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**[Short Communication]**

# Yolk Syncytial Layer Independent Expression of *no tail* (*Brachyury*) or *goosecoid* Genes in Cultured Explants from Embryos of Freshwater Fish Medaka

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**ABSTRACT**—Formation of embryonic axis is an essential step for animal development but its mechanism is not well understood. For axial determination in the fish embryos, participation of the yolk syncytial layer (YSL; a unique multinucleated structure formed in the yolk cell) has been shown. To investigate relationship between the YSL and axial specification, we examined whether or not expressions of the mesodermal marker genes *no tail* (*Brachyury*) or *goosecoid* are dependent on the YSL in explants isolated from the medaka embryos. The results of whole-mount *in situ* hybridization showed that these genes were expressed in the explants irrespective of the YSL, indicating that activation of these genes is a separate process from YSL-dependent axis formation.

## INTRODUCTION

The first patterning step in animal development is establishment of the embryonic axis. Relatively large and transparent teleost embryos provide a good experimental system to study how axial determination occurs in vertebrates. In the matured oocytes of medaka, the animal-vegetal polarity is present as can be recognized from attachment site of long filaments at the vegetal pole, or sperm entry site (micropyle) at the animal pole. And after fertilization, the cytoplasm streams to the animal pole to form a lenticular blastodisc. This animal-vegetal polarity corresponds to the future antero-posterior axis of the embryo (Iwamatsu, 1994; Driever, 1995). Then blastodisc undergoes successive meroblastic cleavages, and at the blastula stage the blastoderm is composed of three domains of cells. They are the enveloping cell layer, the deep cell layer and the yolk cell (Kane *et al*., 1992). Among these, the deep cells will form the entire body of the embryos, whereas the enveloping cells and the yolk cell constitute extra-embryonic lineages. Also at the blastula stage, the nuclei from some of the marginal blastomeres move into the yolk cell and multiply in the cortical cytoplasm to form the yolk syncytial layer (YSL)(Betchaku and Trinkaus, 1978; Kimmel and Law, 1985a).

Fates of the deep cells are not determined at the blastula stage as revealed by their extensive migration and random mixing in zebrafish embryos (Kimmel and Law, 1985b; Warga and Kimmel, 1990). Also, the cells remain totipotent so that transplantation of the blastomeres to recipient embryos resulted in formation of chimeras in the zebrafish (Lin *et al*., 1992) or in the medaka (Wakamatsu *et al*., 1993; Hyodo and Matsuhashi, 1994).

How is the dorso-ventral polarity determined in the radially symmetric blastula-stage embryos? From where the dorsal-inducing signal originates in teleost embryos? Experiments involving embryological manipulations such as microsurgery or transplantation have been performed with some teleost species (Oppenheimer, 1936; Tung *et al*., 1945; Devillers, 1961; Long, 1983; Bozhkova *et al*., 1994; Mizuno *et al*., 1996; Mizuno *et al*., 1997; Yamaha *et al*., 1998) and it is generally considered from these studies that the yolk cell provides the blastoderm with certain substance(s) necessary for embryonic pattern formation (Driever, 1995). Particularly, involvement of the yolk cell or the YSL in specification of the dorsal fate has been demonstrated by Long (1983) and Yamaha *et al*. (1998). When younger rainbow trout blastomeres were transplanted onto gastrula-stage yolk syncytial cells from which the blastoderm had been removed, they developed embryonic structures on top of the host yolk cells with the same polarity as the original host embryos (Long, 1983). And Yamaha *et al*. (1998) showed from similar experiments with the gold fish embryos that during the blastula stage dorsal blastomeres became committed gradually to their dorsal fate

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under the inductive influence of the yolk cell.

In our previous study (Hyodo *et al.*, 1996), we also showed the importance of the YSL for axial patterning of the medaka embryos. We dissected the blastula stage embryos and prepared the explants with or without the upper part of the yolk cell including the YSL. After incubation of these explants in a culture medium, many of them containing YSL developed into fish-like structures with apparent antero-posterior and dorso-ventral axes, but most of those without the YSL developed into irregular aggregates of cells. These results suggested that formation of the YSL was correlated with development of embryonic axial structures. Then we assumed that the mesodermal marker genes *ntl* or *gsc* would be expressed depending on the YSL in explants. In this paper, we examined this possibility by whole-mount *in situ* hybridization using these genes as molecular probes.

## MATERIALS AND METHODS

### Fish and embryos

An orange-colored mutant, one of the body-color mutants of medaka, was used throughout this study. Methods for maintenance of fish, collection and incubation of the embryos were as described previously (Hyodo *et al.*, 1996). Embryos were staged according to Iwamatsu (1994). Embryos at stages 9, 10, 11 were used in this study. The stage 9 embryos were at the late morula stage with blastoderms consisting of 4 to 5 layers of cells. At stage 10, the early blastula, a few rows of the YSL nuclei appeared. And at stage 11, late blastula, the number of YSL nuclei increased to 5 to 6 rows.

### Dissection and incubation of explants

For sterilization, the embryos were soaked for 1 min in a neutral 0.4% NaClO solution and washed with the saline solution. They were then dissected using a pair of small scissors with a 5 mm curved blade. During dissection, most of the yolk was released into the saline. One group of explants was prepared to contain both the blastoderm and the upper part of the yolk cell. This part consisted of membranous material which we refer to as the yolk cell fraction (YCF). The YCF from embryos at stages 10 and 11 contained multinucleated YSL. Another group of explants only contained the blastoderm, from which the YCF was removed.

The explants with or without the YCF were incubated in 199 culture medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 15% fetal calf serum and 50 μg/mL ampicillin. The explants were incubated at 22 to 24°C for 20 to 24 hr until the intact siblings reached stage 18 (formation of eye vesicle) or stage16 (75% epiboly) for detecting expressions of *ntl* or *gsc*, respectively.

### Whole-mount *in situ* hybridization

The medaka *ntl* gene was cloned by K. Araki (National Research Institute of Aquaculture, Mie, Japan)(manuscript submitted) and the *gsc* gene by K. Inohaya (Sophia University, Tokyo, Japan) (Inohaya, 1996). Plasmid DNA's containing these genes were kindly provided by these authors. Digoxigenin-11-UTP labeled antisense probes were synthesized by *in vitro* transcription using linearized plasmid DNAs according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). Because the fish embryos or explants were fragile, most of the steps were carried out using baskets as described by Stachel *et al.* (1993).

The embryos or explants were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C, transferred to 100% methanol and stored at –20°C until further treatment. *In situ* hybridization was carried out as described by Oxtoby and Jowett (1993).

## RESULTS AND DISCUSSION

First, we examined expression patterns of *ntl* and *gsc* genes in control embryos by whole-mount *in situ* hybridization. Expression of *ntl* gene was first detected in a portion of the blastoderm marginin in stage 12 embryos (data not shown). Then, at stage 15 (mid-gastrula), the staining was extended to the whole germ-ring around the blastoderm (Fig. 1A) and a strong signal was noticed at the presumptive dorsal region of the embryo. After appearance of the embryonic body, intense staining was observed at the posterior region of the embryo (Fig. 1B), presumably at the notochord (Schulte-Merker *et al.*, 1992; Schulte-Merker *et al.*, 1994).

Fig. 1C shows a profile of the *gsc* gene expression in a stage 14 embryo (early-gastrula). The staining was at a small portion of the blastoderm margin, the future dorsal region of the embryo (Stachel *et al.*, 1993; Thisse *et al.*, 1994). Then, in the late gastrula embryo, a strong signal was observed at the most anterior portion of the embryonic body (Fig. 1D). These results of *ntl* and *gsc* expressions indicated that these two

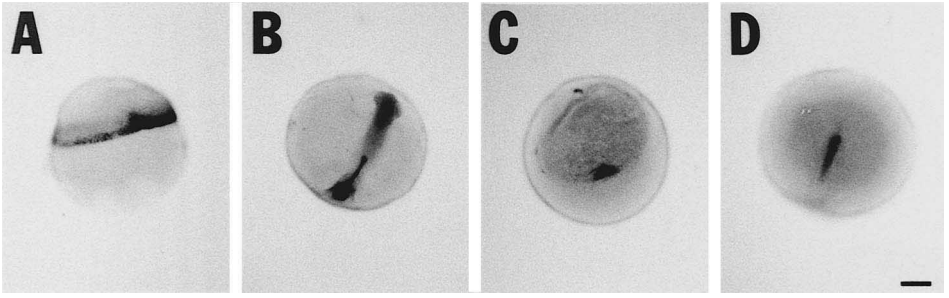


**Fig. 1.** Expression of ntl and gsc genes in medaka embryos by whole-mount *in situ* hybridization. (A) *ntl* gene expression in a stage 15 embryo (mid-gastrula and 40–50% epiboly) viewed from the lateral side. The future embryonic body is slightly to the right. (B) *ntl* gene expression in a stage 21 embryo (6-somite stage), dorso-lateral view; the top is the anterior end. (C) *gsc* gene expression in a stage 10 embryo (early blastula) viewed from the animal pole. (D) *gsc* gene expression in a stage 16 embryo (late gastrula, 75% epiboly), lateral side view; the embryonic body is slightly to the left. The top is the anterior end. Bar is 200 μm.

genes would be good markers for detecting axial specifications.

We have focused our attention on the mechanism which operates for determination of the dorso-ventral axis in medaka embryos, particularly on the relationship between formation of the YSL and axial specification. Our previous study (Hyodo *et al*., 1996) demonstrated that the YSL plays an important role for patterning the axial structure during development of dissected explants. From these observations, we assumed that expression of the mesoderm marker genes *ntl* or *gsc* might be strongly correlated with formation of the YSL. For detection of the *ntl* and *gsc* expressions in the explants, we have used the embryos at stages 9, 10 and 11. We have prepared two groups of explants, one with the yolk cell fraction (YCF), the upper part of the yolk cell, and another without it. In the explants from stage 10 and 11 embryos, the YCF contained the YSL. The explants were incubated in the 199 culture medium for 20 to 24 hr and expressions of the *ntl* and *gsc* genes were analysed by whole-mount *in situ* hybridization. The results showed that most of the explants were positively stained by these probes. Fig. 2 shows *ntl* and *gsc* gene expressions in explants from stage 9 without or with the YCF. Fig. 3 shows *ntl* and *gsc* gene expressions in stage 11 explants without or with the YCF. And Table 1 summarizes the results of this study. The results indicated that expressions of the *ntl* and *gsc* genes in the explants occurred irrespective of the YSL.

Although most of the explants were positively stained, the profiles of explants and staining patterns differed among the stages examined. The shapes of most stage 9 explants were spherical (Fig. 2) while explants at the later stage (Fig. 3) were elongated. When the staining patterns of both genes were compared, they were more intensive and broader in stage 9 explants (Fig. 2) but were restricted to a portion close to one end in stage 11 explants (Fig. 3). The restricted staining profiles at the later stage resembled those of the control embryos. And there was no difference in explant profiles or staining patterns between the explants with or without the YCF.

From the results of this study, we conclude that expression of these genes in the explants was independent of formation of the YSL. And it was also suggested that expression of these genes had been determined at an earlier stage than formation of the YSL. Earlier determination of the expression domains of these genes in the blastoderm seems to be inconsistent with the uncommitted nature of the deep cells or their random mixing in the blastula embryos (Kimmel and Law, 1985b; Warga and Kimmel, 1990). This discrepancy, however, might be explained by considering that determination of gene expression occurs in two steps of signal transfer, first from the yolk cell to the marginal blastomeres, and second from the marginal cells to the deep cells. Recent reports from several laboratories support this model. Concerning the first step, Mizuno *et al*. (1997) and Yamaha *et al*. (1998) showed

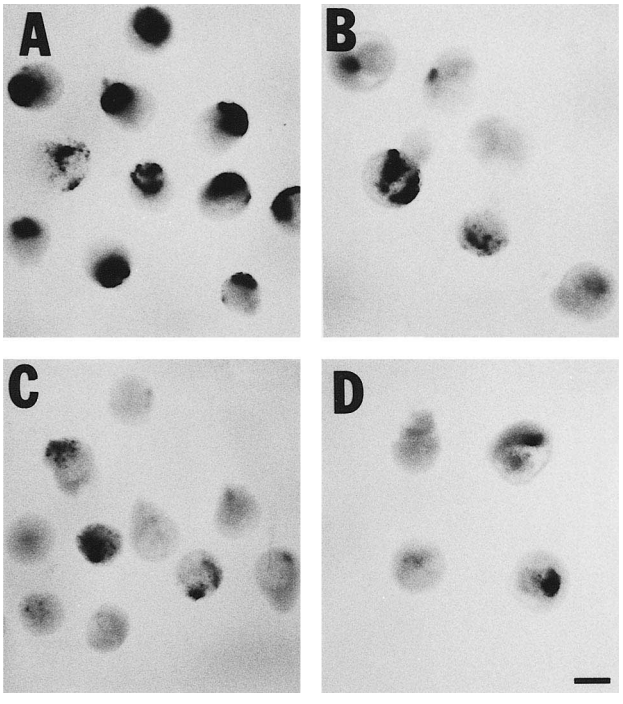


**Fig. 2.** Expression of *ntl* and *gsc* genes in medaka explants from stage 9 embryos by whole-mount *in situ* hybridization. (A) *ntl* gene expression in explants without the YCF. (B) *ntl* gene expression in explants with the YCF. (C) *gsc* gene expression in explants without the YCF. (D) *gsc* gene expression in explants with the YCF. Bar is 100 μm.

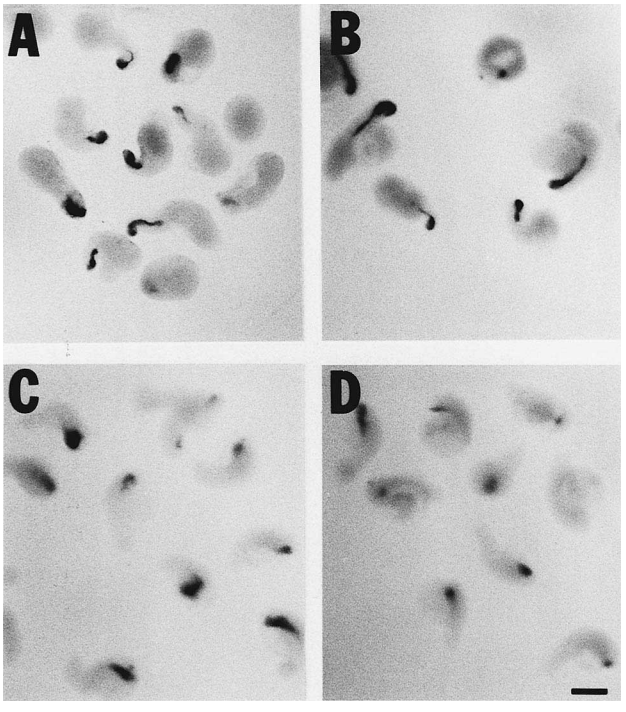


**Fig. 3.** Expression of *ntl* and *gsc* genes in medaka explants from stage 11 embryos by whole-mount *in situ* hybridization. (A) *ntl* gene expression in explants without the YCF. (B) *ntl* gene expression in explants with the YCF. (C) *gsc* gene expression in explants without the YCF. (D) *gsc* gene expression in explants with the YCF. Bar is 100 μm.

**Table 1.** Expression of *ntl* and *gsc* genes in the medaka explants. Positively stained explants (%) and number of explants examined (in parentheses).

| | stage 9 | | stage 10 | | stage 11 | |
|---|---|---|---|---|---|---|
| YCF | – | + | – | + | – | + |
| *ntl* | 86.1 (36) | 52.9 (17) | 78.5 (14) | 100 (12) | 88.2 (17) | 100 (9) |
| *gsc* | 80.0 (15) | 75.0 (8) | 72.7 (11) | 78.6 (14) | 100 (8) | 83.3 (12) |

in gold fish embryos that axial determinants might exist in the vegetal hemisphere and be transmitted to the blastoderm at an early cleavage stage. Earlier commitment of dorsal blastomers was also shown by Yamaha *et al.* (1998). Then, Wilson *et al.* (1995) reported that cytoplasm initially present at the edge of the 1-cell stage blastodisc was transmitted specifically to the marginal blastomeres of the gastrula stage zebrafish embryos.

The second step, inducibility of the mesodermal marker genes in the blastoderm has been demonstrated by Mizuno *et al.* (1996). In their study with zebrafish embryos, the yolk cell without the blastoderm was transplanted on the animal pole region of the host embryo with the YSL facing the host animal pole. In such combined embryos, expressions of several genes including *ntl* or *gsc* were induced ectopically in the host blastoderm. The results indicated that these genes were inducible by signal(s) transmitted, directly in their case, from the yolk cell. Thus, the recent investigations described above are consistent with the two step model of signal transmission.

Even if the mechanism of gene expression could be interpreted by the model, however, the importance of the YSL for axial determination remains. After incubation, the explants without the YSL developed into irregular aggregates of cells (Hyodo *et al.*, 1996) in spite of the fact that both of these genes were expressed (Figs. 2 and 3, Table 1). And the explants containing the YSL developed into fish-like structures with axial polarity (Hyodo *et al.*, 1996). Our studies indicated that YSL has some particular role in the determination or maintenance of axial structure. Recently novel zebrafish genes, a homeobox gene *dharma* (Yamanaka *et al.*, 1998) and a nodal-related gene *squint* ( Feldman *et al.*, 1998), have been identified which were expressed in the YSL, together with the dorsal blastomeres. Microinjection studies (Yamanaka *et al.*, 1998; Feldman *et al.*, 1998) or mutant analysis (Feldman *et al.*, 1998) showed that they function in induction of organizer. The function of the YSL observed in our study might be interpreted by actions of these genes.

Another interesting result in this study was the change in profiles of gene expression domains in the explants. They were broader and stronger in stage 9 explants (Fig. 2) than in those of stage 11 explants (Fig. 3). At the later stage, they were restricted to small regions in the explants. The broader staining pattern in stage 9 embryos seemed to be the result of elevated and unregulated expression, suggesting that these genes at the later stages might be regulated by suppression in the non-expressing regions.

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