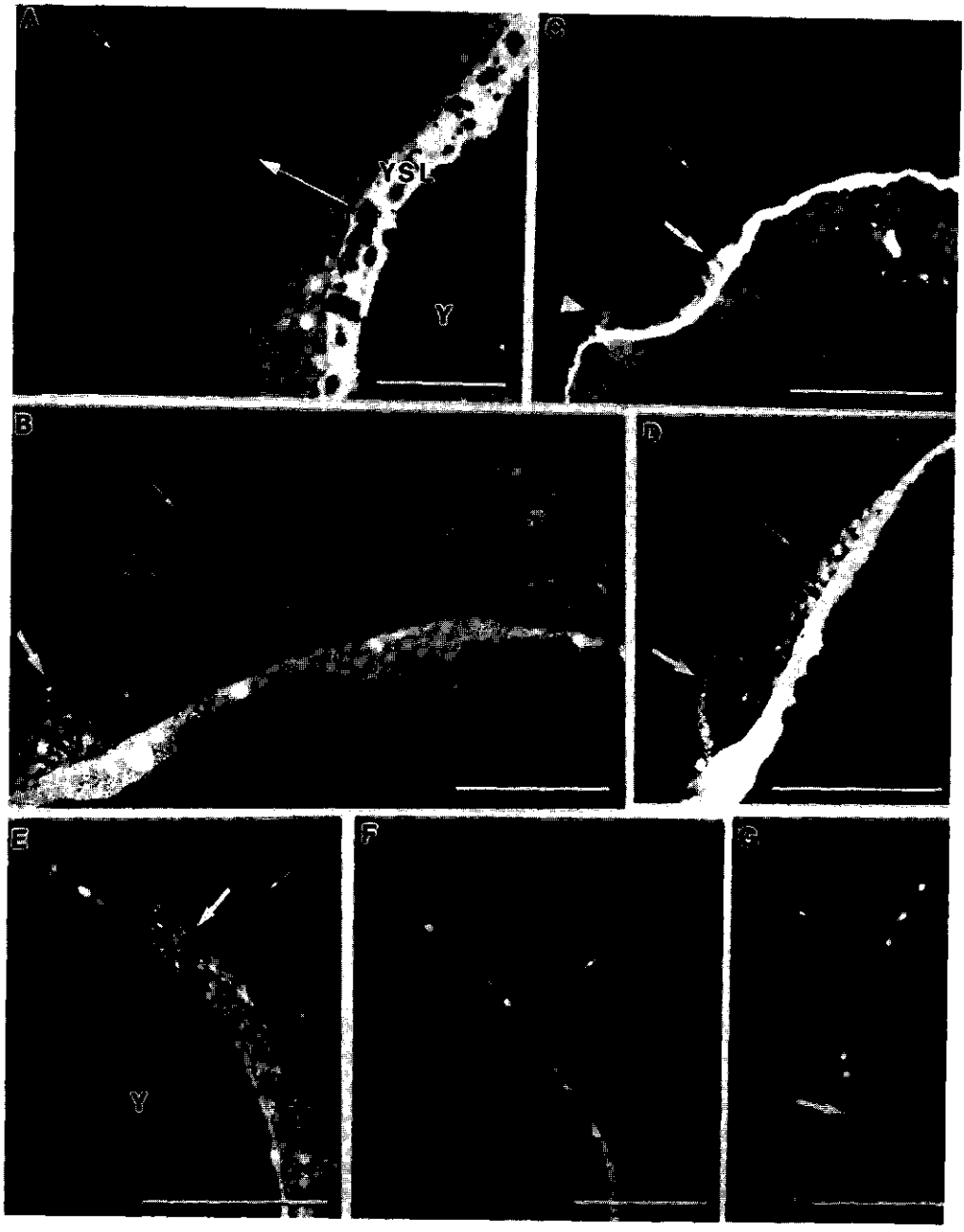


Fig. 1. Schematical drawing of median sections of three different stages of development. (A). The 4 h stage, shortly before the onset of epiboly, just after the formation of the YSL. (B). The 60% epiboly stage. (C). The 90% epiboly stage. DC, deep cells; EVL, enveloping layer; YSL, yolk syncytial layer; Y, yolk; a, animal side; v, vegetal side.

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 most bright 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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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 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%

Figs. 2A-G (pg 72) show 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), showing distinct labeling 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 1/2 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. This section, made perpendicular to the direction of epiboly, shows a part of the labeled ring. In contact with the YSL a group of labeled cells is present (thick arrow). The presence of individually labeled cells in the epiblast is probably caused by cell mixing, which is not yet completely finished at the time of injection. Y, yolk. Scale bar: 100 μm. (F). Injection at 60% epiboly (7 h a.f.) and fixation at 80% 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. (G) 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.

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.

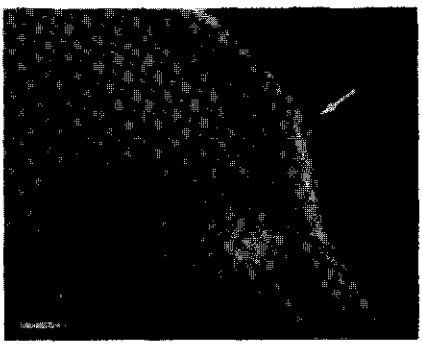


*Fig. 3. Embryo of **B. conchonius** in toto, injected with FC at the 40% epiboly stage and fixed at the 60% epiboly stage. The arrow indicates the presence of a labeled group of cells, at the same location as found after LY injections. The high background is due to the fact that this photograph is a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

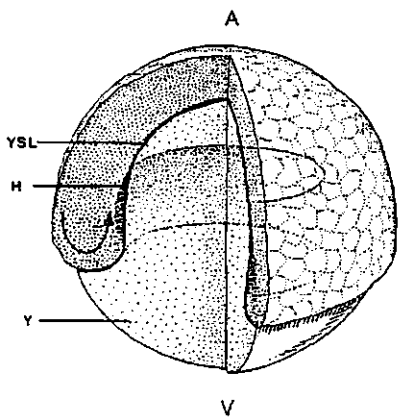


*Fig. 4. Three-dimensional image of a **Barbus conchonius** embryo at 60% epiboly, shortly after the formation of the ring-shaped cell group, the hypoblast (H). It is clear that at the animal side (A) of the fluorescent cell group a sharp boundary is present, while the vegetal side (V) shows a more diffuse boundary. The arrow indicates the involution movement of the cells within the germ ring. Y, yolk; YSL, yolk synsytial layer.*

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),

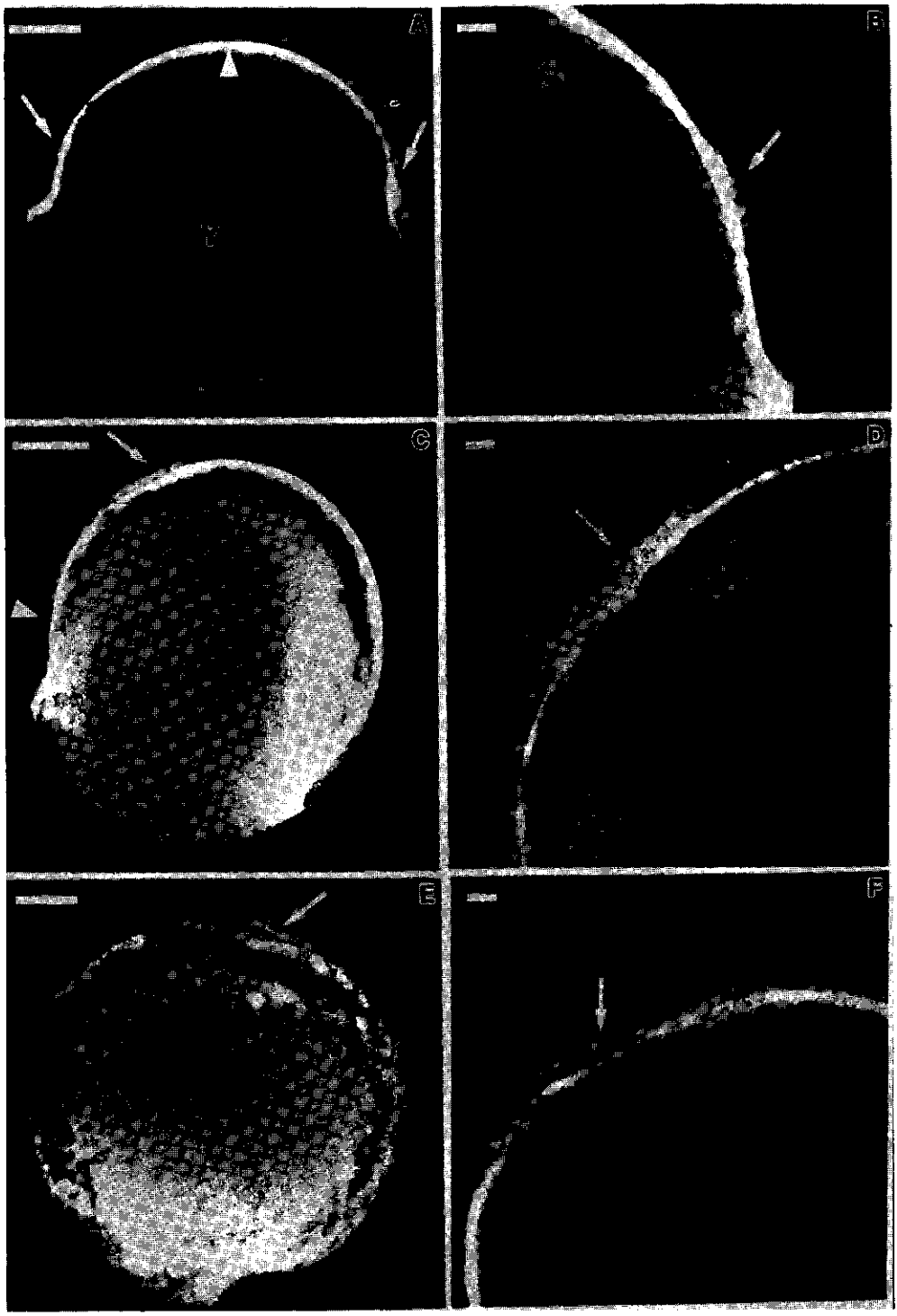
whereas at the opposite side (ventral side) labeled cells disappeared (Fig. 5C). During epiboly the brightness of the fluorescent cell group gradually decreased towards the germ ring, due to the spread out nature of the label. The weakly labeled cells may reflect that connections between labeled and unlabeled cells have been present. The weakly labeled cells may correspond with cells, involuted after dye-uncoupling, or 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 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.* 1992) and the zebrafish (Kimmel and Warga, 1987, 1988). The contrast between labeled and unlabeled neighbouring cells suggests


A
B
C
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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 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 gastrula stage. Although the hypoblast cells of *B. conchonius* appear to form a dye-coupled unit, we can not consider it as a developmental compartment, because these compartments have a precise lineage. Furthermore, because we did not find a strict border at

Fig. 5. (pg 76). 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 into 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 towards 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.

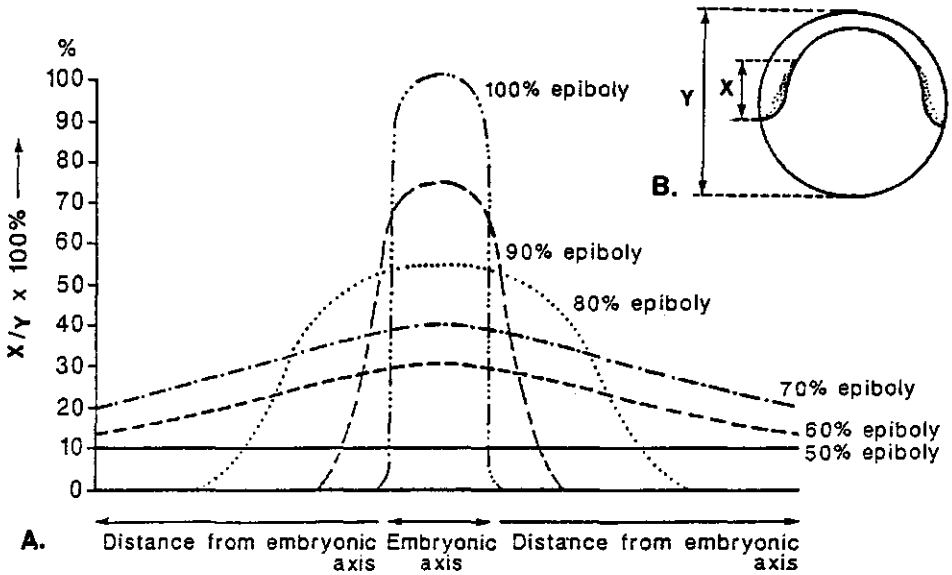

Fig. 6. Model of the change in morphology of the LY-containing cell group (hypoblast) at successive stages of epiboly (A). We measured the relative height of the hypoblast as indicated in (B). This is the height from the "head" to the margin (X) related to the height of the embryo (Y). The relative height 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.

the margin of the dye-coupled hypoblast, this germ layer can not be referred to as a communication compartment.

Between 50% and 100% epiboly, the labeled hypoblast cells migrate towards the dorsally located embryonic axis, thereby changing into a bar-like structure with the highest intensity of label in the future head region. These cells may be the progeny of the first involuted cells since they appear at the onset of gastrulation. Warga and Kimmel (1990) concluded for the zebrafish that the first involuted cells may have an endodermal fate. Possibly, the brightest fluorescing cells in the head represent endoderm, while weakly fluorescing cells, involuted later, might be destined to form the mesoderm. In all stages, a caudalwards decrease in the intensity of the label was observed, probably caused by unlabeled uninvoluted cells joining the hypoblast later. However, the labeling of these cells is too weak to follow their fate in our experiments.

The change in shape of the hypoblast is caused by dorsalwards cell movements, as also described by Warga and Kimmel (1990). This cell migration may go along two different pathways. The pathway may be from the ventral side along the germ ring towards the dorsal side. As a consequence, the cells involuted at the dorsal side will be pushed forward into the rostral direction. The other alternative is that cells migrate from the ventral side directly into the direction of the leading edge of the hypoblast at the dorsal side. We have no evidence as yet for either of these two alternatives.

Acknowledgements

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Chapter 5

INVOLVEMENT OF FIBRONECTIN IN EPIBOLY AND GASTRULATION IN EMBRYOS OF THE COMMON CARP, *CYPRINUS CARPIO*

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INVOLVEMENT OF FIBRONECTIN IN EPIBOLY AND GASTRULATION IN EMBRYOS OF THE COMMON CARP, *CYPRINUS CARPIO*

Summary

The present report firstly describes a pilot study in which, during early development of embryos of the common carp, *Cyprinus carpio*, the cellular adhesion to fibronectin (FN) was blocked by administration of GRGDS peptide (which binds to the FN-receptor). As this treatment resulted in developmental aberrations, suggesting a functional role of FN, the major part of the work, describing the distribution of FN during epiboly and gastrulation, was carried out using immunocytochemical methods.

GRGDS treatment had a concentration dependent effect on development. Incubation of embryos in 1.5 mg/ml from the 32 cell stage onwards, caused a retardation of epiboly, which did not proceed beyond 60%. The embryos did not show involution, as was confirmed by histological study. These preliminary results suggest that FN is involved in both epiboly and gastrulation of carp embryos.

During cleavage no specific extracellular binding of anti-FN antiserum could be observed. FN, however, appeared to be present on a number of cell membranes from early epiboly onwards. After the onset of gastrulation we observed a gradually increasing number of the deepest epiblast cells, showing immunostaining on the part of their surface, facing the yolk syncytial layer (YSL) or the involuted cells. During early epiboly FN was only found in areas in front of the migratory hypoblast cells. Later on, FN partly separated the hypoblast cells from the epiblast cells. At 100% epiboly some contact areas of epiblast and hypoblast showed a discontinuous lining of FN, while other areas appeared devoid of FN. The results indicate that FN is involved in the migration and guidance of hypoblast cells during gastrulation in carp.

Key words: gastrulation, fibronectin, fish development

Introduction

An important morphogenetic process during early development is gastrulation, finally resulting in three distinct germ layers within the embryo. Especially with respect to chick and fish development, also epiboly (blastoderm expansion after meroblastic cleavage) is a crucial morphogenetic event. In a number of species gastrulation appears to be mediated, at least partly, by components of the ECM, especially fibronectin (FN). Using microscopical, immunocytological and gelelectrophoresis techniques, it was demonstrated for amphibians that FN, present as a fibrillar network on the inner cell surface of the blastocoel roof, preceeds and guides mesodermal cell migration (Boucaut

et al. 1990; Winklbauer, 1990; Winklbauer and Nagel, 1991). As reviewed by Boucaut *et al.* (1990) gastrulation in amphibians can be blocked by treatment with either Fab' fragments of anti-FN antiserum, an RGD containing peptide (Boucaut *et al.* 1984, 1985; Smith *et al.* 1990), an anti-integrin antibody or tenascin (interfering with interactions of cells with FN). The RGD peptide interferes with binding of FN (containing an RGD sequence in its cell binding domain) to the cellular FN-receptors (integrins) of many cell types in several species (Yamada and Kennedy, 1984; Ruoslahti and Pierschbacher, 1986; Buck and Horwitz, 1987; Hynes, 1987; Tamkun *et al.* 1986). It is shown for *Xenopus* that treatment with an RGD containing peptide only results in random movements instead of directional movements of mesoderm cells on the blastocoel roof, suggesting that FN may be especially involved in directionality of locomotion (Winklbauer, 1990; Winklbauer and Nagel, 1991).

In vivo and *in vitro* studies in chick embryos demonstrate that the appearance of FN on the basal surface of the epiblast is correlated with mesoderm migration (Critchley *et al.* 1979; Sanders, 1980, 1982; Duband and Thiery, 1982; Harrisson *et al.* 1984a, b, 1985, 1988; Toyoizumi *et al.* 1991; Toyoizumi and Takeuchi, 1992). *In vitro* mesodermal cell spreading can be inhibited by treatment with RGDS or the Fab' fragment of anti-FN antibody (Brown and Sanders, 1991). Besides gastrulation, blastoderm expansion also appeared to be dependent on FN, since this morphogenetic movement was also inhibited by the GRGDS peptide (Lash *et al.* 1990).

The process of epiboly and gastrulation in fish embryos has received relatively little attention hitherto. Consequently, the presence and function of FN is only incidentally studied. Fig. 1 schematically represents successive stages of epiboly and gastrulation of fish embryos. During epiboly the yolk cell becomes surrounded by the cell mass. At the onset of gastrulation (50% epiboly) a germ ring arises, in which, at least in cyprinids, the hypoblast is formed by involution movements of individual cells (Wood and Timmermans, 1988; Warga and Kimmel, 1990). In fishes epiboly and gastrulation can be considered as separate processes, although, at least in cyprinid fishes, they overlap each other in time. Recently, dye-coupling experiments of Gevers and Timmermans (1991) revealed for *Barbus conchonius* that hypoblast cells are at least partly dye-coupled but are dye-uncoupled from the non-involuted epiblast cells, indicating their separate identity. Convergence, movement of hypoblast and epiblast rostrodorsalwards, forming the embryonic shield and later on the embryonic axis (see Warga and Kimmel, 1990; Trinkaus *et al.* 1992), is supposed to be a part of gastrulation.

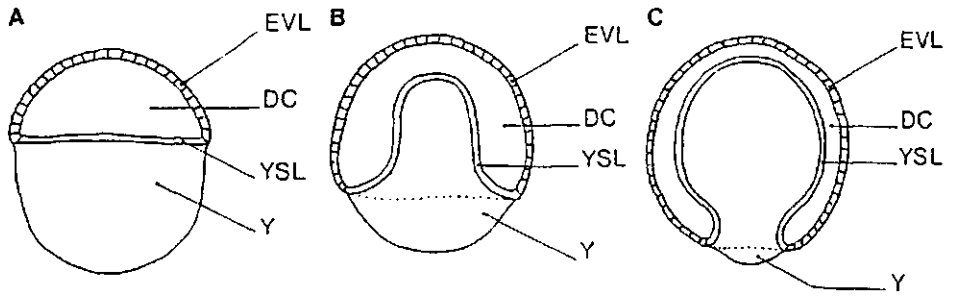


Fig. 1. Schematic drawing of median sections of different stages of epiboly, showing the three cell types of the embryo: enveloping layer (EVL), yolk syncytial layer (YSL) and deep cells (DC). Only the DC contribute to the embryo proper. (A). 0% epiboly stage, shortly after the formation of the YSL. (B). 60% epiboly stage, just after the onset of gastrulation. (C). 90% epiboly stage. Y, yolk.

We are aware of only one abstract publication on the presence of FN in fish embryos (Boulekbache *et al.* 1984). The latter authors report the appearance of FN during early *Salmo* development, using immunocytochemical techniques. They observed a high level of FN in blastula and gastrula stages. During gastrulation FN especially settled in front of the migrating mesoderm.

To establish whether FN plays a role during early development of carp embryos, we firstly performed a pilot experiment, using GRGDS administration. As the results indicated that GRGDS inhibits both epiboly and gastrulation, the major part of the study was concentrated on the distribution of FN, using immunocytochemical methods, during successive stages of cleavage, epiboly and gastrulation.

Material and methods

Embryos

Adult specimens were kept in 800 l tanks at 23°C and fed with Trouvit pellets (Trouvit Forel no. 4, Trouw and Co, Putten, The Netherlands) at a daily rate of 2% of the body weight. 41 hours (h) and 11 h before egg collection adult females of *Cyprinus carpio* were injected intramuscularly with 0.6 mg/kg and 6 mg/kg carp pituitary homogenate (Hydroquest International, Rosemont, NY, USA), respectively. Adult males were injected with 2 mg/kg carp pituitary homogenate 11 h before sperm collection. On the day of fertilization the injected specimens were anaesthesized in 0.02% TMS (Tricaine methane sulphonate, Crescent

Research Chemicals, Arizona, USA) and dry stripped. All further treatments were performed at 25°C. 25 mg eggs were gently mixed with 2.5 ml sperm for 30 sec. Artificial fertilization of control eggs (not to be dechorionated) occurred for 1 min by addition of 0.5% ureum/0.2% NaCl. Subsequently fertilized eggs were washed with ureum/NaCl (6 min) and Cu-free aerated tap water (24 min), treated with tannic acid (0.25 g/l; 10 sec), washed in Cu-free aerated tap water and placed in a column continuously flowed with Cu-free aerated tap water. Treatments with ureum/NaCl and tannic acid prevent sticking together of the embryos by their chorions. Eggs, to be used in our experiments, were artificially fertilized (1 min) in Cu-free aerated tap water and dechorionated with 0.125% trypsin (Sigma) in 10% Holtfreter solution (6 mM NaCl, 0.07 mM KCl, 0.07 mM $CaCl_2$, 0.24 mM $NaHCO_3$) for 30 min. In addition they were carefully washed and not manipulated for at least one hour and a half (until the 4-8 cell stage) since they appeared to be very sensitive to manipulation during early developmental stages. Subsequent development of dechorionated embryos occurred under continuous flow of aerated Holtfreter solution.

GRGDS administration

Embryos were incubated in groups of 10 in 10% Holtfreter solution containing 0.5, 1, 1.5, 2 or 4 mg/ml GRGDS peptide (MW=491, Boehringer Mannheim). As a control these treatments were also performed with two related and roughly similarly sized peptides (RKDVY, M_r=680, Sigma and RFDS, M_r=523, Sigma). Incubation was started at the 32 cell stage, 0% epiboly stage or 40% epiboly stage and finished at 60%, 80% or 100% epiboly by fixation in Bouin's fluid. When in a treated group a significant number of embryos developed similarly as the controls, up to the 100% epiboly stage, a few of them were cultured until hatching stage (52 h after fertilization) to confirm that development was normal. During incubation in the peptide solution successive stages of development were compared with a control group, which only was dechorionated and allowed to develop in Holtfreter solution until fixation. Embryos were sectioned and stained using standard procedures. Sections of embryos of control and peptide treated groups were compared with respect to progress of epiboly and progress of gastrulation (involution). Photographs were made, using a 25 ASA Kodak T-max Black/White film.

Immunocytochemistry

Embryos were fixed for 12 h in 4% paraformaldehyde in Holtfreter solution (pH=7.4) or in Bouin's fluid at the 64 and 512 cell stage and at 0%, 20%, 40%, 50%, 60%, 80% and 100% epiboly (at least 5 embryos of each stage), orientated in agarose (type VII, Sigma), dehydrated in a graded series of ethanol, embedded in paraffin and sectioned at 5 μm. Antisera incubations (diluted in TBS/Triton, containing 0.05M Tris/0.15M NaCl/0.05% Triton-X-100; pH=7.6) and washings (performed between antisera incubations in TBS/Triton, 3x5 min) were carried out at room temperature, except incubation with first antibody. Sections were deparaffinized and endogeneous peroxidase was inhibited (0.2% H_2O_2 in 100% ethanol for 30 min). Sections were preincubated for 20 min with 20% normal goat serum. Afterwards, they were incubated successively in polyclonal anti-FN antiserum (18 h, 4°C, Rabbit-anti-Human FN antiserum, 1:4000, Dakopatts), GAR antiserum (60 min, 1:150, Nordic) and PAP complex (60 min, 1:1000, Nordic) and washed with TBS without NaCl (TB). Subsequent staining was performed in a solution containing 0.02% H_2O_2, 0.025% DAB and 0.25% di-ammonium nickel(II)sulphate-6-hydrate (BDH) in TB. Sections were washed in aqua dest and mounted in entallan using standard procedures. For negative controls anti-FN antiserum or GAR antiserum was replaced by TBS/Triton. Specificity of first antibody was tested by absorption of anti-FN antiserum by FN. 1 μl FN (1 mg/ml) was added to 1 μl anti-FN antiserum (pure). The mixture was diluted to 1:100 and incubated successively for 60 min at 37°C and for 16 h at 4°C. After centrifugation (13000 rpm, 5 min) the supernatant was diluted to 1:4000 and tested on sections. Anti-FN antiserum without antigen was treated and tested the same way as a control on the absorption assay. Intestinal tissue of adult mouse and carp was used as positive control. All negative controls were unstained while sections of intestinal tissue of both carp and mouse showed extensive labeling of the basal membranes, where FN should be expected. Absorption of anti-FN antiserum to FN resulted in unstained sections. Photographs were made, using a 25 ASA Kodak T-max Black/White film.

Results

Effect of GRGDS on development

After incubation in Holtfreter solution containing 0.5 or 1 mg/ml GRGDS, embryonic development, up to hatching, did not show abnormalities, independent on the stage of onset of incubation.

At GRGDS concentrations of 2 and 4 mg/ml, 100% of the embryos

died within 45-75 min, dependent on concentration and the developmental stage at which treatment started. Embryos, fixed before dying, showed a number of leaks within the enveloping layer (EVL), which is normally tightly sealed. In the area beneath a leak a considerable number of deep cells were disconnected and had died.

Treatment with GRGDS at a concentration of 1.5 mg/ml resulted in embryos without dying cells, but showing specific developmental aberrations, when compared to untreated embryos. In general, administration from the 32 cell stage onwards had a severe effect, in contrast to administration, which started at the 0% epiboly or 40% epiboly stage. Apparently, during epiboly penetration of GRGDS through the EVL is limited as no disturbance of development occurred. Thus, only the results from embryos, treated from the 32 cell stage onwards, will be considered here. Until the onset of epiboly no abnormalities were observed. However, epiboly was slowed down and none of the embryos passed the stage of about 60% epiboly (Fig 2A, B). Sections of these embryos, fixed at 20% epiboly (controls were already at the 60% epiboly stage) or at 60% epiboly (controls were already at the 100% epiboly stage), revealed that a hypoblast was not present (Fig 2A, B, C). The YSL, however, had considerably enlarged after GRGDS treatment (Fig. 2B). Furthermore, blastoderm thinning and germ ring formation, as normally occurs during epiboly, was not observed.

Incubation in solutions with control peptides had no effect on development, up to hatching, even at concentrations of 4 mg/ml.

Immunocytochemistry

With respect to fixation, Bouin's fixative lead to better results compared to paraformaldehyde fixative, because the ratio between general background and specific binding of anti-FN antiserum to antigenic determinants was optimal. Therefore, we only consider the results after fixation in Bouin's fluid.

During cleavage stages no immunostaining for FN could be observed in the ECM. However, the cytoplasm of the blastomeres was stained weakly. From early epiboly onwards some weak staining was observed at the outer side of cell membranes (Fig. 3A). No abundant extracellular staining was observed before 50% epiboly. Between 60% and 100% epiboly immunostaining appeared on the surface of the deepest layer of epiblast cells, facing the YSL or the involuted hypoblast cells (we will call this the basal surface of the cells). The number of stained cells gradually increased during the progress of epiboly. At 60% epiboly, shortly after the onset of gastrulation, only a few scattered

labeled epiblast cells were found in the area in front of the migratory hypoblast cells, extending up to the animal pole (Fig. 3B, C). However, staining with anti-FN antiserum was never observed in the area of involution. At 80% epiboly many more epiblast cells, especially those containing lobopodia, were immunostained on their basal surface (Fig. 3D). Vegetally, anti-FN antiserum staining partly separated epiblast cells from hypoblast cells. At 100% epiboly in some areas a discontinuous lining was present between epiblast and hypoblast cells (Fig. 3E), while other areas did not contain labeled epiblast cells. During late epiboly some weak staining could be observed on the surface of the YSL, but it is far less distinct as labeling of the deepest epiblast cells.

During all stages a considerable number of nuclei was labeled, which probably is the result of a cross reaction of the polyclonal anti-FN

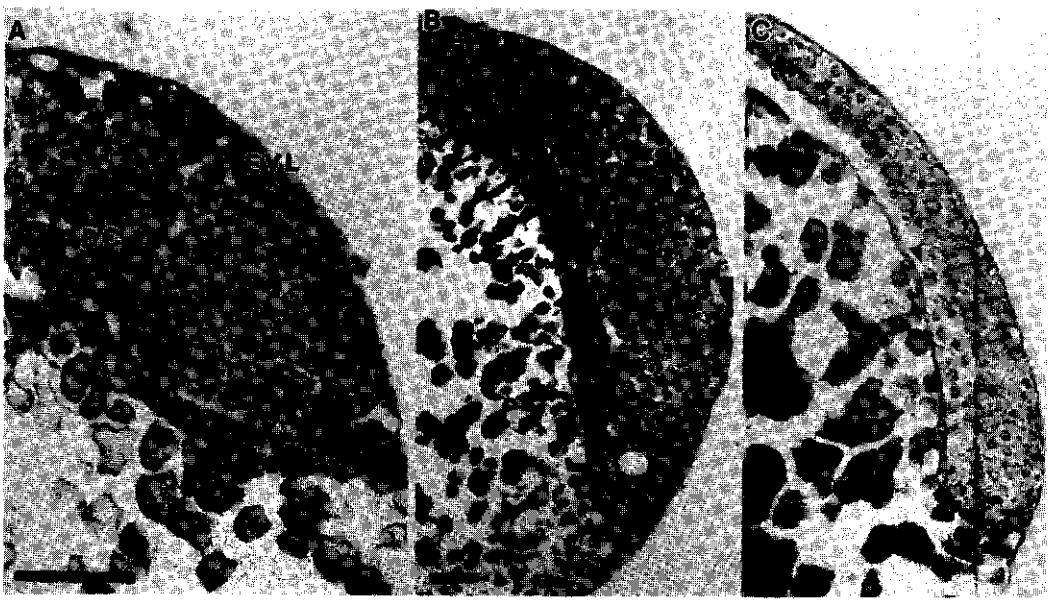


Fig. 2. Median sections of embryos after GRGDS treatment from the 32 cell stage onwards. Abbreviations as described before. Scale bars: 50 μm. (A). Embryo at the 20% epiboly stage, while control embryos already reached the 60% epiboly stage. (B). Embryo at about the 60% epiboly stage, while control embryos already reached the 100% epiboly stage. No hypoblast or involuting cells can be observed. (C). Control embryo at about the 60% epiboly stage. A clear hypoblast can be observed as a separate layer of cells (arrowhead).

antiserum with a nuclear antigenic determinant. Nuclei of intestinal tissue of both carp and mouse were not or only weakly stained. Possibly, the anti-FN antibody cross-reacts with lamin (Skalli and Goldman, 1991), a nuclear intermediate filamant, which may contain sequence homologies with FN. The labeling of nuclei is not given attention in this paper.

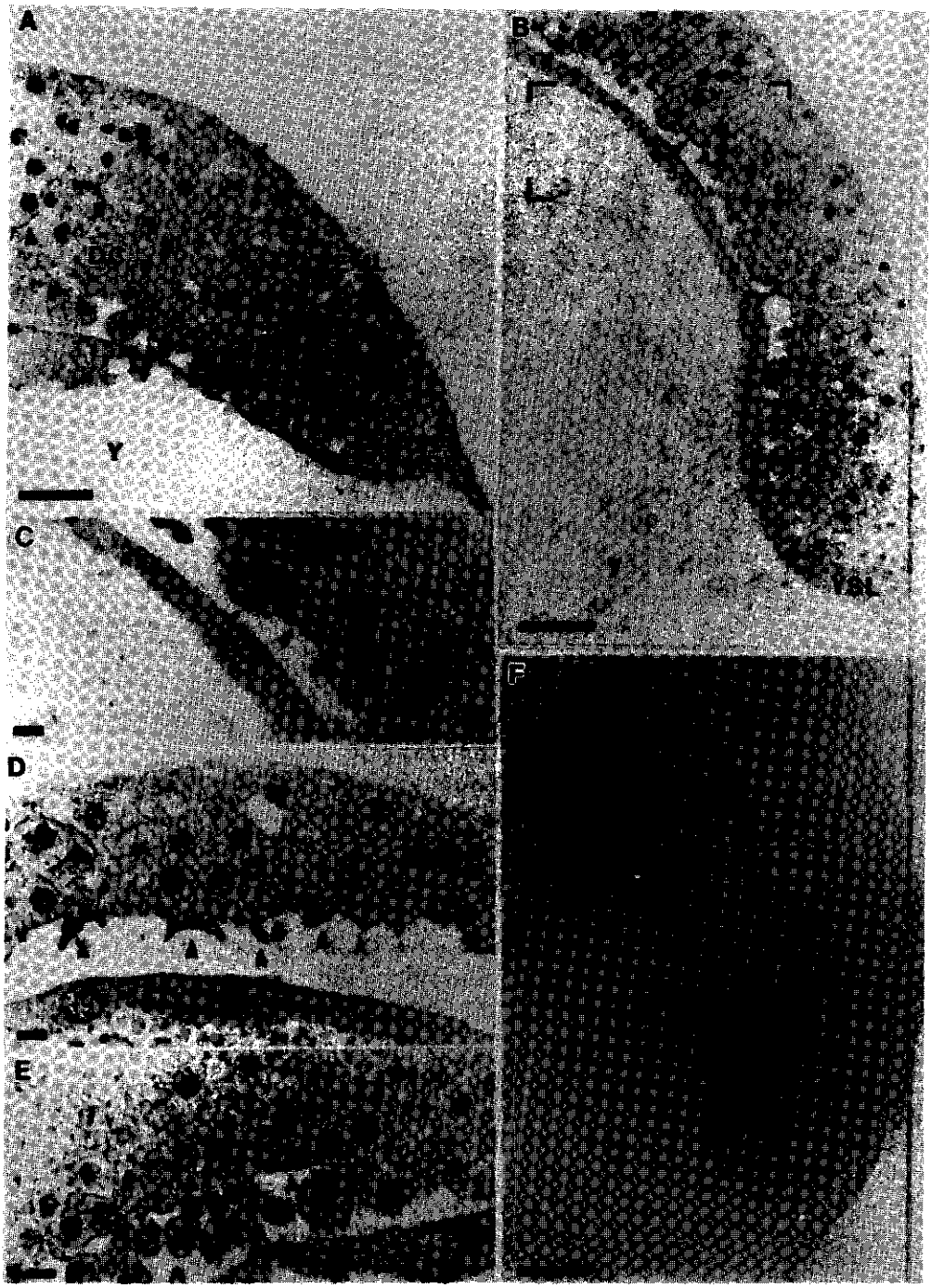
Control sections, lacking incubation with anti-FN antiserum or GAR antiserum, were unstained (Fig. 3F). Sections, incubated with anti-FN antiserum after absorption with FN, also were unstained.

Discussion

The present report describes a pilot experiment, showing an effect of administration of GRGDS peptide. The results clearly suggest that FN is involved in epiboly and gastrulation. Furthermore, using immunocytochemical methods the distribution of FN during epiboly and gastrulation is described. From 60% epiboly onwards, after the onset of gastrulation, extracellular FN appeared on the basal surface of the epiblast cells, facing the YSL. The labeled epiblast cells increased in number during the progress of gastrulation, although even at 100% epiboly some areas lacked FN staining.

The light staining within the blastomeres during cleavage and blastula stages may indicate that FN or its precursors are already present in the cytoplasm but not yet secreted. However, low levels of (extracellular) FN may be not immunocytochemically detectable. The presence of relatively low levels of FN either in the cytoplasm or already located extracellularly during cleavage and blastula stages was also reported for amphibians (Boucaut and Darribère, 1983; Darribère *et al.* 1984; Lee *et al.* 1984) and chick (Harrisson *et al.* 1988; Raddatz *et al.*

Fig. 3. (pg 93). Anti-FN antibody staining on median sections of embryos during several stages of epiboly. Scale bars: 50 μm (A, B, F) or 10 μm (C. D, E). Abbreviations as described before. (A). 20% epiboly stage. Cell membrane staining becomes distinct at some locations (arrowheads). (B). Stage of about 60% epiboly, shortly after the onset of involution. A number of epiblast cells, facing the YSL, start expressing FN on their basal surface (arrowheads). (C). Detail of (B)(arrowheads). (D). 80% epiboly stage. Immunostaining is present on the deepest epiblast cells, especially those containing lobopodia (arrowheads). (E). 100% epiboly stage. Immunostaining is present between epiblast and migrating hypoblast cells (arrowheads). (F). Negative control of a 60% epiboly embryo, in which anti-FN antibody incubation is replaced by TBS/Triton. The same results were obtained after absorption of anti-FN antiserum with FN before immunostaining.


A
B
C
D
E
F

1991). In amphibians, also FN receptors have been already found on cells of the blastula stage (Darribère *et al.* 1988).

In carp embryos a weak FN staining between the cells was observed during epiboly and GRGDS treatment resulted in retardation of epiboly. This may suggest that FN is involved in epiboly. Also in chick embryos blastoderm expansion appears to be mediated by FN, which is presumably secreted by the edge cells, utilizing this as a substratum during blastoderm expansion (Lash *et al.* 1990). It is reported for *Xenopus* that the rate of synthesis of FN increases dramatically at the midblastula transition, but the glycoprotein is immunocytochemically detectable on sections only from early gastrulation onwards (Lee *et al.* 1984). In this respect it is interesting that the period of formation of the YSL and the onset of epiboly in fish embryos is, according to Kimmel (1989), comparable with the midblastula transition of *Xenopus*. The presence of FN on a number of cell membranes during epiboly of carp embryos can be considered as a preparation for the next period, during which FN may function in directional cell migration (see below).

During gastrulation of carp embryos FN could not be observed at the margin, where involution occurs. This might suggest that FN is not involved in the involution movements. Smith *et al.* (1990) reported for *Xenopus* that gastrulation is not simply initiated by control of FN synthesis. Sanders and Prasad (1991) demonstrated for chick embryos that TGF-ß could modulate the ECM, which may influence gastrulation. Possibly, some factors like TGF-ß, activin, FGF and collagen (see Smith, 1989; Slack, 1990) may induce mesodermal cells and trigger involution movements. This might result in or coincide with the appearance of FN on epiblast cells, making hypoblast migration and further involution possible.

During the progress of gastrulation FN appeared in an increasing area on the basal surface of the epiblast cells, facing the YSL ("blastocoel roof"). especially those containing lobopodia. Early in gastrulation FN staining only was present on a small number of epiblast cells, some of them located in front of the involuted hypoblast cells. During late gastrulation of carp embryos many FN expressing YSL-facing epiblast cells with lobopodia are present, suggesting a role of FN in migration of hypoblast cells. The presence of FN, preceeding contacts with involuted cells was also reported for amphibians (see Boucaut *et al.* 1990) and chick (Critchley *et al.* 1979; Duband and Thiery, 1982; Harrisson *et al.* 1984a, b, 1985, 1988; Sanders, 1980, 1982; Brown and Sanders, 1991), suggesting that FN mainly guides the direction of locomotion of involuted cells. GRGDS treatment of carp embryos prevented the formation of two

separate layers of tissue (epiblast and hypoblast), indicating an inhibition of involution and/or an inhibition of directional migration after involution. FN might also be involved in the formation of the embryonic shield, since convergence is a part of gastrulation. The involvement of FN during embryonic shield formation was also suggested for *Salmo* by Boulekbache *et al.* (1984). In *Xenopus*, however, convergent extension appeared not to be blocked by an RGD containing peptide (Smith *et al.* 1990).

The presence of FN, as immunocytochemically detected, can not in all cases be completely correlated with the effects of GRGDS treatment. This might be partly explained by inhibition of the cellular adhesion to laminin, another glycoprotein of the ECM (Horwitz *et al.* 1985; Grant *et al.* 1989; Aumailley *et al.* 1990), although RGD has been shown to be only weakly active in inhibiting functional interactions of cells with laminin (Yamada and Kennedy, 1987). Laminin and FN often appear to be codistributed (Nakatsuji *et al.* 1985; Darribère *et al.* 1986; Zagris and Chung, 1990). However, Brown and Sanders (1991) were unable to demonstrate any effect on the mesoderm cells of chick by injecting either anti-laminin or YIGSR peptide (cell binding site of laminin), while anti-FN or RGDS peptide significantly reduced the number of cells attached to the basement membrane. This may suggest that interactions between the integrin receptor and FN play a more important role during gastrulation than laminin-receptor interactions. Both the appearance of laminin and a more detailed study of developmental effects of GRGDS peptide will be subject of further study.

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Chapter 6

GENERAL DISCUSSION

GENERAL DISCUSSION

Facts and hypotheses about PGCs in vertebrates

The result of development is an individual with both somatic cells (for vegetative functions) and germ cells (for generative reproduction). In species showing a preformistic origin of at least germ cells (nematodes, insects, anuran amphibians) specific cytoplasm, germinal plasm, is present in the oocyte. In species showing an epigenetic determination of (also) germ cells (urodelan amphibians, birds, mammals), germ cells appear as just one of the many cell types generated during embryogenesis, and germ cell characteristics appear later during development (see Nieuwkoop and Sutasurya, 1979). Because of the two different types of germ cell origin throughout the animal kingdom, germinal plasm of preformistically arisen PGCs is considered to be different from nuage (a term introduced by André and Rouiller, 1957), appearing in PGCs at later developmental stages. However, both are ultrastructurally comparable and considered to be characteristic for germ cells (see Eddy, 1975; Nieuwkoop and Sutasurya, 1979). Nuage material is also termed "germinal dense bodies", "nucleolus-like bodies", "electron opaque substance" or "mitochondria-associated granular material". As described by Satoh (1974) for several stages of germ cell development of *Oryzias latipes*, nuage appears as "germinal dense bodies, often interspersed with large aggregations of mitochondria, not bound by a membrane, and consisting of an interwoven meshwork of fibrils". A largely similar description is given by Eddy (1975) for the germinal plasm.

Since in the amphibian order both the preformistic and the epigenetic type of PGC formation are present, it is important to know what type of PGC formation occurs in the evolutionary lower class of fishes. The presence of germinal plasm or nuage-like structures may be helpful to recognize PGCs in embryos.

In *Barbus conchonius* the first cells containing some perinuclear nuage or germ plasm-like electron dense material are observed after completion of epiboly (10 h a.f.; Chapter 2). This fact, in addition to the indeterminate germ cell line in *Barbus conchonius* (Chapter 3), may indicate that we have to do with an epigenetic origin of PGCs in cyprinid fishes, and suggests the perinuclear dense material to represent nuage. Therefore, the latter term will be used below.

During the early developmental stages of *Barbus conchonius*, nuage in PGCs is located near the nuclear envelope and has a granular structure, highly comparable to the structure of the chromatin material within the nucleus. In urodelan embryos it has been observed that during

successive stages of development the structure and location of the nuage material changes: the granules increase in density and are translocated towards mitochondrial clusters (Nieuwkoop and Sutasurya, 1979). A slight increase in density of the granules is also observed in *Barbus conchonius* between 10-12 h and 24 h a.f. As reported for the closely related common carp, *Cyprinus carpio*, germ cells of later stages possess mitochondrial clusters intermingled with electron dense material (Van Winkoop *et al.* 1992). A comparable ultrastructural change of the "germinal dense bodies" during successive stages of development is described for PGCs of *Oryzias latipes* (Hamaguchi, 1985). Thus, the morphology and dynamics of nuage of urodeles and (at least) cyprinid and cyprinidont fishes appears to be very much alike.

The exact composition of the nuage material is not known. The presence of RNA and proteins in nuage of adult and embryonic germ cells has been reported in a number of species (Dixon, 1981; Eddy and Ito, 1971; Takeuchi and Takeuchi, 1982). With respect to germ cells of fishes, Toury *et al.* (1977) and Azevedo (1984) reported the ribonucleoproteinaceous character of nuage in spermatocytes and previtellogenic oocytes of adult fishes. Clérot (1979) concluded, from experiments using labeled uridin and amino acids that nuage in germ cells of adult cyprinid fishes also contained RNA and proteins. His study on the kinetics of incorporation of both precursors makes likely that at least part of the nuage is derived from nucleocytoplasmic transfer.

The function of nuage is also unknown. Although its presence can be used to identify PGCs, it is not clear whether it is involved in the determination or differentiation of PGCs. This should be investigated by transplantation of PGCs to ectopic locations or to embryos of different developmental stages. These experiments have not been reported for fish hitherto. Transplantation of PGCs in fish embryos is difficult to perform, due to the low number of these cells and their embedding within somatic tissue.

Based on its composition, a number of suggestions have been made with respect to the cell-biological role of nuage. In fishes, the majority of the work has been performed with germ cells of adult specimens. Azevedo (1984) stated for previtellogenic oocytes that nuage represents the RNA and protein necessary for the cytoplasmic ribosomal maturation. The increase of the volume of the granular component during oocyte growth and the observed increase in ribosomal number support this suggestion. The close association of nuage with mitochondria made Azevedo (1984) to hypothesize that nuage may be involved in the transfer of genetic information or proteins into the mitochondria. Clérot (1979) described its transfer between nucleus and cytoplasm in cyprinid fishes, possibly indicating that nuage may function

as part of an extranuclear mechanism to control cellular processes. Hogan (1978) suggested for embryos of *Oryzias latipes* that nuage may play a key role in mitochondriogenesis.

In *Barbus conchonius* the earliest detected PGCs, observed after completion of epiboly (10 h a.f.), are located within mesodermal tissue and they make contact with endodermal cells (Chapter 2). The location of PGCs at the stage of first identification, as well as the indeterminate germ cell line (Chapter 3) again suggest an epigenetic formation of PGCs and point to a mesodermal origin. However, Hamaguchi (1982, 1985) reported for the teleost *Oryzias latipes* the earliest presence of PGCs within the endoderm. In other vertebrate classes the origin of PGCs appears to be variable too. In urodelan amphibians the number of generated germ cells is positively correlated to the amount of induced mesoderm, indicating that the germ cell line originates in cells, already determined as mesoderm (see Dixon, 1981). In anurans, however, the germ cell line appears to contain material from the vegetative pole and the origin of the germ cells may be traced from the entoderm (Nieuwkoop and Sutasurya, 1979). Concerning birds and mammals germ cells appear to arise from the epiblast (Eyal-Giladi *et al.* 1981; McLaren, 1983; see also Nieuwkoop, 1991) and, as reported for mouse embryos, are possibly one of the first mesodermal cell lineages (derived from the epiblast), set aside during gastrulation (Ginsburg *et al.* 1990). It can be hypothesized that the fact that, in many vertebrates, PGCs can only be recognized at relatively late and possibly significantly different developmental stages, results in correspondingly different conclusions concerning their origin.

In conclusion, germ cell formation in most vertebrates appears to occur epigenetically, and the question of the origin of these cells still has to be answered. However, in a number of vertebrates PGCs may originate within the mesoderm. Among the latter are the urodeles and probably the cyprinids, including *Barbus conchonius*.

In addition to the evidence for epigenetic formation of PGCs of *Barbus conchonius* within the mesoderm, the presence of contacts between the earliest PGCs and endodermal cells also supports evidence for the homology between cyprinid fishes and urodeles. Possibly these endodermal cells induce PGCs, as is also reported for urodelan embryos (Nieuwkoop and Sutasurya, 1979).

Based on the epigenetic as well as preformistic origin of germ cells within the amphibian order, Nieuwkoop and Sutasurya (1976) proposed a polyphyletic origin of the recent amphibians from different groups of osteichtyan fishes. Since also different germ cell origins in fishes have been reported (see Johnston, 1951), this hypothesis remains interesting. Furthermore, PGCs of chelonian reptiles (turtles) seem to have an

endodermal origin (Fujimoto *et al.* 1979), as anurans. Based on our results, one can hypothesize a common phylogenetic origin of cyprinids and urodeles. The anurans could be part of the other branch of the suggested evolutionary bifurcation, showing endodermal origin of PGCs, which may also hold for a number of teleosts and reptiles. It could be interesting to perform studies on the germ cell line in fishes with an endodermal PGC origin: possibly, their germ cell line arises in a preformistic way as is reported for anuran amphibia. The above hypothesis may be very speculative. Furthermore, ontogenetic features may not be clearly correlated with phylogenesis at all. During the process of evolution certain changes in early ontogeny may have taken place in different kinds of animals without a change in the final body plan of the adult, if the specimen survives. These changes may not have been selected out because selection pressure was on the adults which may not have been very different from the original individuals.

Determination of somatic cells and the restriction of cell fate

In addition to the study on the origin of PGCs, also somatic cell lineages are followed from the 64 cell stage onwards until somite stages (12 h a.f.; Chapter 3). Strong evidence is obtained for *Barbus conchonius* that at the 64 cell stage cells are not yet committed to their fate. This is also indicated by the finding that more-or less randomly a cell migration process (cell mixing) starts at the onset of epiboly. The future location of cells and therefore their presumptive fate is highly unpredictable. However, we found a slight difference between the fate of the upper layer cells (ULC) and lower layer cells (LLC) of the 64 cell stage, indicating that cell mixing may not be completely at random. Apparently the chance to be committed to a certain fate differs slightly between the ULC and LLC progeny.

The presence of unpredictable cell fates during early stages of development, as found for *Barbus conchonius*, was also described in other fish species. Cell mixing in blastula stages is reported for *Brachydanio rerio* (Kimmel and Warga, 1987a), *Oryzias latipes* (Kageyama, 1977), *Fundulus heteroclitus* (Trinkaus and Lentz, 1967), *Notobranchius korthausae* (Lesseps *et al.* 1979) and *Salmo gairdneri* (Ballard, 1973a, b). The indeterminate fate of early cleavage cells of zebrafish embryos is demonstrated by Streisinger *et al.* (1989), who reported that in the 32 cell stage almost every blastomere has some descendants that participate in the formation of the pigmented retina. Similar results with respect to indeterminate fate during early cleavage stages are found for mammals

(Gardner and Lawrence, 1985; Winkel and Pederson, 1988). In *Xenopus* fate maps of cleavage stages can be made, due to relatively limited cell mixing (Moody, 1987; Dale and Slack, 1987; Wetts and Fraser, 1989).

From the end of cell mixing onwards, predictability of cell fates should increase considerably. It is not certain when this stage is reached in fish embryos. In zebrafish, individual cells of the early gastrula generate clones of progeny that are confined within single tissues, but segmental restrictions have not been found (Kimmel, 1989; Kimmel and Warga, 1986, 1987a,b, 1988; Kimmel *et al.* 1988, 1990). For instance, a clonal group of cells in the early gastrula eventually generated muscle fibres distributed over 11 segments (Kimmel *et al.* 1988). This indicates that even after the onset of gastrulation, cells may intermingle with one another, but the extent of mixing gradually decreases. As a result cell mixing of DCs was never observed between the epiblast and hypoblast (Warga and Kimmel, 1990). Our dye-coupling studies in *Barbus conchonius* revealed that the hypoblast was dye-uncoupled from the epiblast, indicating their separated identity (Chapter 4; see below). We never observed labeled cells in between the two germ layers. However, as in zebrafish, possibly cell mixing in *Barbus conchonius* may still occur after the onset of gastrulation, but be restricted to a germ layer, and probably later on to a separate part of that germ layer, which may coincide with newly formed (dye-coupled) compartments within the hypoblast or epiblast. Such a subdivision of a communication compartment, suggested to be coincident with restricted fate, is also reported for the postimplantation mouse embryo (Lo and Gilula, 1979), molluscs (Serras *et al.* 1989, 1990) and insects (Weir and Lo, 1982). In conclusion, cell fate restrictions in fish embryos may occur gradually, starting at the onset of gastrulation. Fate maps should be made from this stage onwards.

Ballard (1973a, b; 1981; 1982) proposed after implantation of chalk particles in the blastula stage and the subsequent analysis of their position after gastrulation that the fate map of fishes might be three dimensional. The fate map made by Kimmel *et al.* (1990) for the zebrafish did not confirm this conclusion. These authors report that DCs located near the animal pole of the early gastrula give rise to ectodermal fates. Cells near the blastoderm margin have endodermal and mesodermal fates. Furthermore, dorsal cells give rise to dorsal and anterior structures, while ventral cells have dorsal, ventral and posterior fates. The relative moment of involution is related to fate (Warga and Kimmel, 1990). Boundaries of germ layer fields at late blastula stage overlap to a considerable extent, but less than clones initiated at earlier stages. Kimmel *et al.* (1990) suggested that the zebrafish fate map is organized similar to that of *Xenopus*, but with its vegetal pole opened up

and the yolk cell inserted at this location. According to the supposed mesodermal epigenetic PGC origin of *Barbus conchonius* it may be worthwhile to compare the fate map of cyprinid fishes with urodelan fate maps instead of that of *Xenopus* (anures). The fate map for zebrafish as made by Kimmel *et al.* (1990), might be more comparable with the fate map for urodeles (Delarue *et al.* 1992).

Possible mechanisms of undirectional migration of single cells

Several authors suggested explanations with respect to the causal analysis of cell mixing, but until now this question is not resolved. This problem involves the question why cells start to move at a certain developmental stage, the question why they move without directionality, and the question of the developmental significance.

With respect to the first question Trinkaus (1990) hypothesized that "cells have to learn how to move". Weliky and Oster (1990) made a mechanical model for *Fundulus heteroclitus*, in which cell rearrangements were explained by a disbalance of forces. The rapidly changing space between EVL and YSL during epiboly might cause such a disbalance for the DCs. On the other hand, a kind of disbalance between the cells may be a prerequisite for a rapidly changing space (see below). Newport and Kirschner (1982) suggested for the *Xenopus* embryo that cell migration normally begins as the cells have reached a critical ratio of nucleus to cytoplasm. Cell movement in *Barbus conchonius* might be explained by the mechanical model, in addition to the ratio of nucleus to cytoplasm, since cleavage continues up to the early epiboly stage.

Considering the second part of the problem, undirectional cell movement, Trinkaus (1985) reviewed that the factors that would give directionality to moving cells are steric hindrance, chemotaxis, contacts among cells and electrical fields. He reported that in *Fundulus heteroclitus* directional cell migration of cultured embryonic cells coincides with electrical coupling between the cells, which may suggest a role for an electrical signal with respect to movement in the same direction. Absence or lack of detection of direction controlling factors may cause cell mixing in a system where cells are forced to move. Trinkaus (1985) proposed that cells fail to show contact inhibition because they are separated from each other by intervening extracellular matrix. Undirectional migration of cells in *Barbus conchonius* may be explained by the failure of cells to receive an inhibiting or direction controlling signal: after the onset of epiboly dye-coupling between the DCs disappeared (Chapter 4), which may cause the immediate start of movement. Furthermore, the presence of fibronectin (FN) between the cells (Chapter 5; see below), likely as part of a more complex

extracellular matrix, is in accord with the hypothesis of Trinkaus. The matrix might prevent cells to receive possible signals for migration directionality. FN, known to play a role in guidance of moving cells in amphibians, might be distributed randomly in these early stages, resulting in random movements. Individual cells might be "guided" towards every location within the embryo (Chapter 5).

Evidence for the developmental significance for cell mixing is not yet present, but this phenomenon might exist because it creates a more flexible mass of tissue, which is necessary for the epibolic spread (see below).

Intercellular communication related to cell fate

Injections of LY into the YSL of *Barbus conchonius* during several stages of epiboly revealed that at the onset of gastrulation the involuting hypoblast cells become labeled, leaving the epiblast unlabeled (Chapter 4). The labeled part of the hypoblast can be followed as a separate cell group during successive stages of gastrulation and convergence. At 60% epiboly and later the blastodermal cells, involuting at that stage, are dye-uncoupled from the YSL.

Reduction of gap junctional communication between cells or groups of cells at specific developmental stages is generally considered to play an important role in restriction of developmental signalling (Bennett and Spray, 1985). Evidence for the potential regulatory role of gap junctional interaction is given by experiments with gap junction antisense RNA or antibodies: injection into early cleavage cells of embryos of different species caused aberrant malformations (Warner *et al.* 1984; Fraser *et al.* 1987; Lee *et al.* 1987; Serras *et al.* 1988).

In addition to the transfer of low molecular weight substances, gap junctions may also be involved in electrical communication between cells. Therefore, dye transfer experiments often are accompanied by a study on the electrical coupling of the dye-uncoupled cell groups. In a number of species (fish, *Xenopus*, mouse), however, dye-uncoupled cell groups remain electrically coupled, possibly caused by charge transfer through the extracellular space (Kimmel *et al.* 1984; Warner *et al.* 1984; Lo and Gilula, 1979). Because the presence or absence of electrical coupling between dye uncoupled cell groups is not clearly correlated with cell fate, we did not perform such experiments in *Barbus conchonius*. Therefore, it can not be concluded that the dye-uncoupled hypoblast and epiblast are also electrically uncoupled.

The question remains what is the developmental significance of the dye-coupling beteen YSL and hypoblast during early gastrulation, and the subsequent uncoupling in *Barbus conchonius*. A hypothetical

substance may be transferred from the YSL towards the blastoderm cells. This may result in the induction of the cells to an endodermal/mesodermal fate (Warga and Kimmel, 1990), in the onset of involution movements of the cells in order to form germ layers (Wood and Timmermans, 1988; Warga and Kimmel, 1990) or in the onset of convergence movements towards the presumptive location of the embryonic axis. Theoretically, all three possibilities for the developmental role of the YSL during early gastrulation may occur: the YSL may be involved in endoderm/mesoderm induction, resulting firstly in involution and secondarily in the establishment of the location of the embryonic shield.

Experimental evidence for the possible role of the YSL in axis formation is given by Long (1983), who suggested that the YSL of trout had the role of directing the cell movements, which establish the embryonic shield during morphogenesis. He transplanted a radially symmetrical early blastodisc (blastula stage) of a trout embryo on the YSL from a gastrulating embryo from which the blastodisc was removed. This experiment resulted in the formation of an embryonic axis at the same relative location as the embryonic shield was formed before the removal of the blastodisc of the fish gastrula. Thus, instructions to control the plane of symmetry may pass from the YSL to the blastodisc. Similar instructions in *Barbus conchonius* may be correlated to the transfer of label from the YSL to the hypoblast during early gastrulation. After transfer of this hypothetic inductive substance from the YSL towards the blastoderm, cells may "know their goal" and YSL and blastoderm can be gap junctionally uncoupled (dye uncoupled). With respect to cyprinids, the function of the YSL in axis establishment still has to be revealed. As yet no experimental evidence has been obtained in fish embryos for a role of the YSL in involution or induction at the stage of early gastrulation. This needs to be elucidated.

Involvement of FN during epiboly and gastrulation

Using a polyclonal anti-FN antibody, immunostaining of carp embryos was negative during cleavage stages and only appeared on a number of cell membranes from early epiboly onwards. After the onset of gastrulation the number of deepest epiblast cells, immunostained on their basal surface, gradually increased. At early stages of gastrulation these cells were found in front of the migratory hypoblast cells, but later on they separated the hypoblast cells from the epiblast. Pilot experiments, using a GRGDS peptide (blocking the adhesion of FN to its receptor) resulted in inhibition of both epiboly and gastrulation, indicating a role for FN in processes involved in these movements.

According to Betchaku and Trinkaus (1978), epibolic spread is passive for the EVL and DCs and occurs in response to pull exerted by the independently expanding YSL. Using immunocytological methods, in carp embryos FN was not found to be located within the YSL or on its cell membrane. How then can the GRGDS peptide inhibit epiboly? During epiboly cells intermingle extensively with each other (Chapter 2) and a number of cells weakly express FN on their cell surface (Chapter 5). The cell mass must be very flexible to respond to the tremendous change in shape within a relatively short time. Possibly, FN on the cell membranes is involved in cell mixing, creating a flexibility in location and shape of the cells. This flexibility might be necessary to allow the relatively rapid expansion during epiboly. Administration of the GRGDS peptide would change the flexible system into a rigid one by inhibiting cell mixing, due to the impossibility of the cells to interact with FN. The rigid tissue mass would not have the potency to change shape so rapidly, and, thus, would not respond to the pull, exerted by the YSL. In order to confirm this hypothesis, it should be interesting to inject cell lineage tracers before or during GRGDS treatment, and observe whether cells become intermingled with each other.

During the progress of gastrulation, FN was found between the epiblast and the migratory hypoblast cells. The presence of FN, preceeding the migratory presumptive mesodermal cells, is also reported for amphibians (see review by Boucaut *et al.* 1990; Nakatsuji *et al.* 1985) and chick (Harrisson *et al.* 1988), and suggests a guidance function. Similarly, in fish embryos, FN may also function as guidance for migrating hypoblast cells. Furthermore, FN was not observed at the margin, where involution occurs, indicating that FN is not involved in the guidance of involuting cells. Possibly, involution movements are mediated by another mechanism, or cells move rostrodorsally as a result of ongoing involution. Apparently, FN guidance becomes more important after cells have been involuted and have to migrate directionally. FN may be also involved in convergence, movement towards the embryonic shield (future embryonic axis, Trinkaus *et al.* 1992), of the hypoblast cells. Involvement of FN in embryonic axis formation is also suggested for *Salmo* (Boulekbache *et al.* 1984). However, in *Xenopus* convergent extension is not blocked by an RGD containing peptide (Smith *et al.* 1990). The blastoderm of carp embryos, treated with GRGDS, homogeneously surrounded the yolk (maximal 60%), even when control embryos already had arrived at the 100% epiboly stage. This may indicate a lack of involution and convergence. The latter is probably due to the disturbance of migration of hypoblast cells. Although in cyprinid fishes epiboly and gastrulation can be considered to be separate processes, it can not be excluded that the

influence of GRGDS on gastrulation may be a secondary effect of the inhibition of epiboly. Therefore, it should be interesting to study the effects of GRGDS on gastrulae of fish species, in which epiboly and gastrulation are completely separated in time (annual fishes; see Van Haarlem, 1981).

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Chapter 7

SUMMARY

SUMMARY

Cell fates can be established either by preformation or by epigenesis. With respect to primordial germ cells (PGCs) it has been shown that the Amphibia exhibit both types of cell fate establishment. Therefore, it is important to study the germ cell origin of the evolutionary lower class of fishes.

In order to study the origin and cell fate establishment of a specific cell type, two approaches are possible. Firstly, its morphological characteristics may be studied, and traced back to the earliest possible developmental stage. Using this approach, we studied in *Barbus conchonius*, a cyprinid fish, the presence, location and morphology of cells, containing nuage, an ultrastructural electron dense perinuclear material, generally accepted to be characteristic for germ cells (Chapter 2). The results show that nuage containing cells, PGCs, could be found from 10 h after fertilization (a.f.) onwards (around 100% epiboly). They translocate between 10 h and 12 h a.f. from a position within the mesoderm towards a position between mesoderm and yolk syncytial layer (YSL). However, PGCs remain separated from the YSL by extensions of endodermal cells. The location of PGCs at the stage of first identification may indicate that they originate within the mesoderm. Contacts with endodermal cells may be involved in the formation of their characteristics.

A second approach for studying the origin of PGCs concerns the labeling of blastomeres during cleavage stages and following their progeny up to the stage of morphological recognition of PGCs (Chapter 3). Using this method, a cell lineage tracer, Lucifer Yellow - Dextran (LY-D), was injected into individual blastomeres of the 64 cell stage of *Barbus conchonius* embryos. Study of the fate of their progeny revealed that a lower layer cell (LLC) could give rise to both somatic cells and a number of PGCs (in about 25% of the cases), while injections into upper layer cells (ULC) only resulted in labeled somatic cells. The distribution of somatic progeny after injection of a certain blastomere was unpredictable with respect to both the later location within the embryo and the tissue type of its descendants. Unpredictable cell lineages were probably due to extensive intermingling of cells during epiboly. The results suggest that the formation of PGCs as well as that of somatic cells is an epigenetic process. As mentioned above, the PGCs may originate within the mesoderm (Chapter 2).

Since in *Barbus conchonius* cells apparently are not yet committed to their fate during cleavage and start extensive intermingling during

epiboly, the question arose when cell fate restrictions occur (Chapter 4). These restrictions may also be important for the determination of a diversity of cell types, including PGCs. Changes in communication properties between cells or cell groups may be correlated with differences of their developmental pathways. Since it was known that during early epiboly certain deep cells are dye-coupled to the yolk syncytial layer (YSL), injections of Lucifer Yellow (LY) into the YSL at several stages of epiboly were used in order to study changes in communication properties. The formation of a group of dye-coupled cells, indicating a group of gap junctionally communicating cells, was described. At the onset of epiboly LY appeared to be transferred from the YSL to all blastodermal cells. Between 40% and 60% epiboly we observed a ring-shaped group of labeled cells, which probably had involuted during early gastrulation. This cell group correlated with the leading edge of the hypoblast, and was dye-uncoupled from the uninvoluted epiblast. From 60% epiboly onwards the blastodermal cells were dye-uncoupled from the YSL. Between 50% and 100% epiboly the ring-shaped labeled hypoblast was, due to convergent cell movements, gradually transferred towards a dorsally located bar-like structure. Gap junctions appear to connect cells with the same fate. Consequently, the appearance of part of the hypoblast as a dye-coupled cell layer, which is dye-uncoupled from the epiblast, may correlate with early restriction in cell fate. On the other hand, gap junctions between YSL and DC may be involved in the transfer of an inducing signal at the onset of gastrulation.

Apparently, it is only from gastrulation onwards that developmental pathways of cell groups gradually disperse. Since cell determination appeared to be highly regulative, knowledge of the control of the directional mass cell migration during gastrula stages may be important for understanding cell fate establishment. Because fibronectin (FN) appeared to be involved in the guidance of migrating cells in amphibian embryos, the presence of FN during epiboly and gastrulation was studied in embryos of the common carp (*Cyprinus carpio*; Chapter 5). However, in order to establish that FN is also involved in early fish development, we first performed a pilot study in which the functional role of FN was blocked by treatment with GRGDS, interfering with interactions between FN and its receptor. Since this treatment resulted both in retardation of epiboly, and a lack of involution, it became interesting to study the localization of FN during respective stages. Using immunocytological methods, FN first appeared present on a number of cell membranes during early epiboly. During the progress of gastrulation (from 50% epiboly onwards) we observed a gradually increasing number of epiblast

cells, which were labeled on their YSL facing surface. They were first present in front of the migratory hypoblast cells and later partly separated the hypoblast cells from the epiblast. The results suggest that also in cyprinid fishes FN may be involved in the guidance of gastrulating cells.

In conclusion, PGCs in embryos of cyprinid fishes can be recognized from late gastrula stages onwards by the presence of nuage. They are supposed to arise epigenetically from the mesoderm, possibly by inducing influence of endodermal cells. Determination processes of both PGCs and somatic cells probably do not start until early gastrulation (50% epiboly). FN may be involved directly (induction processes) or indirectly (morphogenetic movements resulting in induction) in epigenetic processes.

Chapter 8

SAMENVATTING

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Het lot van cellen kan worden vastgelegd via preformatie (d.m.v. segregatie van determinanten in de onbevruchte eicel) of via epigenese (d.m.v. induktieprocessen tijdens de ontwikkeling). Voor wat betreft de primordiale geslachtscellen (PGCs) is aangetoond dat binnen de klasse van de amfibiën beide wijzen van herkomst van geslachtscellen voorkomen. Om die reden is het van belang om de oorsprong van de geslachtscellen te bestuderen in de evolutionair lagere klasse van de vissen.

Om de oorsprong en het vastleggen van het lot van een specifiek celtype te bestuderen, zijn er twee benaderingen mogelijk. Als eerste benadering kunnen de morfologische kenmerken en het vroegste ontwikkelingsstadium waarop deze kenmerken verschijnen, bestudeerd worden. Om deze reden hebben wij in een karper-achtige vis, de prachtbarbeel (*Barbus conchonius*), de aanwezigheid, de plaats en de morfologie bestudeerd van cellen, die nuage bevatten (Hoofdstuk 2). Nuage is elektronendicht perinucleair materiaal, waarvan algemeen wordt aangenomen dat het karakteristiek is voor de geslachtscellen. De resultaten toonden aan dat nuage-bevattende cellen, PGCs, aanwezig zijn vanaf 10 uur na de bevruchting (n.b.), vlak na 100% epibolie. De PGCs verplaatsten zich tussen 10 en 12 uur n.b. vanaf een positie in het mesoderm naar een positie tussen het mesoderm en de "yolk syncytial layer" (YSL), een meerkernig cytoplasma van de dooiercel. Echter, de PGCs bleven gescheiden van de YSL door uitlopers van endoderm cellen. De positie van de vroegst herkenbare PGCs suggereert een mesodermale herkomst. Kontakten met endodermale cellen zijn mogelijk betrokken bij de vorming van de kenmerken van de PGCs.

Een tweede benadering om de oorsprong van de PGCs te bestuderen, is het labelen van blastomeren tijdens een vroeg klievingsstadium en het vervolgen van de nakomelingen tot aan het stadium waarop de PGCs morfologisch herkend kunnen worden (Hoofdstuk 3). Injektie van Lucifer Yellow - Dextraan (LY-D) in blastomeren van het 64 cellig stadium van *Barbus conchonius* embryos toonde aan dat de afstammelingen van de onderste cellaag, naast somatische cellen, in 25% van de gevallen enkele gelabelde PGCs bevatten. Injekties in de bovenste cellaag resulteerden slechts in gelabelde somatische cellen. De verspreiding van gelabelde nakomelingen na injektie van een bepaalde blastomeer was onvoorspelbaar voor wat betreft de toekomstige plaats binnen het embryo of het weefseltype waarvan de cellen deel zouden gaan uitmaken. Deze onvoorspelbaarheid

werd waarschijnlijk veroorzaakt door een intensieve dooréénmenging van de cellen tijdens de epibolie. De resultaten suggereren dat de vorming van zowel PGCs als somatische cellen een epigenetish proces is. Zoals eerder verondersteld, zullen de PGCs dan waarschijnlijk in het mesoderm gevormd worden (Hoofdstuk 2).

Het bovenstaande verschaft duidelijke evidentie dat cellen nog geen vastgestelde ontwikkelingsrichting hebben tijdens klievingsfasen en de vroege epibolie. De vraag rijst dan vanaf welk ontwikkelingsstadium embryonale cellen van *Barbus conchonius* geleidelijk beperkt worden in hun ontwikkelingspotentie (Hoofdstuk 4). Dit stadium zou immers ook belangrijk kunnen zijn voor de determinatie van PGCs. Verandering in kommunikatie tussen cellen of celgroepen wordt verondersteld gekorreleerd te zijn met het ontstaan van verschillen in hun ontwikkelingsrichting. Omdat bekend was dat tijdens de vroege epibolie diepe cellen gelabeld (Lucifer Yellow, LY) kunnen worden via de YSL (waarschijnlijk via gap junctions), hebben we LY geinjiceerd in de YSL tijdens verschillende stadia van de epibolie met het doel celkommunikatie kompartimenten te bestuderen. De vorming van een groep van cellen, die met elkaar maar niet met een daarbuiten gelegen groep van cellen kommuniceerden, werd beschreven. Aangetoond werd dat bij het begin van de epibolie alle blastodermcellen gekoppeld zijn. Tussen 40% en 60% epibolie, wanneer de gastrulatie begint, ontstond een ring-vormige groep van gelabelde cellen, die overéénkwam met het vroegst ingerolde deel van de hypoblast. Deze hypoblastcellen waren LY-ontkoppeld van de niet ingerolde epiblast. Vanaf 60% epibolie waren alle blastoderm-cellen LY-ontkoppeld van de YSL. Door convergentie-bewegingen tussen 50% en 100% epibolie veranderde de gelabelde ringvormige groep van cellen geleidelijk in een rostrodorsaal gelegen staafvormige struktuur, onderdeel vormend van de embryonale as. De beschreven restriktie van celkommunikatie tussen hypoblast en epiblast via gap junctions zou gekorreleerd kunnen zijn met vroege restrikties in ontwikkelingspotentie. Aan de andere kant kunnen gap junctions tussen YSL en presumptieve hypoblastcellen betrokken zijn bij het transport van een inducerend signaal aan het begin van de gastrulatie.

Blijkbaar gaan pas vanaf de gastrulatie ontwikkelingswegen van cellen en celgroepen geleidelijk divergeren. Omdat de determinatie van cellen in sterke mate epigenetisch is, is te verwachten dat celbewegingen tijdens de gastrulatie een belangrijke rol spelen bij deze ontwikkelingsdivergentie. Omdat bij amfibiën fibronectine (FN) betrokken is bij celmigratie processen, hebben wij de aanwezigheid van FN tijdens epibolie en gastrulatie bestudeerd bij embryos van de karper,

Cyprinus carpio (Hoofdstuk 5). Echter, eerst is een pilot studie uitgevoerd, waarbij de funktionele rol van (eventueel aanwezig) FN geblokkeerd werd door behandeling met GRGDS, een peptide dat de FN-receptor bezet. Met GRGDS behandelde embryos vertoonden een vertraagde epibolie en vormden geen hypoblast. Daardoor was het van belang om de lokalisatie van FN tijdens de vroege ontwikkeling van visse-embryos te bestuderen. Met behulp van immunocytochemische methoden is vastgesteld dat FN voor het eerst verscheen op een aantal celmembranen tijdens de vroege epibolie. Tijdens de gastrulatie (vanaf 50% epibolie) vertoonde een geleidelijk toenemend aantal epiblastcellen aan de YSL zijde een positieve reaktie. Deze cellen bevonden zich eerst vòòr de migrerende hypoblast cellen en later tussen de hypoblast cellen en de epiblast. De resultaten maken aannemelijk dat ook bij de karper FN betrokken is bij migratie van de ingerolde hypoblast cellen.

Samenvattend kunnen we concluderen dat PGCs in embryos van cyprinide vissen herkend kunnen worden vanaf late gastrula stadia door de aanwezigheid van nuage. Waarschijnlijk ontstaan ze epigenetisch vanuit het mesoderm, mogelijk via inducerende invloed vanuit het endoderm. Determinatie processen van zowel PGCs als somatische cellen lijken niet eerder dan de gastrulatie aan te vangen. FN is betrokken bij de geleiding van celbewegingen van hypoblast cellen tijdens de gastrulatie. Daarom zou FN direkt (induktie) of indirekt (celbewegingen leidend tot induktie) betrokken kunnen zijn bij epigenetische processen.

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CURRICULUM VITAE

Petra Gevers werd op 18 augustus 1961 te Leiden geboren. In 1973 begon zij de Middelbare school opleiding op het Marnix van St. Aldegonde-college te Haarlem, alwaar in 1980 het VWO-b diploma behaald werd. Van 1980 tot 1983 werd de opleiding Fysiotherapie gevolgd op Academie Leffelaar te Amsterdam. In 1983 werd begonnen met de biologie studie aan de Vrije Universiteit te Amsterdam. De afstudeervakken hadden betrekking op de neuro-endocriene regulatie van het eileg-gedrag van mollusken (VU, Amsterdam, Vakgroep Organismale Dierkunde, sektie Histologie) en de vroege ontwikkeling van mollusken (RUU, Utrecht, Vakgroep Experimentele Dierkunde, sektie Ontwikkelingbiologie). Het doctoraaldiploma werd in 1988 behaald, waarna zij werd aangesteld als onderzoeker-in-opleiding (OIO) op een door BION gesubsidieerd onderzoeksprojekt, uit te voeren op de Landbouwuniversiteit te Wageningen bij de vakgroep Experimentele Diermorfologie en Celbiologie (EDC), sektie Ontwikkelingsbiologie. De resultaten van bovengenoemd projekt zijn beschreven in dit proefschrift. Tijdens deze aanstelling werden verschillende onderwijs-elementen gevolgd, te weten "Methoden en Technieken in de Celbiologie" te Amsterdam, "Moleculaire Ontwikkelingbiologie" te Wageningen, "Experimental embryology in marine plants and animals" te Banyuls-sur-Mer (Frankrijk) en Proefdierkunde te Nijmegen.