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Embryonic Stages from Cleavage to Gastrula in the Loach *Misgurnus anguillicaudatus*

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ABSTRACT—Early developmental staging from the zygote stage to the gastrula is a basic step for studying embryonic development and biotechnology. We described the early embryonic development of the loach, *Misgurnus anguillicaudatus*, based on morphological features and gene expression. Synchronous cleavage was repeated for 9 cycles about every 27 min at 20°C after the first cleavage. After the 10th synchronous cleavage, asynchronous cleavage was observed 5.5 h post-fertilization (hpf), indicating the mid-blastula transition. The yolk syncytial layer (YSL) was formed at this time. Expressions of *goosecoid* and *no tail* were detected by whole-mount *in situ* hybridization from 6 hpf. This time corresponded to the late-blastula period. Thereafter, epiboly started and a blastoderm covered over the yolk cell at 8 hpf. At 10 hpf, the germ ring and the embryonic shield were formed, indicating the stage of early gastrula. Afterward, the epiboly advanced at the rate of 10% of the yolk cell each hour. The blastoderm covered the yolk cell completely at 15 hpf. The embryonic development of the loach resembled that of the zebrafish in terms of morphological change and gene expression. Therefore, it is possible that knowledge of the developmental stages of the zebrafish might be applicable to the loach.

Key words: loach, developmental stages, mid-blastula transition (MBT), *goosecoid*, *no tail*

INTRODUCTION

The staging of early embryogenesis is a basic step for developmental biology. Developmental stages based on morphological features have been reported in many fish species (*Fundulus heteroclitus*; Armstrong *et al*., 1965, salmonid fishes, *Salmo gairdneri* and *Salvelinus fontinalis*; Ballard, 1973, medaka, *Oryzias latipes*; Iwamatsu, 1994, American shad, *Alosa sapidissima*; Shardo, 1995, ice goby (shiro-uo), *Leucopsarion petersii*; Arakawa *et al*., 1999, and others), but detailed staging based on developmental genetics has been studied only in zebrafish *Danio rerio* (Kimmel *et al*., 1995) and goldfish *Carassius auratus* (Yamaha *et al*., 1999).

According to Yamaha *et al*. (1999), a blastodisc cleaves synchronously in early embryonic development after fertilization, then mitotic divisions occur asynchronously. Such a turning point in cell division is referred to as the mid-blastula transition (MBT), which is characterized by cell cycle lengthening, loss of synchronism in cell divisions, activation of transcription and appearance of cell motility in *Xenopus* (Newport and Kirschner, 1982) and zebrafish (Kane and Kimmel, 1993). After MBT, the yolk syncytial layer (YSL) is formed and epiboly begins. The specification occurs in several regions of the blastoderm due to induction from the yolk cell (Mizuno *et al*., 1996; Ober and Schulte, 1999) and the expression of zygotic genome in the proliferated blastomeres (e.g., *no tail* at blastoderm margin, Schulte-Merker *et al*., 1992; *goosecoid* at the dorsal side of blastoderm, Stachel *et al*., 1993). When embryos initiate gastrulation, morphogenetic movements occur and then germ layers are formed. On the other hand, experimental embryological studies have revealed that embryonic cells before the gastrula stage have a high regulative ability (Ho and Kimmel, 1993).

Gene transfer (medaka; Ozato *et al*., 1992, zebrafish; Linney *et al*., 1999, rainbow trout, *Oncorhynchus mykiss*; Yoshizaki *et al*., 2000, mud loach, *Misgurnus mizolepis*; Nam *et al*., 2001), nuclear transplantation (European loach, *Misgurnus fossilis*; Gasaryan *et al*., 1979, medaka; Waka-

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matsu *et al.*, 2001, zebrafish; Lee *et al.*, 2002) and germ line chimera (zebrafish; Lin *et al.*, 1992, medaka; Wakamatsu *et al.*, 1993, goldfish; Yamaha *et al.*, 2001, rainbow trout; Takeuchi *et al.*, 2001, loach, *Misgurnus anguillicaudatus*; Nakagawa *et al.*, 2002) have been developed and performed in several fishes from the viewpoints of basic biology as well as advanced aquaculture. These biotechnological techniques are essentially applicable to the early embryos before gastrulation because the developmental fate of the blastomeres has not yet been committed; thus various types of embryo manipulation are possible. Therefore, it is necessary to identify the embryonic stages of the target species for further biological practices.

In the present study, we describe the early embryonic stages from cleavage to the gastrula period of the loach *Misgurnus anguillicaudatus*, based on morphological features, cytological observation, histological results and whole-mount *in situ* hybridization.

MATERIALS AND METHODS

Egg and sperm

Parental fish of the loach, *Misgurnus anguillicaudatus*, were caught from paddy fields and water ways in Kita-village, Sorachi county, Hokkaido prefecture, Japan by the Loach Farming Cooperation of Kitamura during the spawning period from June to July

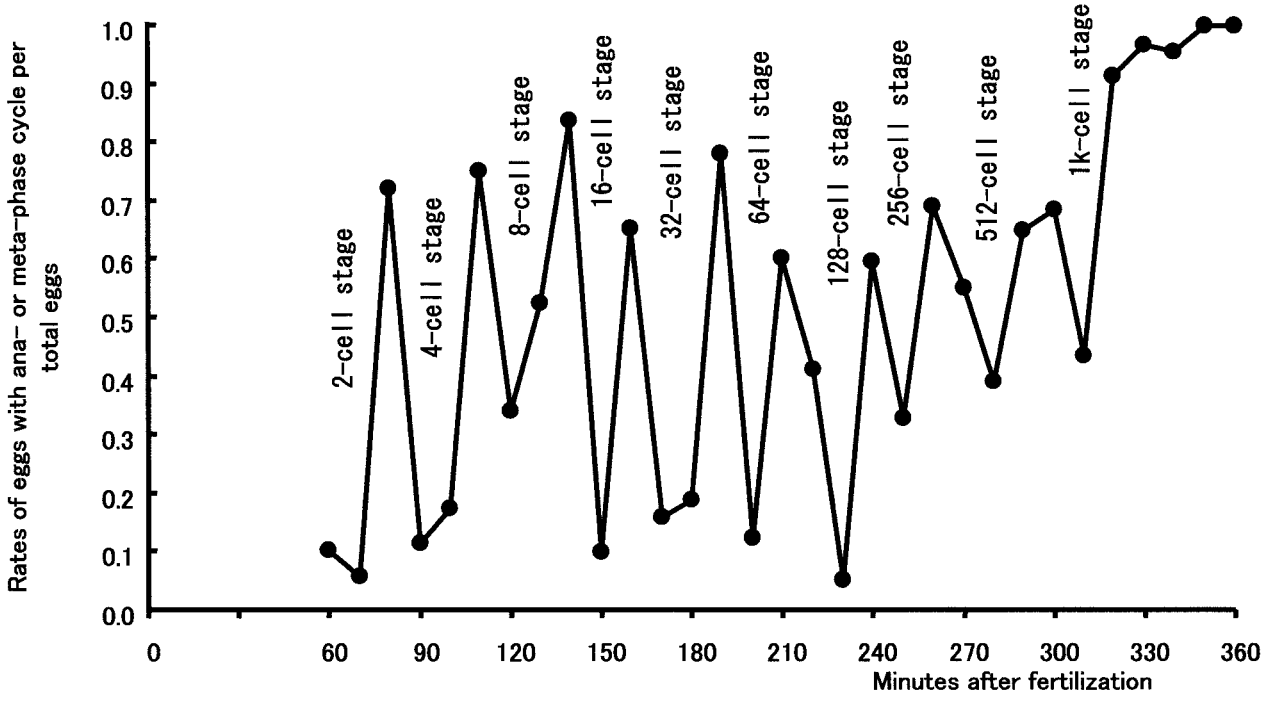


Fig. 1. Transition from synchronous to the asynchronous cleavage cycle between 60 and 360 min post-fertilization (mpf). Eggs were fixed with glutaraldehyde every 10 min from 60 to 360 mpf and their cell division stage was determined. The frequency of eggs with meta- or anaphase blastomeres per total eggs in each sample was plotted according to the advancement every 10 min. Synchronous cleavage was repeated 9 times after 2 cell stages. After the 1 k cell stage, asynchronous cleavage was observed.

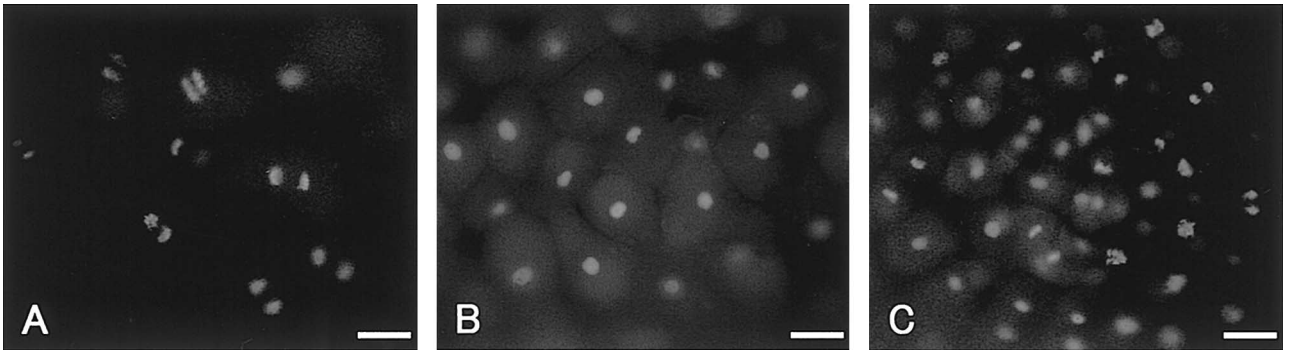


Fig. 2. Fluorescent micrographs of cleavage cycle in embryos. Eggs were fixed with glutaraldehyde, stained with DAPI, and observed under a fluorescent microscope. (A) Synchronous anaphase in the 9th cycle (260 mpf). (B) Synchronous S phase in the 10th cycle (280 mpf). (C) Asynchronous blastomeres with the 11th cleavage cycle (330 mpf). Scale bars = 50 μm

2001. We induced ovulation and spermiation to collect gametes by intraperitoneal injections of Human Chorionic Gonadotropin (20 IU/g body weight for males and females). Eggs were manually stripped on polyvinyl chloride film. Sperm was collected into hematocrit blood drawing tubes and diluted 1: 20 with loach physiological saline (7.5 g/L NaCl, 0.2 g/L KCl, 0.2 g/L $MgCl_2$ and 0.4 g/L $CaCl_2$, pH 7.8 by $NaHCO_3$). Eggs were inseminated with diluted sperm and fertilized by tap water in glass petri dishes at 20°C. Fertilized eggs were dechorionated by treatment with Ringer's solution (7.5 g/L NaCl, 0.2 g/L KCl and 0.2 g/L $CaCl_2$) containing 0.1% trypsin and 0.4% urea for about 10 min. Dechorionated eggs were transferred to Ringer's solution containing 1.6% albumen, and were incubated at 20°C after the transfer.

Visualization of nuclei

About fifty dechorionated eggs were fixed every 10 min from 60 to 360 min post-fertilization (mpf) with 2% glutaraldehyde in phosphate-buffered saline (PBS) overnight. Fixed embryos were stored in PBS at 4°C. After the removal of the blastodisc from the yolk cell, the nuclei were stained with 5 mg/ml 4'-6-diaminido-2-phenylindole (DAPI) dissolved in 10 mM Tris-HCl (pH 7.4), 5mM EDTA, and 0.15 M NaCl for 1 h or more. The blastodiscs were then washed with PBS, and observed from an animal pole under a fluorescence microscope (Olympus Model BH-2).

Histology

Embryos were fixed with Bouin's fixative for 3 h. Fixed embryos were stored in 80% ethanol and then dehydrated by butyl alcohol series and embedded in paraffin blocks. Serial sections were cut at 8 μm thickness and stained with hematoxylin-eosin.

Whole-mount *in situ* hybridization (WISH) analysis

From 5 to 15 h post-fertilization (hpf), dechorionated eggs were fixed every hour with 4% paraformaldehyde in PBS for 30 h. Fixed embryos were stored in 100% methanol at –20°C. WISH was performed by staining with a single color in the blastula stage, and with

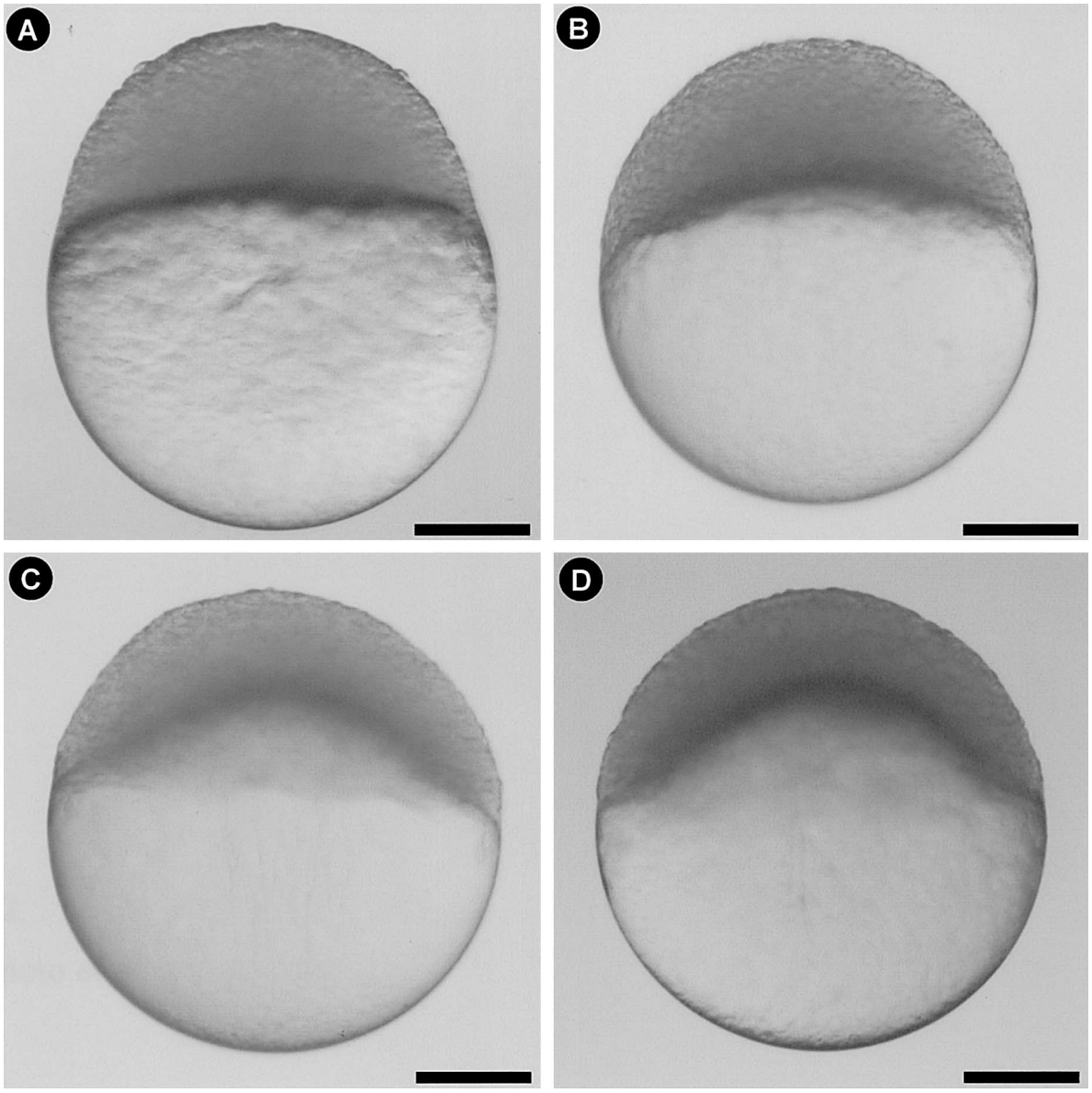


Fig. 3. Morphological change during the late-blastula stage. External appearance of the embryo at 6 (A), 7 (B) and 8 hpf (C, D). Scale bars = 200 μm.

two colors in the gastrula stage according to Jowett and Lettica (1994) with slight modifications.

goosecoid (*gsc*) (Stachel *et al*., 1993) and *no tail* (*ntl*) (Schulte-Merker *et al*., 1992) of zebrafish were used as dorsal mesodermal and pan-mesodermal markers, respectively (courtesy of Dr. H. Takeda; *ntl* was originally cloned by Dr. S. Schulte-Merker and *gsc* by Drs. M. Tada and N. Ueno). For two-color *in situ* hybridization, RNA probes were labeled with digoxigenin for *gsc* and with fluorescein isothiocyanate (FITC) for *ntl*.

RESULTS

Morphological changes in early development

The first cleavage occurred about 60 min post-fertilization (mpf) at 20°C. Thereafter, cleavages occurred about every 27 min until 310 mpf (Fig. 1). Cells divided synchronously in a single blastodisc until the 10th cleavage (Fig. 1), however, the synchronism was slightly different between the marginal part and the animal pole of the blastodisc after approximately the 8th cleavage. The adjacent blastomeres showed a synchronous cell cycle. After 320 mpf, the cells divided asynchronously, even in the adjacent blastomeres of a single blastodisc (Fig. 2).

Blastomeres of the marginal part of the blastodisc connected to the cytoplasm of yolk cell before 4 hpf. At 5 hpf, nuclei in this region started to proliferate mitotically without cytoplasmic division, forming the external yolk syncytial layer (E-YSL). At the center of the bottom of the blastodisc, a multinucleate cytoplasmic layer was not observed until 5 hpf. Some blastomeres in this region were stained more deeply with hematoxylin than those of the overlaying deep blastomeres. After 6 hpf, syncytial cytoplasm was observed in the central part of the bottom of the blastoderm, and thus the internal yolk syncytial layer (I-YSL) was formed. Thereafter, the blastoderm was separated from yolk by the YSL.

After 7 hpf, the shape of the embryo changed from ellipsoidal (Fig. 3A) to spherical (Fig. 3B), afterwhich epiboly began. The I-YSL began to bulge toward the animal pole, and the marginal part moved toward the vegetal pole of the

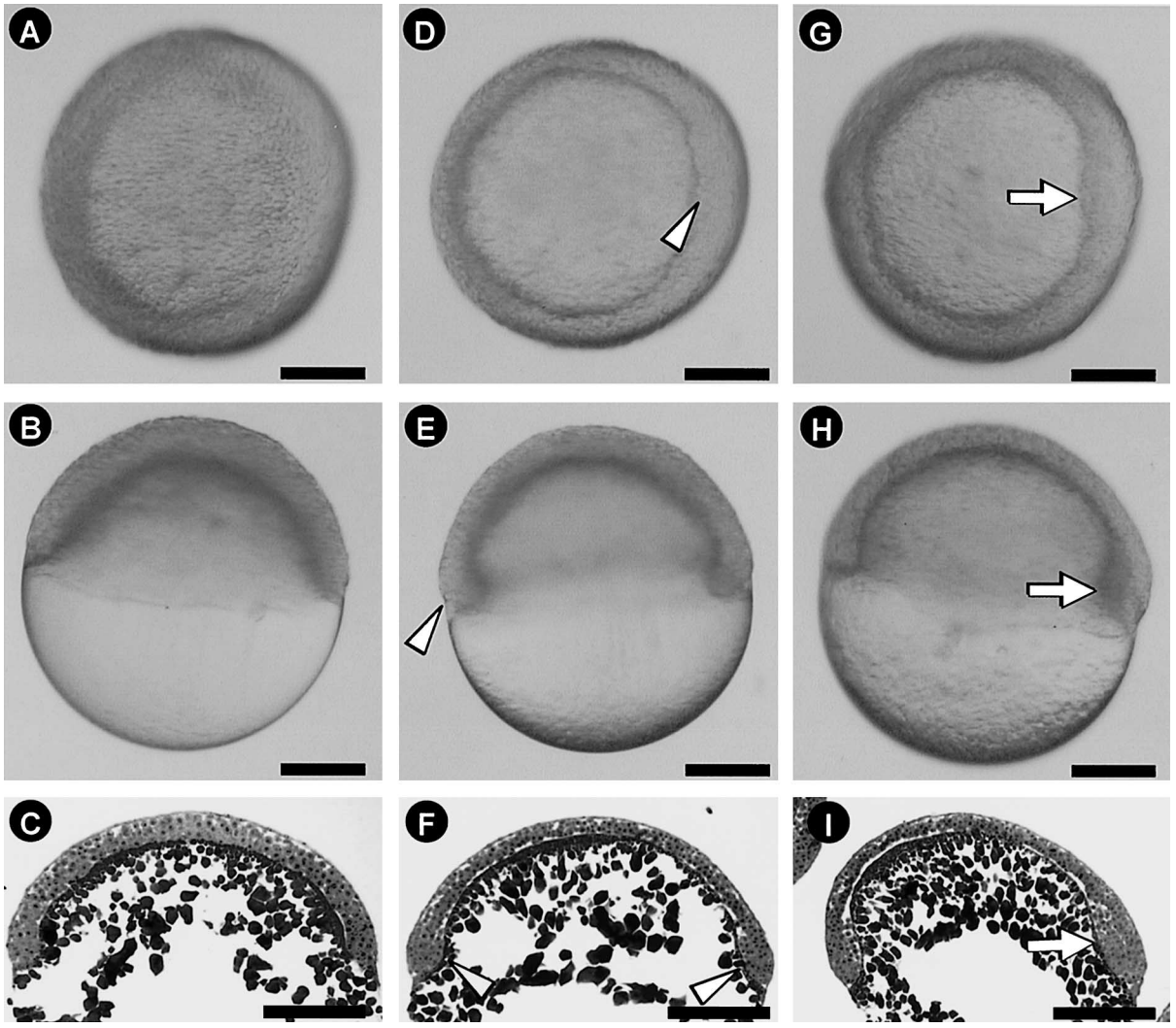


Fig. 4. Germ ring and embryonic shield formation in the early gastrula period. (A-C) 50% epiboly at 9 hpf. (D-F) Germ ring at 10 hpf. (G-I) Embryonic shield at 10 hpf. (A), (D) and (G) are animal pole views of each embryo. (B), (E) and (H) are lateral views of each embryo. (C), (F) and (I) are histological views of each embryo. Arrowheads indicate the germ rings. Arrows indicate the embryonic shields. Scale bars = 200 μm.

yolk cell during epiboly. At 8 hpf, the blastoderm formed a dome-like shape by bulging of the I-YSL toward the animal pole (Fig. 3C). In some embryos, the blastoderm covered approximately 30% of the yolk cell with nearly uniform thickness (Fig. 3D).

After 9 hpf, the blastoderm covered half of the animal hemisphere of the yolk cell (Fig. 4A, B and C). At 10 hpf, the marginal region of the blastoderm thickened and formed a germ ring (Fig. 4D, E and F). Almost simultaneously, the embryonic shield was formed (Fig. 4G, H and I). The exact time of embryonic shield formation was not determined. During these events, epiboly was temporarily arrested. After the shield formation, epiboly continued. At 15 hpf, the blastoderm covered the yolk cell completely.

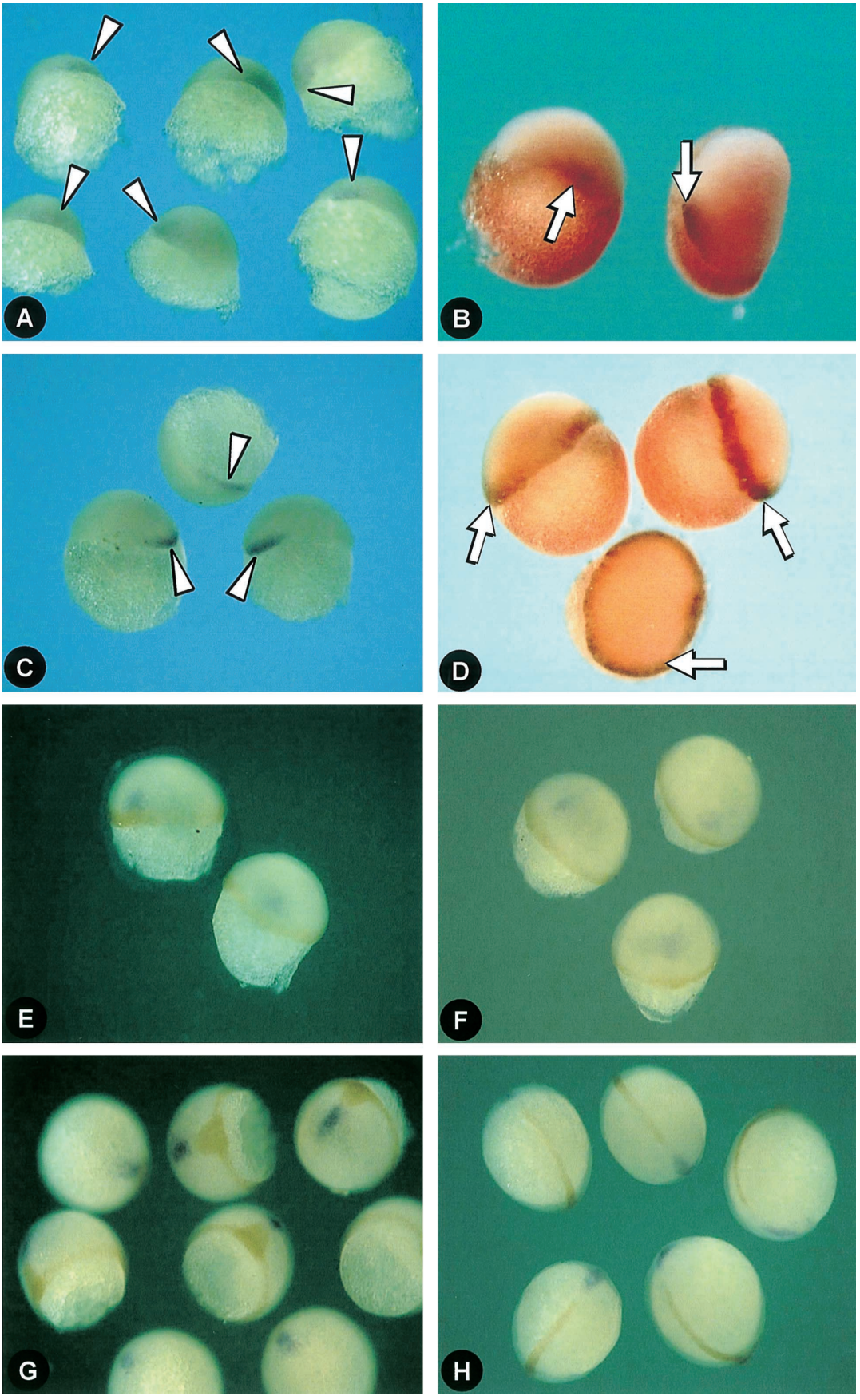
We observed that a difference in developmental rate arose among siblings during embryonic development. It was already observed during synchronous cleavages, while clearly at 10 hpf. In this time, several embryos had already finished the formation of the embryonic shield, whereas others had not. Thus, at 15 hpf, some embryos did not achieve 100% epiboly. These differences in developmental rates among siblings tended to increase with the progress of embryonic development.

gsc and *ntl* expression during development

gsc and *ntl* transcripts were first detected at a part of the marginal blastoderm at 6 hpf by WISH (Fig. 5A and B). *gsc* showed graded expression on one side of the embryo (Fig. 5A). Thereafter, at 8 hpf, *gsc* expression was localized to part of the blastoderm margin (Fig. 5C), but *ntl* expression expanded over the entire blastoderm margin (Fig. 5D). During gastrulation, expressing regions of *gsc* and *ntl* were separated. However, *gsc*- and *ntl*-expressing regions overlapped at 9 hpf (data not shown). At 10 hpf, *gsc*-expressing regions were shifted toward the animal pole of the embryo, separating from *ntl* expressing regions (Fig. 5E). On the other hand, *ntl*-expressing regions showed no shift (Fig. 5E). Then, at 11 hpf, when *gsc*-expressing regions moved toward the animal poles, *ntl*-expressing regions thickened in the dorsal region (Fig. 5F). At 12 hpf, expression of *ntl* expanded to the presumptive notochord (Fig. 5G). Subsequently, *gsc* and *ntl* expressed independently without overlapping (Fig. 5G). Finally, at 15 hpf, i.e. 100% epiboly, the leading edge of the *gsc*-expressing region reached the ani-

Table 1. Developmental stages of loach embryos from 0 ~ 15 h at 20°C.

Period, Stage name	Time	Notes
Zygote 1-cell	0 h 0 min	
Synchronous cleavage period		
2-cell	1 h 10 min	
4-cell	1 h 30 min	
8-cell	2 h	
16-cell	2 h 30 min	
32-cell	2 h 50 min	
64-cell	3 h 20 min	
128-cell	3 h 50 min	
256-cell	4 h 10 min	
512-cell	4 h 40 min	
	5 h	E-YSL formation
1k-cell	5 h 10 min	
Mid-blastula transition (MBT)	5 h 20 min	Asynchronous cleavage occurring
Late-blastula period		
Oblong	6 h	I-YSL formation, initiation of *gsc* and *ntl* expression at a part of marginal blastoderm
Sphere	7 h	
Dome, 30% epiboly	8 h	Epiboly beginning, *ntl* expressing at marginal blastoderm
Gastrula period		
50% epiboly	9 h	
Germ ring, embryonic shield	10 h	*gsc* expressing region moving to the animal pole
60% epiboly	11 h	*gsc* expressing region separated from *ntl* expressing region
70% epiboly	12 h	*ntl* expression expanding presumptive notochord
100% epiboly	15 h	



mal pole, and *ntl* was expressed in the presumptive notochordal region (Fig. 5H).

DISCUSSION

The developmental process in the loach was already reported by Watanabe *et al.* (1948). They observed the development from zygote to hatch, however they did not describe particular characteristics of the embryos from cleavage to the gastrula period in detail. In teleosts, characteristic indexes of these periods have been determined only in two species, zebrafish (Kimmel *et al*., 1995) and goldfish (Yamaha *et al*., 1999). Consequentially, the period before gastrulation can be divided into two periods, the synchronous cleavage period and the late-blastula period, by the MBT. After this period, embryos enter the gastrula period when characteristic germ layers are formed by morphogenetic movements. According to the observations of the present study, developmental schedules from cleavage to gastrula in the loach *M. anguillicaudatus* are defined as the following periods and summarized in Table 1.

The synchronous cleavage period and the MBT: Early embryonic development is characterized by rapid and synchronous cleavage to proliferate cell number. This stage corresponded to the period from 1 cell to 1k cells in the loach. Synchronous cleavage was repeated for 9 cycles about every 27 min interval after the first cleavage, thus the first asynchronous cleavage occurred at the 11th cell cycle. Thus, we determined that the MBT occurred at 320 mpf after the 1k-cell stage, because different cell cycles were observed even in the adjacent blastomeres of a single blastoderm. The MBT is characterized by loss of synchronism in cell divisions. In zebrafish and goldfish, asynchronous cleavage begins at the 10th cell cycle (Kane and Kimmel, 1993; Yamaha *et al*., 1999). The number of synchronous cleavages is regulated by the nucleo-cytoplasmic ratio (Newport and Kirschner, 1982; Kane and Kimmel, 1993). The loach, zebrafish and goldfish taxonomically belong to the same order, Cypriniformes. However, the relative DNA content of the loach was about two-thirds of the other two species (loach; Zhang and Arai, 1996, zebrafish and goldfish; Ciudad *et al*., 2002). Therefore, it seems that the number of synchronous cleavages in loach embryos might require an increase of one cell cycle to achieve the threshold nucleo-cytoplasmic ratio at the MBT, as compared with zebrafish and goldfish. Furthermore, it was reported that the E-YSL was formed roughly at the time of the MBT in zebrafish (Kimmel *et al*., 1995; Kimmel and Law, 1985), *Fundulus* (Trinkaus, 1992) and goldfish (Yamaha *et al*., 1999). Similarly, in the loach the E-YSL was formed at around MBT.

Late-blastula period: In the loach, this period corresponded to the time from 6 to 9 hpf. In zebrafish, the expression of *gsc* as a dorsal mesodermal signal and *ntl* as a pan-mesodermal signal was first detected at the late-blastula stage (Stachel *et al*., 1993; Schulte-Merker *et al*., 1992; Schulte-Merker *et al*., 1994). In the expression of these genes, the E-YSL plays an important role in the induction of the blastoderm for mesendoderm (Mizuno *et al*., 1996; Chen and Kimelman, 2000; Ober and Schulte, 1999). In the loach, as the expression of mesodermal marker genes *gsc* and *ntl* was detected after 6 hpf, we determined that the onset of the late-blastula period occurred at this time. The YSL consists of two regions, the E-YSL and the I-YSL. The YSL plays an important role in epiboly (Solnica-Krezel and Driever, 1994). During epiboly, the E-YSL spreads along the yolk surface toward the vegetal pole with the enveloping layer attached to it (Trinkaus, 1984), while the I-YSL bulges toward the animal pole (Topczewski and Solnica-Krezel, 1999). In zebrafish (Kimmel *et al*., 1995) and goldfish (Yamaha *et al*., 1999), it has been reported that the I-YSL is formed after E-YSL formation. Also in the loach, the I-YSL was histologically observed at 6 hpf after E-YSL formation.

The late-blastula period in zebrafish is subdivided to 'oblong', 'sphere', 'dome' and '30% epiboly' periods by external appearance (Warga and Kimmel, 1990). The same morphological features as zebrafish were also observed in the loach. Therefore, we applied these terms to the subdivided late-blastula period of the loach. At 6 hpf, loach embryos resembled zebrafish embryos in the 'oblong' stage in external appearance and the start of marker gene expression. Thus, we decided that loach embryos in 6 hpf corresponded to the 'oblong' stage (Fig. 3A). In the same way, most embryos at 7 hpf were staged 'sphere' (Fig. 3B). In zebrafish, 'dome' and '30% epiboly' stages are characterized by bulging of the I-YSL surface and the beginning of epiboly (Warga and Kimmel, 1990) and *ntl* expression in the blastoderm margin (Schulte-Merker *et al*., 1992). Since these features were apparently observed in the loach embryos at 8 hpf, we concluded that this time was the 'dome' or '30% epiboly' stage. However, we could not determine an accurate 'dome' or '30% epiboly' stage because these stages simultaneously occurred at 8 hpf.

Gastrula period: Gastrula was staged between 9 and 15 hpf. The beginning of movements to form the inner layer of cells, so called involution, defined the onset of gastrulation. Subsequently, formation of the germ ring and the

Fig. 5. *ntl* and *gsc* expression detected by whole-mount *in situ* hybridization from 6 to 15 hpf. (A) *gsc* is graded expression in part of the blastoderm at 6 hpf (arrowheads). (B) *ntl* is expressed in a local marginal region of the blastoderm at 6 hpf (arrows). (C) *gsc* expression is localized along the margin of the blastoderm at 8 hpf (arrowheads). (D) *ntl* is expressed all around the blastoderm margin at 8 hpf (arrows). (E–H) *gsc*- and *ntl*-expressing regions are indicated by blue and brown, respectively. (E) The *gsc*-expressing region migrates toward the animal pole, but *ntl* expresses in the germ ring at 10 hpf. (F) The *gsc*-expressing region completely separates from the *ntl*-expressing region at 11 hpf. (G) The *ntl*-expressing region is expanded to the presumptive notochord at 12 hpf. (H) *gsc* is expressed in the prechordal plate and *ntl* is expressed in the presumptive notochord at 15 hpf.

embryonic shield was recognized as the external characteristic of the gastrula. With regards to zebrafish, gastrulation occurs at 50% epiboly. The formation of the germ ring and the embryonic shield of goldfish (Yamaha *et al*., 1999), medaka (Iwamatsu, 1994), *Fundulus* (Armstrong and Child, 1965) and salmonid fishes (Ballard, 1973) occurs before 50% epiboly. In the loach, gastrulation occurred at 9 hpf on 50% epiboly. The germ ring and embryonic shield were formed within 1 h after 50% epiboly. Epiboly continued after the temporal arrest of embryonic shield formation. Epiboly advanced at a rate of 10% of the yolk cell each hour. Finally, epiboly ended when the blastoderm completely covered the yolk plug. Thus, we defined 100% epiboly at 15 hpf. However, asynchrony of development was observed in all developmental stages. This asynchrony became more obvious as time passed. This phenomenon has been observed in zebrafish (Kimmel *et al*., 1995) and goldfish (Yamaha *et al*., 1999). It is thought that this problem could be explained by slight differences in incubations condition or developmental abilities of individual embryos.

The course of morphological change and gene expression in the development of the loach resembled those observed in zebrafish, though developmental speed and incubation temperature were different between the two species. Thus, the knowledge of the developmental stages of the zebrafish may applicable to the loach.

In this study, we defined developmental indexes of the early embryonic stages in the loach based on morphological features and gene expression. This information advances the biotechnology of the loach, as embryos can be used at their optimum development stage for manipulation. It was shown that the developmental fate of the blastomere has not been determined before gastrulation in zebrafish (Ho and Kimmel, 1993). In the loach, the period from 5 to 8 hpf seems to be suitable for blastoderm manipulation. For further progress of the biotechnology of the loach, it is necessary to clarify the germ cell lineage to pick up primordial germ cells selectively, as well as to clarify the pluripotency of embryonic cells in several developmental stages to use donor cells.

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