Int. J. Dev. Biol. 45: 661-668 (2001)

Original Article

Mesoderm formation by isolated and cultivated 8-cell stage blastomeres of the teleost, *Leucopsarion ptersii* (shiro-uo)

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ABSTRACT Isolation of cleavage-stage blastomeres and the study of their developmental potential has been used extensively for analyzing the mechanisms of embryogenesis in vertebrates, including amphibians and echinoderms. We devised a method to isolate 8-cell stage blastomeres in the teleost, shiro-uo, by utilizing its unique cleavage pattern of the horizontal 3rd cleavage plane. Removal of all the upper blastomeres at the 8-cell stage allowed almost normal embryogenesis from the remaining lower blastomeres and yolk cell mass. Isolated upper or lower blastomeres formed vesicles and spherical bodies, which later showed morphological changes during cultivation. Mesoderm formation was detected not only in the cultivated lower blastomeres or whole blastomeres but also in the upper blastomeres isolated from the yolk cell mass at the 8-cell stage, although at a lower frequency than the lower blastomeres. These results indicated the presence of very early signaling for mesoderm induction, which is independent from the currently postulated signals from the yolk syncytial layer at later stages. This also indicated non-equivalence or differentiation of the blastomeres from the very early cleavage stage in teleost embryos.

KEY WORDS: *ice goby (shiro-uo), 8-cell stage, isolated blastomeres, mesoderm induction, teleost embryogenesis*

Introduction

Isolation of cleavage-stage blastomeres and analysis of their developmental potentials are effective ways to resolve mechanisms of early development such as mesoderm induction, organizer formation and axis formation. Such isolation experiments have provided important information about echinoderm and amphibian embryogenesis (Driesch, 1892; Hörstadius, 1928, 1939; Spemann, 1938; Kageura and Yamana, 1986; Kageura, 1995).

In teleost fishes, isolation of blastomeres at early cleavage stages is difficult because early cleavages produce one layer of blastomeres up to the 16 or 32-cell stages and all blastomeres are connected to the yolk cell. Shiro-uo embryos, however, have a unique cleavage pattern among fish embryos and it resembles that of echinoderm and amphibian embryos (Nakatsuji *et al.,* 1997; Arakawa *et al.,* 1999). The shiro-uo (ice goby), *Leucopsarion petersii*, belongs to the most advanced and largest group of the teleosts, the Perciformes group, and inhabits the inshore area of Japan. During the initial steps of embryogenesis, the first two vertical cleavages produce 4 blastomeres on the yolk surface at the 4-cell stage. The third cleavage is horizontal in all blastomeres, and it produces two tiers of blastomeres at the 8-cell stage. The forth cleavage plane appears vertically in all blastomeres at the16-cell stage (Arakawa *et al.,* 1999).

We took advantage of this unique cleavage pattern and devised an isolation method of the 8-cell stage blastomeres to examine their developmental potency. Upper blastomeres, lower blastomeres or all the blastomeres could be isolated from the yolk cell mass at the 8-cell stage. After cultivation, they formed vesicles or spherical bodies and later showed morphological changes reminiscent of activin-treated animal cap explants of amphibian embryos (Asashima *et al.,* 1990). In such explants, *ntl* gene expression was detected by whole mount *in situ* hybridization analysis not only in the isolated lower blastomeres but also in the isolated upper blastomeres, thus indicating the occurrence of mesoderm formation in blastomere explants, without exogenous stimulation signals.

Abbreviations used in this paper: FGF, fibroblast growth factor; *ntl, no tail;* PCR, polymerase chain reaction; YSL, yolk syncytial layer.

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0214-6282/2001/$25.00

Printed in Spain
www.ijdb.ehu.es

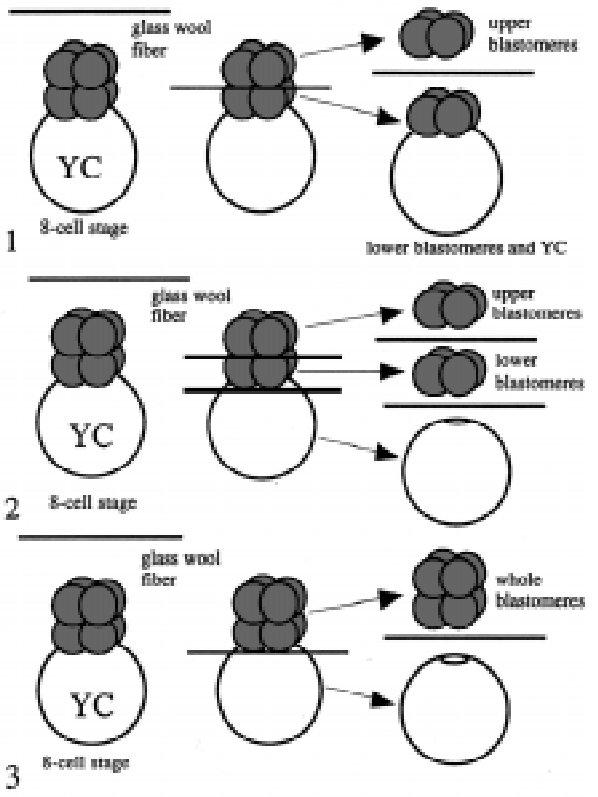

Fig. 1. Method of blastomere isolation. *Mechanical isolation methods of blastomeres at 8-cell stage. Larger circles indicate yolk cell mass (YC), while smaller circles indicate blastomeres.* **(1)** *Isolation of upper 4 blastomeres.* **(2)** *Isolation of upper and lower 4 blastomeres.* **(3)** *Isolation of whole blastomeres (all the 8 blastomeres) from yolk cell mass.*

Results

Isolation of the 8-cell stage blastomeres

We devised a mechanical isolation method for blastomeres at the 8-cell stage by modifying the method using a glass wool fiber, which was used in experiments with goldfish (Yamaha *et al.,* 1997, 1998; Mizuno *et al.,* 1997) (Fig. 1). Isolation of all the upper blastomeres from 8-cell stage embryos was achieved by pressing a glass wool fiber along the third cleavage plane. Also, isolation of all the lower blastomeres was achieved by pressing a glass fiber along the joint plane with the yolk cell mass after removal of the upper blastomeres. Severe damage or disruption of blastomeres by the operation was avoided by using a medium containing albumin.

Explant culture from isolated blastomeres

After removal of all the upper blastomeres, the development of the remaining lower blastomeres and yolk cell mass was examined. A slightly smaller size was exhibited but basically normal embryogenesis was observed (Fig. 2, right column). Twenty out of 21 operated embryos completed axial formation and almost normal development. After the operation, a vertical cleavage produced 8 blastomeres, followed by the next horizontal cleavage and apparently normal cleavage and blastula stages. Epiboly started, and the embryonic shield and axial structures formed normally. The duration of epiboly, however, was prolonged for approximately 3 h when compared with the intact embryos. We obtained similar results when all the upper blastomeres were removed at the 16-cell stage (data not shown).

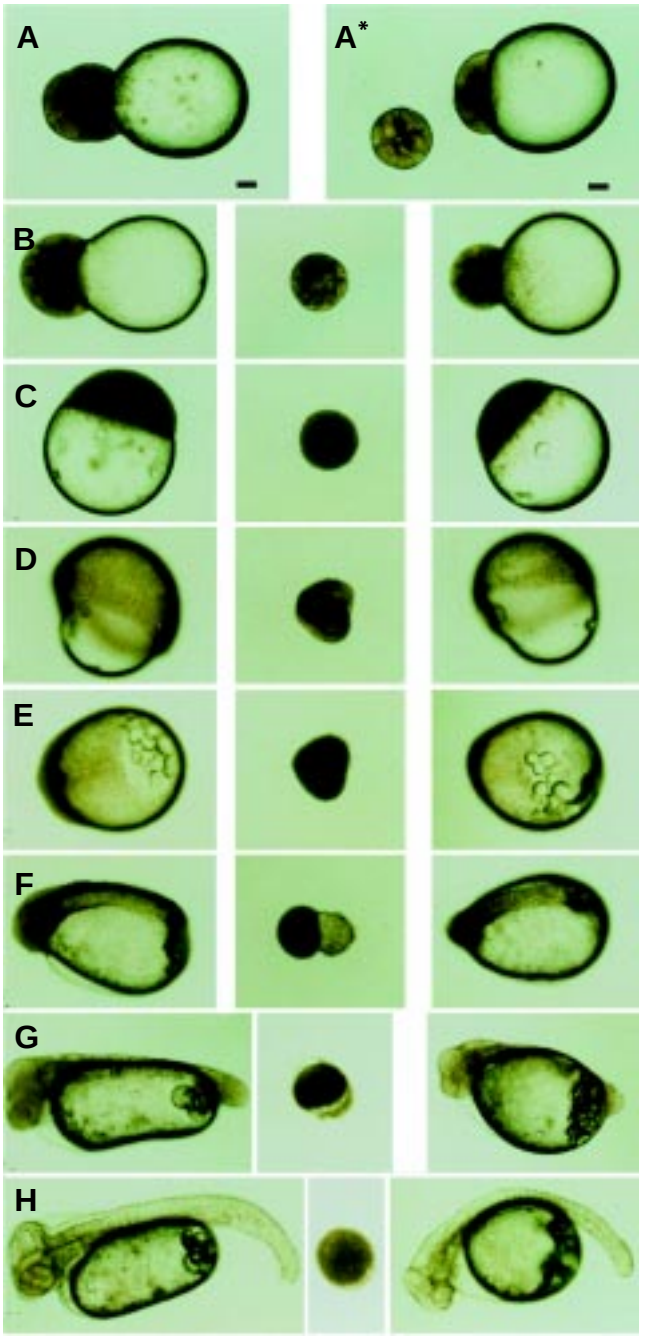

Fig. 2. Developmental potential after blastomere isolation. *Morphogenesis of* **(middle column)** *isolated upper blastomeres and* **(right column)** *remaining lower blastomeres and yolk cell mass . An 8-cell stage embryo* **(A)** *before and* **(A*)** *after isolation of all upper blastomeres.* **(Left column)** *Development of an intact embryo .* **(B)** *32-cell stage,* **(C)** *10%-epiboly stage,* **(D)** *50%-epiboly stage,* **(E)** *100%-epiboly stage,* **(F)** *3-somite stage,* **(G)** *10-somite stage, and* **(H)** *26-somite stage. Isolated upper blastomeres (middle column) developed to a vesicle and spherical body and exhibited elongation. Embryogenesis of the remaining lower blastomeres and yolk mass (right column) was almost normal. Bars, 100 μm.*

Isolated upper blastomeres continued cleavage and formed vesicles or spherical bodies at stages corresponding to the 50%-epiboly stage of the control embryos. These vesicles had resemblance to the animal cap explants of amphibian embryos or permanent blastulae formed by the animal pole blastomeres of echinoderm embryos (Fig. 2D). Then, they exhibited signs of morphogenesis by elongation or formation of a protrusion at a stage corresponding to the 3-somite stage of intact embryos. This morphogenesis resembled that of activin-treated animal cap explants of amphibian embryos (Fig. 2F). Fig. 3 shows the frequencies of the explants that showed protrusion or elongation and those remaining as simple vesicles or spherical bodies 3 days after the operation, which corresponds to the 21- to 24-somite stages of intact embryos. Isolated whole blastomeres (indicating all the 8 blastomeres without the yolk mass) showed the highest frequency (85.7%) of morphogenesis, while isolated upper blastomeres showed the lowest frequency (22.7%).

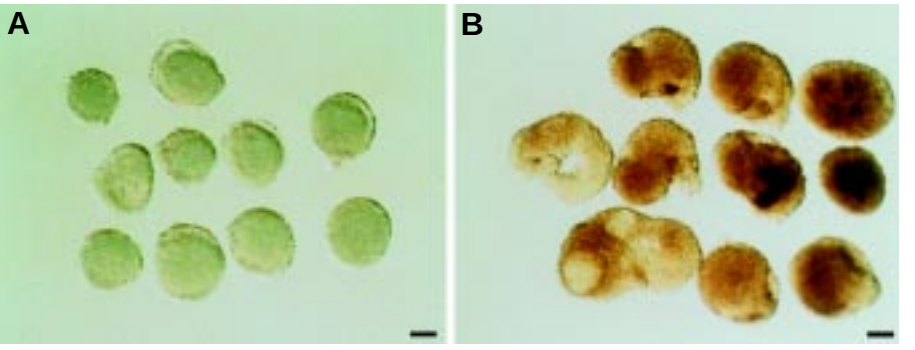

Fig. 4. Development of explants from mid-blastulae. *Morphogenesis of explants of* **(A)** *upper-half blastoderm and* **(B)** *lower-half blastoderm 3 days after operation at the mid-blastula stage. Explants of upper-half blastoderm formed only vesicles, while some of the lower-half blastoderm exhibited elongation. Bars, 100μm.*

Similar isolation experiments were carried out at the mid-blastula stage for comparison. Development of isolated upper-half blastoderm, lower-half blastoderm or whole blastoderm (indicating the isolated intact blastoderm without yolk mass) were examined up until 3 days, when intact embryos were at the 26- to 28-somite stages (Fig. 4). The isolated upper-half blastoderm formed only vesicles, while some of the isolated lower-half blastoderm exhibited elongation after formation of vesicles. All of the isolated whole blastoderm exhibited elongation (Fig. 3). It is worth noting that none of the upper-half blastoderm isolated at the mid-blastula stage showed elongation or protrusion, while 22.7% of the upper blastomeres isolated at the 8-cell stage exhibited morphogenesis.

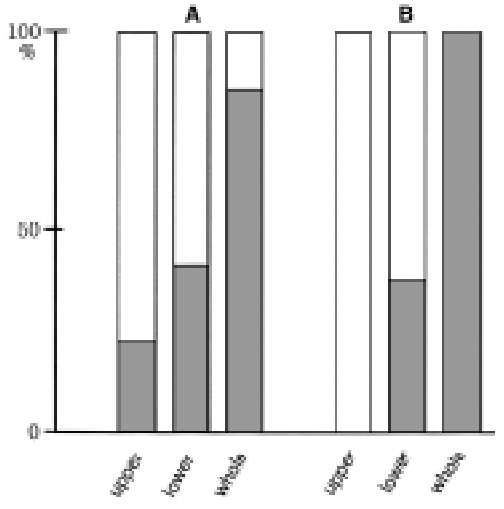

Fig. 3. Frequency of explants showing morphogenesis. (A) *Frequencies of explants showing elongation or protrusion (closed bars), or those remaining as a vesicle or spherical structure (open bars), 3 days after operation at the 8-cell stage. No. of explants examined were 44 (upper blastomeres), 17 (lower blastomeres) and 7 (whole blastomeres).* **(B)** *The same frequencies 3 days after operation at the mid-blastula stage. No. of samples were 21 (upper-half blastoderm), 21 (lower–half blastoderm) and 20 (whole blastoderm; indicating the isolated intact blastoderm without yolk mass).*

Mesoderm formation in explants

Histological examination of the isolated upper blatsomeres after cultivation for 24 h, when intact embryos were at the 50%-epiboly stage, indicated the presence of a cavity in the center of a spherical structure, which had a similarity with the animal cap explants of amphibian embryos and the permanent blastulae formed by isolated animal pole blastomeres of echinoderm embryos (Fig. 5). Furthermore, some of the elongated explants from upper blastomeres of 8-cell stage embryos contained morphologically differentiated cell masses, including those resembling blood cells, in the spherical body at 56 h after the isolation, when intact embryos were at the 13 to 14-somite stage (Fig. 5). Such differentiated explants showed positive bands for the GATA-3 gene expression in the RT-PCR analysis using a set of primers specific for the shiro-uo GATA-3 mRNA, thus indicating the hematopoietic differentiation (Neave *et al.,* 1995; Manaia *et al.,* 2000).

In order to analyze morphogenesis in the explants at a molecular level, expression of the panmesodermal maker gene *ntl (no tail)* was examined. A 320 bp cDNA fragment of shiro-uo *ntl* was amplified from cDNA of early somite stage embryos using degenerate primers designed to a conserved region in the T-box between human and amphyoxius brachyury. The amino acid sequence encoded by the shiro-uo *ntl*cDNA fragment (Fig. 6) was 92% and 87% identical with human and amphyoxius brachyury, respectively. Whole-mount *in situ* hybridization was carrying out using digoxigenin-labelled antisense riboprobes prepared from the cDNA fragment. We first examined *ntl* expression in intact embryos. Expression patterns were almost the same as those in the zebrafish (Schulte-Merker *et al.,* 1992, 1994). During the doming and early gastrulation stages, expression of *ntl* mRNA was detected in the marginal zone of blastoderm forming a ring (data not shown). At the 80% epiboly stage, hypoblast cells of the germ ring showed the expression of *ntl*mRNA (Fig. 6A). At the bud stage, cells in the presumptive notochord and tailbud expressed higher levels of the *ntl*mRNA (Fig. 6B). During the somite stages, the expression was elongated along the anterior-posterior axis and highest at the posterior end (Fig. 6C).

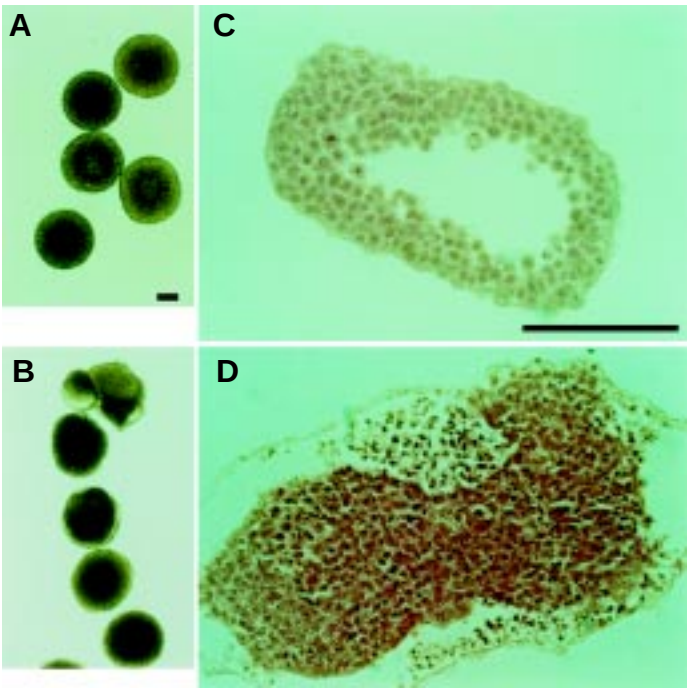

Fig. 5. Histology of explants. *Whole mount observation and histological sections of the isolated upper blastomeres of 8-cell stage embryos after cultivation for* **(A,C)** *24 h, showing cavity formation and* **(B,D)** *56 h, showing the presence of differentiated cell masses).* A,B *and* C,D *are at the same magnification. Bars 100*μ*m.*

Whole-mount *in situ* hybridization of the explants of isolated blastomeres also indicated expression of *ntl* mRNA. At 26 h after isolation of blastomeres at the 8-cell stage, which corresponds to the 80% epiboly stage, not only the explants of the whole blastomeres but also those of the upper blastomeres exhibited *ntl* expression (Fig. 6D). In the explants of upper blatomeres, *ntl* expression first appeared as a deformed ring and later converged to an end of the explant (Fig. 6 D,E,F). Explants of whole blastomeres also showed expression in a similarly restricted region (data not shown). Histological sections of *ntl* stained explants, at a stage corresponding to the bud stage of intact embryos, exhibited expression in a cell mass that included the inner cells (Fig. 6G).

Approximately 10% of the explants of upper blastomeres showed *ntl* expression, which corresponded to approximately half of the explants that exhibited morphgenesis (Fig. 7). In the explants of whole blastomeres, about 38% of all the samples examined showed *ntl* expression (Fig. 7).

To characterize ectodermal differentiation, we examined the expression of *keratin* mRNA in these explants, when intact control embryos were at the 11-somite stage. *Keratin* mRNA expression was detected in the surface cell layer of the spherical bodies, indicating differentiation of the epidermis on the explant surfaces (Fig. 8).

Discussion

In the present study, we developed a method to analyze developmental potentials of the blastomeres isolated from the early cleavage stage embryos of the teleost *Leucopsarion ptersii* (shiro-uo). The mechanical isolation method with a glass wool fiber, used previously for goldfish embryos (Yamaha *et al.,* 1997, 1998; Mizuno *et al.,* 1997), was successfully applied to shiro-uo embryos without causing cell damage.

Our results indicated that the blastomeres of 8-cell stage embryos already possess a signal or factor to induce mesoderm formation. Also, unequal partitioning or strength of such factors exists between the upper and lower tier blastomeres in 8-cell stage embryos (Fig. 9).

Isolation experiments of 8-cell stage blastomeres of the echinoderm by Hörstadius (1928, 1939) indicated that the 4 animal blastomeres form only hollow balls of ciliated epidermal cells, but the 4 vegetal blastomeres can develop into slightly abnormal embryos. These studies established the idea that a developmental gradient exists along the animal-vegetal axis in early echinoderm embryos and that the vegetal half contains the organizing center of embryogenesis.

Extensive blastomere isolation experiments were carried out using *Xenopus laevis* embryos at 2-, 4-, or 8-cell stages (Kageura and Yamana, 1983; Kageura and Yamana, 1986; Kageura, 1995). For example, reconstituted embryos containing two animal, one dorsovegetal and one ventrovegetal blastomeres of 8-cell stage embryos developed into complete tadpoles (Kageura and Yamana, 1986). Furthermore, a minimal set of one dorsovegetal, two ventrovegetal, and eight animal blastomeres from 32-cell stage embryos was enough for complete tadpole development (Kageura, 1995). These results indicate that blastomeres at early cleavage stages possess different developmental capacities and that both animal and vegetal blastomeres are required for embryogenesis. Also, in *Xenopus laevis,* the partitioning of morphogenetic information into animal quartets by the horizontal third cleavage plane results in gravity-dependent differential morphogenesis and gene regulation (Chung *et al.,* 1994). In fish embryos, however, it has not been clear whether blastomeres at early cleavage stages are equivalent or possess any developmental differences to each other.

The present isolation experiments of upper and lower blastomeres at the 8-cell stage of shiro-uo embryos enabled investigation of their developmental potency. Thus, elongation of the explants occurred at a higher rate in the isolated lower blastomeres than the upper blastomeres. Such elongation is morphologically similar to that of the animal cap of amphibian embryos when the mesoderm induction occurs (Asashima *et al.,* 1990; Smith *et al.,* 1990). Also, the expression of a panmesodermal maker *ntl* (Schulte-Merker *et al.,* 1992, 1994) was detected in the explants of blastomeres isolated at the 8-cell stage. These results indicate that signals for subsequent mesoderm induction are already present in the blastomeres in unequal amounts or strength at the early-cleavage stage in shiro-uo embryos.

One of the first intercellular signaling evens in the vertebrate embryo leads to mesoderm formation and the determination of the dorso-ventral axis. Molecular mechanisms and factors involved in mesoderm induction are well defined in amphibians. The Nieuwkoop center located in the dorso-vegetal blastomeres of cleavage stage amphibian embryos plays a central role in the induction of dorsal mesoderm and formation of the Spemann organizer (Nieuwkoop, 1969; reviewed in Kodjabachian and Lemaire, 1998).

In teleosts such as the zebrafish, the functional equivalent of the amphibian Spemann organizer is the dorsal embryonic shield at the gastrula stage (Schier and Talbot, 1998). Results of yolk cell transplantation experiments at the mid-blastula stage of zebrafish

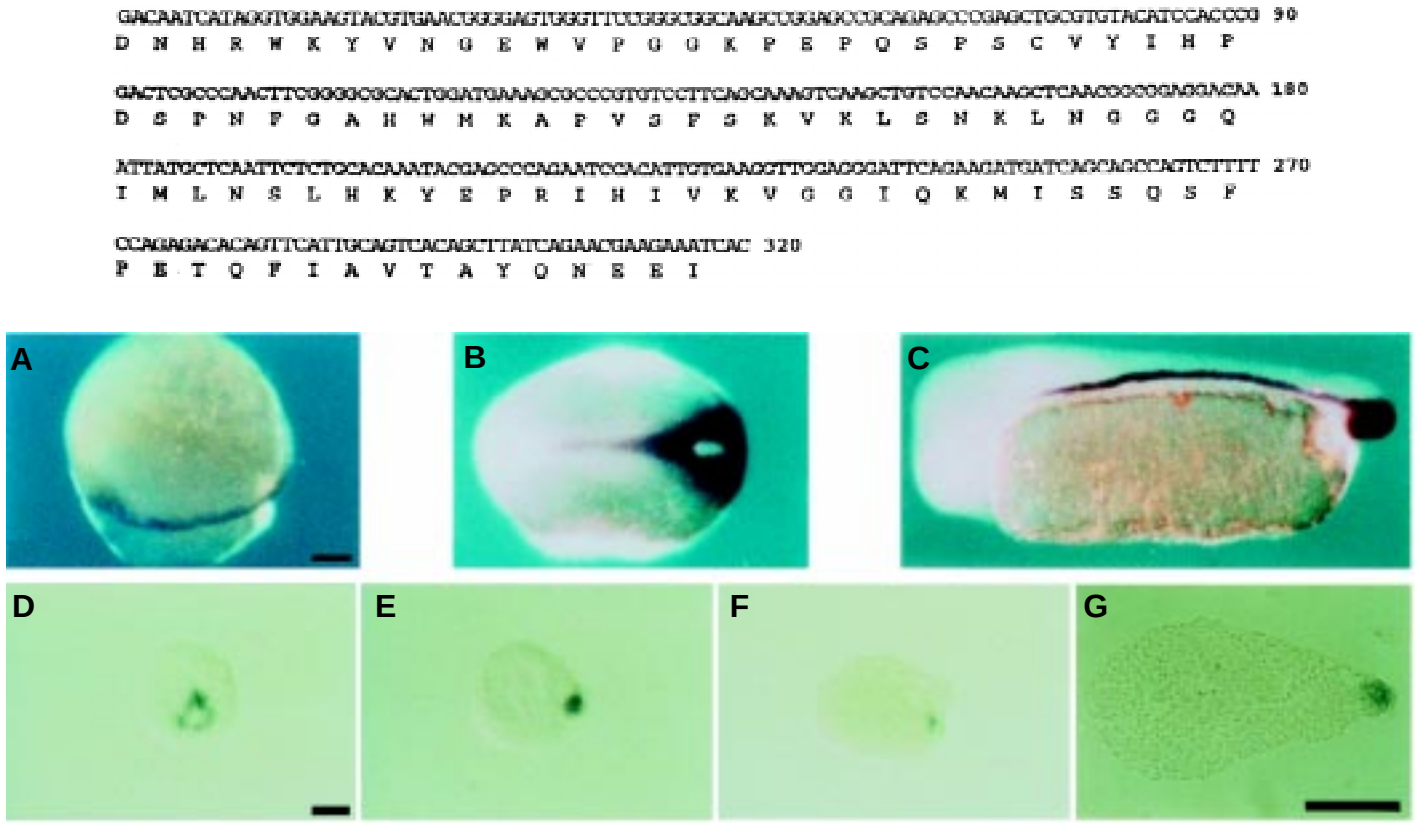

Fig. 6. Expression of *ntl* in explants. (Top) *Nucleotide and predicted amino acid sequences of the ntl cDNA fragment isolated from shiro-uo embryos.* **(A,B,C)** *Whole-mount in situ hybridization samples of ntl expression in intact shiro-uo embryos at the* **(A)** *80%-epiboly,* **(B)** *bud, or* **(C)** *9-somite stages.* **(D,E,F)** *Whole-mount in situ hybridization of ntl expression in the explants of upper blastomeres at the time corresponding to* **(D)** *80%-epiboly,* **(E)** *bud stage, or* **(F)** *4-somite stage of intact control embryos.* **(G)** *A histological section of a sample similar to (E). A,B,C and D,E,F are at the same magnification. Bars 100μm.*

embryos indicated that mesoderm-inducing signals are derived from the yolk cell and passed through the YSL to the blastoderm cells (Mizuno *et al.,* 1996, 1999). Furthermore, *dharma* and *nieuwkoid* gene expression suggests that the yolk cell and YSL have mesoderm-inducing capacity and that the dorsal YSL region corresponds to the Nieuwkoop center (Yamanaka *et al.,* 1998; Koos and Ho, 1998). Analysis of the expression of *ntl* in *janus*-mutant zebrafish embryos indicates that mesoderm induction depends on a marginal position. The yolk cell is also involved in the formation of dorsoventral asymmetry (Abdelilah and Driever, 1997). In medaka embryos, *ntl* and *gsc* gene expression in explants that had been isolated from the YSL during the late-morula to late–blastula stages suggested that mesoderm formation can occur in the absence of the YSL from these stages (Hyodo *et al.,* 1999). The requirement of both the unidentified inducing signal released from YSL for the formation of ventrolateral mesendoderm and the YSL-independent inductive signals for dorsal mesoderm was demonstrated in zebrafish using RNase injection technique (Chen and Kimelman, 2000).

In case of shiro-uo embryos, cultivated blastomeres isolated from the yolk cell at the 8-cell stage, without stimulation by exogenous inducing factors, exhibited elongation that is a typical sign of mesoderm induction in amphibian animal cap assays. Furthermore, the panmesodermal maker *ntl* was expressed in these explants. Also, we found indication of the hematopoietic differentiation. These results indicate that much earlier events than previously thought are involved in mesoderm induction, although we could not find out whether these explants contained dorsal mesoderm or not. Our data suggest the presence of an early signaling mechanism that is independent of the YSL activity at later stages. Alternatively, it might be possible that the mesoderm inducing signals of the YSL originate in blastomeres at early cleavage stages and are later transferred to the YSL, because the YSL is originally derived from the blastomeres. The last unlikely possibility is that expression of *ntl* and mesoderm formation occurred in the explants by artificial signaling that differs from normal mesoderm induction.

Interestingly, none of the upper-half blastoderm isolated at the mid-blastula stage showed elongation or protrusion, while the upper blastomeres isolated at the 8-cell stage exhibited morphgenesis. A study of the spatial and temporal regulation of the *Xenopus ntl* promoter using transgenic embryos showed that repression plays an important role for the restriction of gene expression to the mesoderm (Lerchner *et al.,* 2000). A similar repression mechanism may be involved in the different results of expants isolated at different stages of shiro-uo embryos, such that a local restriction of mesoderm differentiation may develop in the animal blastomeres during developmental progression.

Fate mapping and gene expression analyses in zebrafish showed that differential *noda*/signals may determine localization

of the organizer before gasturulation (Gristsman *et al.,* 2000). In *Xenopus,* mesoderm induction was mediated by a gradient of multiple *nodal*-related signals released by endoderm at the blastula stage (Agius *et al.,* 2000), and they are probably initiated by activin-like molecules (Osada and Wright, 1999).

Mesoderm-inducing upstream factors, such as FGFs and activin are present in unfertilized eggs and early amphibian embryos (Kimelman *et al.,* 1988; Asashima *et al.,* 1991), and determination of the dorso-ventral axis begins soon after fertilization. Also, cytoplasmic maternal determinants play important roles in the patterning of mesoderm in amphibians (Lemaire and Gurdon, 1994; Ding *et al.,* 1998; Darras *et al.,* 1997; Marikawa *et al.,* 1997). In ascidian embryos, maternal gene products are segregated within the endoplasm (Imai *et al.,* 1999). In the shiro-uo, factors for mesoderm induction may have translocated from the yolk cell to the blastomeres by the 8-cell stage. Alternatively, the early blastodisc cytoplasm may already contain factors for mesoderm formation. We have scarce knowledge about differential distribution of mRNAs or proteins among blastomeres at the early cleavage stages in teleosts embryos. Identification of maternal factors and functional analysis of zygotically expressed genes before the mid-blastula transition (MBT) in teleost embryos could be greately facilitated by using shiro-uo embryos, because their blastomeres are suitable for isolation or ablation experiments at very early cleavage stages.

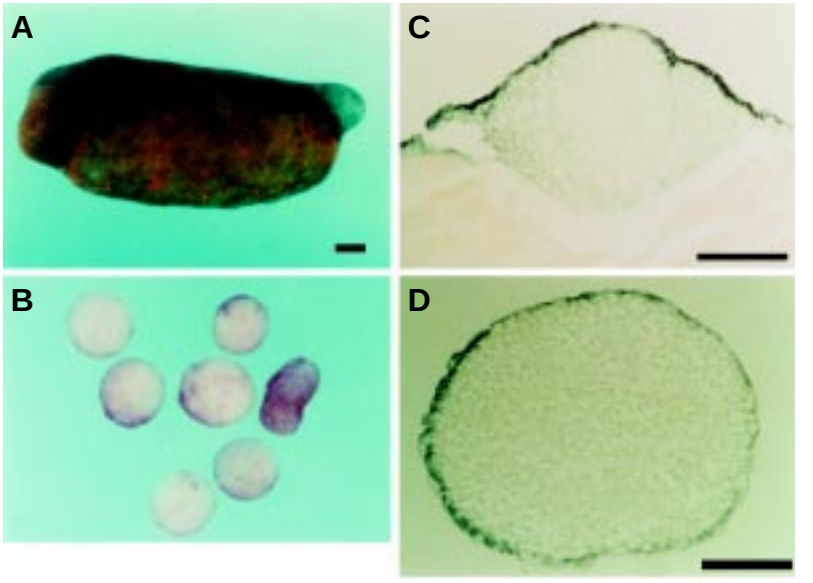

Fig. 8. Expression of *keratin* in explants. (A,B) *Whole-mount in situ hybridization using antisense keratin RNA probes and* **(C,D)** *histological sections. (A,C) A control embryo at the 11-somite stage and (B,D) explants of upper blastomeres. A and B are at the same magnification. Bars; 100μm.*

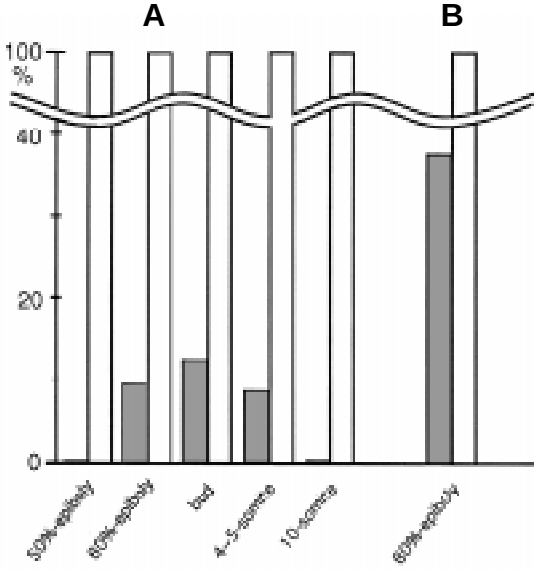

Fig. 7. Frequency of *ntl* expression in explants. (A) *Frequencies (closed bar) of ntl expression in the explants of upper blastomeres examined by in situ hybridization. ntl expression was examined at stages corresponding to the 50%-epiboly (n=25), 80%-epiboly (n=21), bud (n=24), 4~5-somite (n=23) and 10-somite (n=10) stages of intact embryos.* **(B)** *The same frequency in the explants of whole blastomeres (n=8) at the time corresponding to 80%-epiboly. All of the control intact embryos examined at the corresponding stages exhibited ntl expression as shown by open bars.*

Materials and Methods

Fertilized eggs

Adult shiro-uo, *Leucopsarion petersii,* which had been ascending a river for spawing when caught by fishermen, were purchased from the beginning of January to June. They were maintained, keeping females and males separate, in aquariums at 19°C. Adult males were maintained in the dark for 2 weeks to bring about gonadal maturation, while adult females were injected with 25 units of gonadotropin (Teikokuzouki) to cause ovulation within 10 days. Maturation and ovulation were identified through the transparent body wall. As previously described (Arakawa *et al.,* 1999), eggs were mixed with sperm in diluted (10%) sterile medium M199 (Gibco). Fertilized eggs and embryos were incubated in the same diluted M199 solution in sterilized plastic petri dishes at 19°C.

Isolation of blastomeres

Mechanical isolation of blastomeres at the 8-cell stage was performed by modifying the method previously described (Mizuno *et al.,* 1997). All isolation procedures were performed at 19°C in a 100 mm plastic petri dish coated with 2 mm of 0.8 % agar and filled with sterilized Ringer's solution (128 mM NaCl, 2.8 mM KCl, 1.8 mM CaCl2) supplemented with 1.6 % albumin, 100 units/ml penicillin and streptomycin, and 0.0001 % methylene blue. Firstly, the chorion was removed manually from 8-cell stage embryos by fine forceps. Then, isolation of all the upper blastomeres was achieved by pressing a glass wool fiber gently along the third cleavage plane (Fig. 1). Isolation of all the lower blastomeres was achieved by pressing a glass wool fiber gently along the joint plane with the yolk cell mass and slowly towards the agar bed after removal of the upper tier. Also, the whole blastomeres consisting of all the 8 blastomeres were separated from the yolk cell mass in a similar way. Isolation of the upper-half and lower-half blastoderm of blastula stage embryos was performed as for the 8-cell stage embryos. Explants made in this manner were transferred into fresh sterilized solution and cultured for several days at 19°C.

Histological examination

Embryos were fixed with Bouin's fixative solution for 2 h. Dehydration in a butanol series, embedding in paraffin and sectioning were carried out

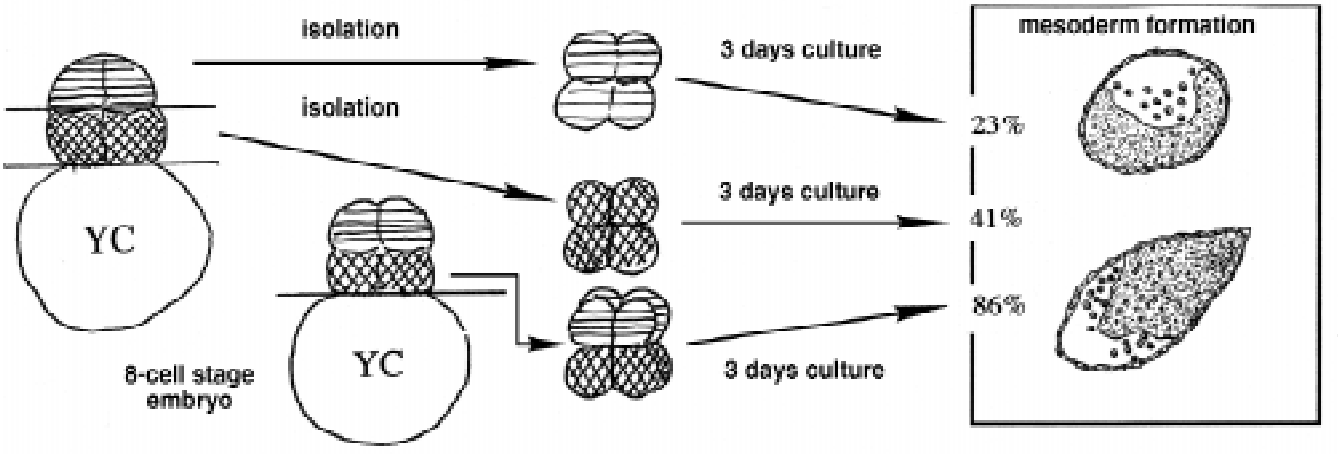

Fig. 9. A diagram to summarize results of the explant culture of blastomeres isolated from 8-cell stage embryos.

following conventional methods. Sections were cut at 8 μm and stained with hematoxylin-eosin.

RT-PCR analysis

The mRNAs were isolated from each of the differentiated explants at 3~4days after the isolation of upper blastomeres from 8-cell stage embryos using the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech). The first strand cDNA was synthesized using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech). 1 μl of first strand cDNA was amplified with Taq polymerase (Takara).

A pair of primers: 5'-TATCTCCGCAACGCCTGCGGTCTC-3') and 5'-TAGTAGAGGCCACAGGCGTTACAC-3') were used for amplifying a191 bp fragment of shiro-uo GATA-3 gene. Amplification was performed for 30 cycles at 60°C.

PCR amplification of the shiro-uo ntl cDNA fragment

mRNAs were isolated from 30 shiro-uo embryos at 4~5-somite stages using the QuickPrep Micro mRNA Purification Kit (Amasham Pharmacia Biotech), and synthesized to double-strand cDNA using the Time Saver cDNA Synthesis Kit (Amasham Pharmacia Biotech). To amplify cDNA fragments of *ntl*, degenerate PCR primers were designed to conserved regions, DNHRWKY and YQNEEIT, between human (Accession No. O15178) and amphioxus (Accession No. Q17134) brachyury, as follows; 5'-GA (Y) AA (Y) CA (Y) (M) G (NC) TGGAA (R) TA-3' (upper) and 5'-GT (D) AT (Y) TC (R) TT (Y) TG (R) TA-3' (lower). The expected length of PCR products was 320 bp. The PCR product of the expected length was agarose-gel purified, ligated to the pCRII vector (Invitrogen), and sequenced using the Big Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin Elmer Biosystem).

Whole-mount in situ hybridization

Staged embryos and explants were fixed overnight at 4°C with 4 % paraformaldehyde in 50 % diluted PBS, rinsed, dehydrated through a methanol series, and stored in absolute methanol at –20°C. A Digoxigenin (Dig) –labeled antisense riboprobe for *ntl*was generated from the *ntl*cDNA clone in pCRII using the DIG RNA Labeling Kit (Roche), as previously described (Suzuki *et al.,* 1998). A *Keratin* Dig-probe was prepared from a 358 bp of flounder type-I *keratin* (Accession No. AB049616) cDNA fragment. Whole-mount *in situ* hybridization was performed as described by Jowett and Lottice (1994).

Acknowledgement

We thank Drs. H. Takeda and T. Mizuno for helpful discussion, and Mr. M. Hamaguchi, T. Kon, A. Korenori, T. Matsuura, and K. Toritake for supplies of fish. This work was supported in part by High-technology Research Center Program of the Ministry of Education, Culture, Sports, Science and Technology, and Tokai University.

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Received: January 2001
Accepted for Publication: March 2001