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Grafting of Center Cells of Horseshoe Crab Embryos into Host Embryos at Different Developmental Stages

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ABSTRACT—The center cells of horseshoe crab embryos at the early gastrula stage induced secondary embryos after grafting into embryos at same stage and into late blastula stages. However, when the center cells of embryos at early gastrula stage were grafted into embryos at the stage of early cleavage, secondary embryos were not formed. Secondary embryos were not induced after grafting center cells into embryos at the late gastrula stage or after. These results indicate that the center cells of the early gastrula cannot induce secondary embryos at stages other than the early gastrula and late blastula.

However, center cells of embryos at stages later than the early gastrula did induce secondary embryos. These center cells of later embryos were grafted into embryos at the early gastrula and late blastula stage, and secondary embryos were induced. This indicates that center cells of embryos after the early gastrula stage retain the ability of embryonic induction.

INTRODUCTION

The mechanism of induction by the primary organizer is now one of the most exciting problems to be analysed in developmental biology [1, 9, 10, 13, 14]. However, reports of the primary organizer in animals except Chordata are sparse [2, 6, 7].

Surface cleavages occur in horseshoe crab embryos as in other Arthropoda, and the blastopore appears at the early gastrula stage. Surface cells migrate toward the blastopore and enter there. A cell mass is formed beneath the blastopore at the early gastrula stage (Fig. 1). The cell mass at the gastrula stage is called the primary cumulus in spider embryos. Morphological observations and previous experimental studies have shown that the germ disc is formed around the cell mass, and that cell mass is later situated at the

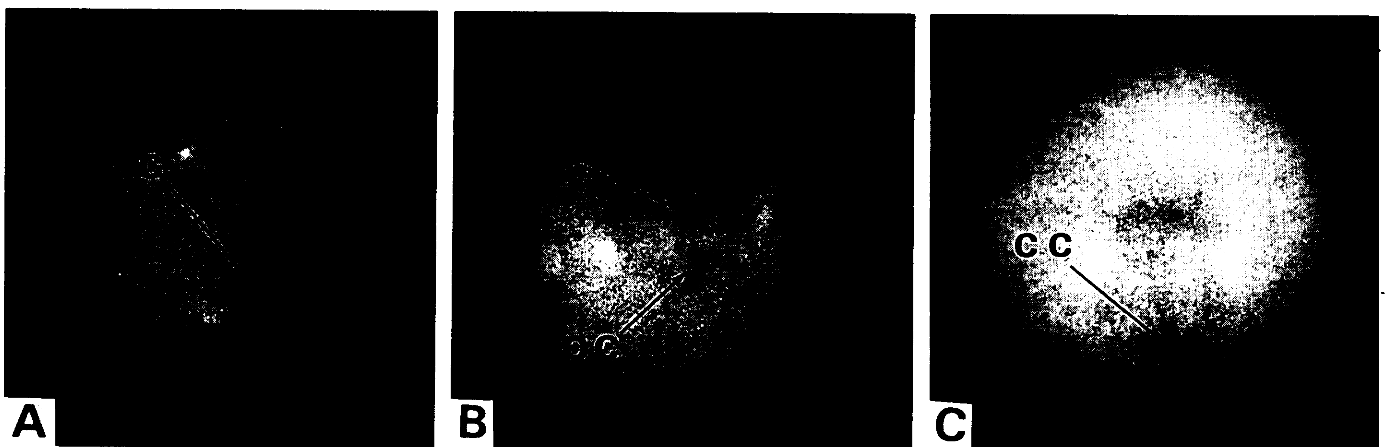

FIG. 1. A: An embryo of horseshoe crab at the early gastrula stage (Stage 7). B: An embryo of horseshoe crab at the late gastrula stage (Stage 10). C: An embryo of horseshoe crab at the early neurula stage (Stage 12). CC; center cells. PC; posterior cumulus.

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posterior end of the ventral plate (the embryonic area) and successively forms segment primordia [3, 4, 5, 12]. The cell mass at the posterior end of the embryonic area is sometimes called the growth zone.

If the cell mass of the embryo at the early gastrula stage is electrically cauterized, the treated embryo cannot develop further. If the cell mass is divided into two or three pieces by electrocauterization or cell dissociation, the treated embryo develops into double or triple embryos [8, 11]. These experiments suggest that the cell mass plays a central role in embryogenesis, and that it is similar to the mesodermal teloblast of Annelida and Crustacea. Therefore the mass is referred to as the center cells. To find out more about the role of the center cells, we attempted grafting experiments [6, 7]. We found that secondary embryos could be induced by interspecific grafts of center cells, indicating that center cells have the capacities of primary induction.

It would be very interesting to know whether the center cells (the primitive cumulus) of the early gastrula can induce secondary embryos in embryos at different stages. Can the center cells (the growth zone) of stages later than the early gastrula induce secondary embryos in embryos at the early gastula stage? This paper deals with this question.

MATERIALS AND METHODS

Adult horseshoe crabs, *Tachypleus* (*Carcinosporpius*) *rotundicauda,* were collected in the Gulf of Siam in Thailand and sent to Japan. *Limulus polyphemus* were obtained from Woods Hole Marine Laboratory in Massachusetts, the Duke University Marine Laboratory in North Carolina, and the Gulf Specimen Company in Florida. They were transferred to Shizuoka University where the present study was conducted. Eggs were fertilized by artificial insemination. The developmental stages were identified according to the normal criteria [12].

For the grafts, a small hole (diameter about 0.05–0.075 mm) was opened in the chorion of host horseshoe crab embryos at the late blastula (Stage 6) and early gastrula stage (Stage 7). The hole was positioned on the side opposite to the center cells. The center cells and other tissues were cut off the donor embryos, and inserted through the hole. In the case of embryos at stages earlier than the gastrula, the surface layer and surface cells of the same volume as the grafted center cells were removed at the early gastrula stage. For the injection of homogenized center cells, center cells of 25–50 eggs at different stages were removed and homogenized in 0.5 ml distilled water or sea water. The concentration of homogenates of center cells from embryos at Stage 7 was about 0.5–5.0 mg (dry weight)/ml. Absorption granules (Collagen, Spongel [Yamanouchi Co., Tokyo]) were added to the resulting homogenates, and the solutions were again homogenized. More than 10^{-6} ml of solution including the granules was then injected into host embryos at different stages, on the side opposite to the center cells. The treated embryos were cultured in a small laboratory dish in about 10 ml sea water containing antibiotics such as 0.5 μg/ml streptomycin and 0.5 unit/ml penicilline.

Normal and treated embryos were vitally stained with 1/20,000–1/400,000 neutral red and observed using a streomicroscope. Secondary embryos were judged to be at stage 20, Stage 21 (the stage of hatching), and the stage after hatching. When the treated embryo had an extra ventral plate (embryonic area), where there were often extra appendages, it was assumed a secondary embryo had been formed.

Some normal and treated embryos were fixed in Bouin's or Carnoy's solution, embedded in celloidin and paraffin and sectioned at 5–10 μm. The sections were stained with Mayer's haematoxylin and eosin.

RESULTS

Grafts of center cells at the early gastrula into embryos at same stage

The grafts of center cells were made in the American horseshoe crab, *Limulus polyphemus* and in the Asian species, *Tachypleus rotundicauda*. The results were similar in both species (Table 1). Although the experiments were performed using mainly *Limulus polyphemus,* the results of experiments using both species are de-

TABLE 1. Results of transplantation of crude center cells from early gastrulae (Stage 7) into embryos at the same stage

DONOR→HOST	Total number of operated on embryos	No of embryos developed upto St. 20 (percent of the total)	No of embryos with secondary embryos (percent of the developed)
Limulus → *Limulus*	162	83 (51.2)	18 (21.7)
Tachypleus → *Tachypleus*	191	20 (10.5)	4 (20.0)
Limulus → *Tachypleus*	166	22 (13.3)	3 (13.6)
Tachypleus → *Limulus*	211	38 (18.0)	6 (15.8)
Non-center cells → *Limulus* [Control]	107	47 (43.9)	0 (0.0)

TABLE 2. Results of transplantation of crude center cells and injection of homogenized center cells. The center cells at Stage 7 were grafted into embryos at the same stage

METHODS OF GRAFTINGS	Total number of operated on embryos	No of embryos developed upto St. 20 (percent of the total)	No of embryos with secondary embryos (percent of the developed)
TRANSPLANTATION	1536	188 (12.2)	37 (19.7)
INJECTION	569	243 (42.7)	23 (9.5)
Injection of homogenized non-center cells [Control]	121	72 (59.5)	1 (1.4)

The control for transplantation experiments is shown in Table 1.

TABLE 3. Grafts of center cells from early gastrulae (Stage 7) into embryos at the late blastula stage (Stage 6) and Stage 7

STAGE OF HOST EMBRYOS	Total number of operated on embryos	No of embryos developed upto St. 20 (percent of the total)	No of embryos with secondary embryos (percent of the developed)
Late blastula (Stage 6)	1878	504 (26.8)	71 (14.1)
Early gastrula (Stage 7)	1503	504 (33.5)	60 (11.9)
Injection of sea water [Control]			
Stage 6	362	137 (37.8)	0 (0.0)
Stage 7	906	373 (41.2)	1 (0.3)

scribed together.

The rate of formation of secondary embryos following the injection of homogenized center cells was lower than that resulting from the transplantation of intact center cells (Table 2). However, as the injection method is simpler, the following section describes the results obtained by this technique.

When the center cells were grafted into embryos at the late blastula stage (Stage 6), secondary embryos were induced at a rate similar to that in embryos at the early gastrula stage (Stage 7) (Table 3 and Fig. 2).

The secondary embryos induced after grafting

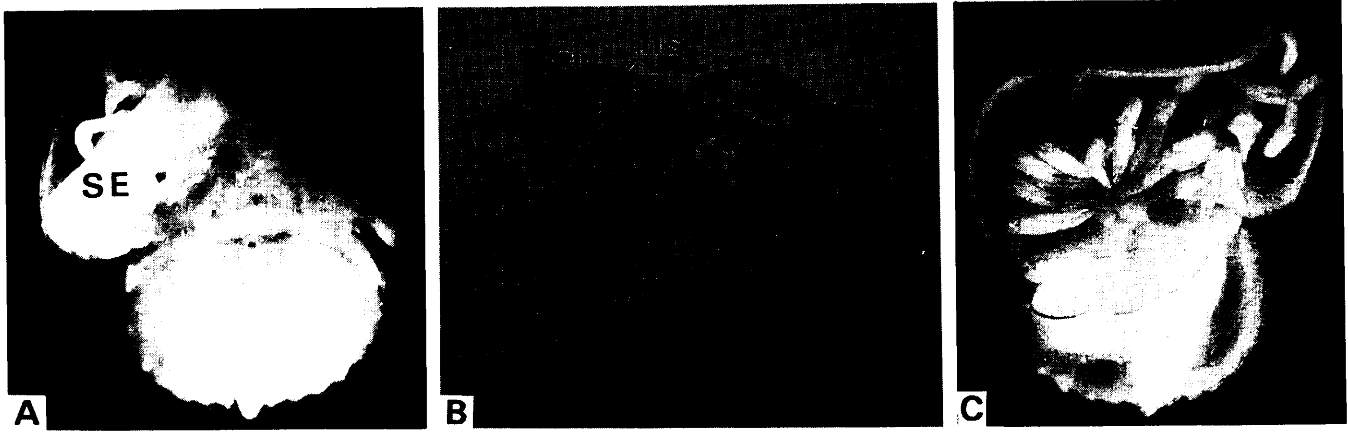

FIG. 2. A: Secondary embryo after gafting of the cortical cytoplasm (surface layer) of an embryo at Stage 3 into an embryo at Stage 7. B: Histological features of the secondary embryo. C: Secondary embryo after grafting of the midgut gland (liver) of an adult into an embryo at Stage 7. SE; secondary embryo. ns; nervous system.

center cells from the early gastrula stage (Stage 7) into embryos at the same stage had the following characteristics. The size and structure of secondary embryos differed. The smaller ones had only a small ventral plate without appendages. The larger ones had the same size and form as the host embryos. The alimentary canals and hearts of secondary embryos often fused with those of the host embryos. The medial body axis of the secondary embryo was sometimes oriented in the opposite direction to that of the host embryo. These characteristics differed from those of double and triple embryos induced by electrocauterization and cell dissociation [11]. In the latter case, the posterior ends of induced double and triple embryos faced towards the point where the center

TABLE 4. Grafts of center cells from early gastrulae (Stage 7) into embryos at different stages

STAGE OF HOST EMBRYOS	Total number of operated on embryos	No of embryos developed upto St. 20 (percent of the total)	No of embryos with secondary embryos (percent of the developed)
Immediately after fertilization	40	21 (52.5)	2 (9.5)
Early cleavage (Stages 1 & 2)	230	62 (27.0)	0 (0.0)
Late cleavage (Stages 3 & 4)	118	30 (25.4)	1 (3.3)
Early blastula (Stage 5)	149	66 (44.3)	2 (3.0)
Late blastula and early gastrula (Stages 6 & 7)	1005	194 (19.3)	26 (13.4)
Late gastrula and early neurula (Stages 10 to 12)	57	29 (50.9)	0 (0.0)
Later stages (Stages 14 to 17)	26	18 (69.2)	0 (0.0)
Injection of sea water [Control]			
Immediately after fertilization	59	24 (40.7)	2 (8.3)
Stages 1 & 2	113	13 (11.5)	0 (0.0)
Stages 3 & 4	111	41 (36.9)	0 (0.0)
Stage 5	55	26 (47.3)	0 (0.0)
Stages 6 & 7	1268	510 (40.2)	1 (0.2)
Stages 10 to 12	349	231 (66.2)	0 (0.0)
Stages 14 to 17	307	274 (89.3)	0 (0.0)

cells had been. In the secondary embryos resulting from grafting, the clear junction part was often observed in the region between the host embryo and the secondary one. These characteristics were not observed in the double and triple embryos induced by electrocauterization and cell dissociation. More detailed characteristics of the secondary embryos have been described in the previous paper [6].

Grafts of center cells from the early gastrula into embryos at different stages

When the center cells (primitive cumulus) of embryos at the early gastrula stage (Stage 7) were homogenized and injected into embryos at early cleavage stages (Stage 1 and Stage 2), secondary embryos were rarely induced, except in embryos injected immediately after fertilization (Table 4).

For the control experiments, we opened small holes in the chorions of embryos at different developmental stages, without grafting. In these cases, secondary embryos were not induced, except in embryos immediately after fertilization. When the host embryos were operated on immediately after fertilization, 21 of 40 treated embryos (52.5%) developed, and 2 of them (9.5%) had secondary embryos. The proportion of treated embryos which developed secondary embryos did not differ significantly from that of the control embryos with small holes but no grafts.

When the host embryos were in the late cleavage stages (Stage 3 and Stage 4) and the early blastula stage (Stage 5), secondary embryos were induced at a very low rate (Fig. 3). The characteristics of the secondary embryos did not differ from those of secondary embryos induced after grafting center cells of early gastrulae into embryos at the early gastrula stage (Stage 7).

The center cells of early gastrulae (Stage 7) were grafted into embryos at stages later than Stage 7 (Table 4). Secondary embryos were never formed.

Grafts of surface cells at stages earlier than gastrulae into eggs at early gastrula stage

The grafting of cortical cytoplasm (surface layer) and surface cells (blastoderm cells) from unfertilized eggs and embryos at the early cleavage stages (Stage 1 and Stage 2) into the embryos at late blastula and early gastrula stages (Stages 6 and 7) never resulted in the formation of secondary embryos (Table 5). The grafting of center cells of embryos at late cleavage stages (Stage 3 and Stage

TABLE 5. Grafts of the cortical cytoplasm (surface layer), surface cells and center cells at different stages into late blastulae (Stage 6) and early gastrulae (Stage 7). The whole tissue of larvae and different tissues from adults were also grafted into embryos at the same stages

STABE OF DONOR EMBRYO		Total number of operated on embryos	No of embryos developed upto St. 20 (percent of the total)	No of embryos with secondary embryos (percent of the developed)
Unfertilized eggs		76	25 (32.9)	0 (0.0)
Early cleavage	(Stages 1 & 2)	120	113 (94.2)	0 (0.0)
Late cleavage	(Stages 3 & 4)	116	66 (56.9)	5 (7.6)
Blastula	(Stages 5 & 6)	159	84 (52.8)	3 (3.6)
Early gastrula	(Stages 7)	1153	238 (20.6)	28 (11.8)
Late gastrula	(Stages 9 to 11)	360	95 (26.4)	9 (9.5)
Later stages	(Stages 13 to 20)	629	294 (46.7)	29 (9.9)
1st & 2nd larvae		179	61 (34.1)	9 (14.8)
Tissues of adults	cartilage	238	76 (31.9)	4 (5.3)
	midgut gland	55	14 (25.5)	4 (28.6)
	ovary	110	42 (38.2)	5 (11.9)
	blood	80	39 (48.8)	0 (0.0)

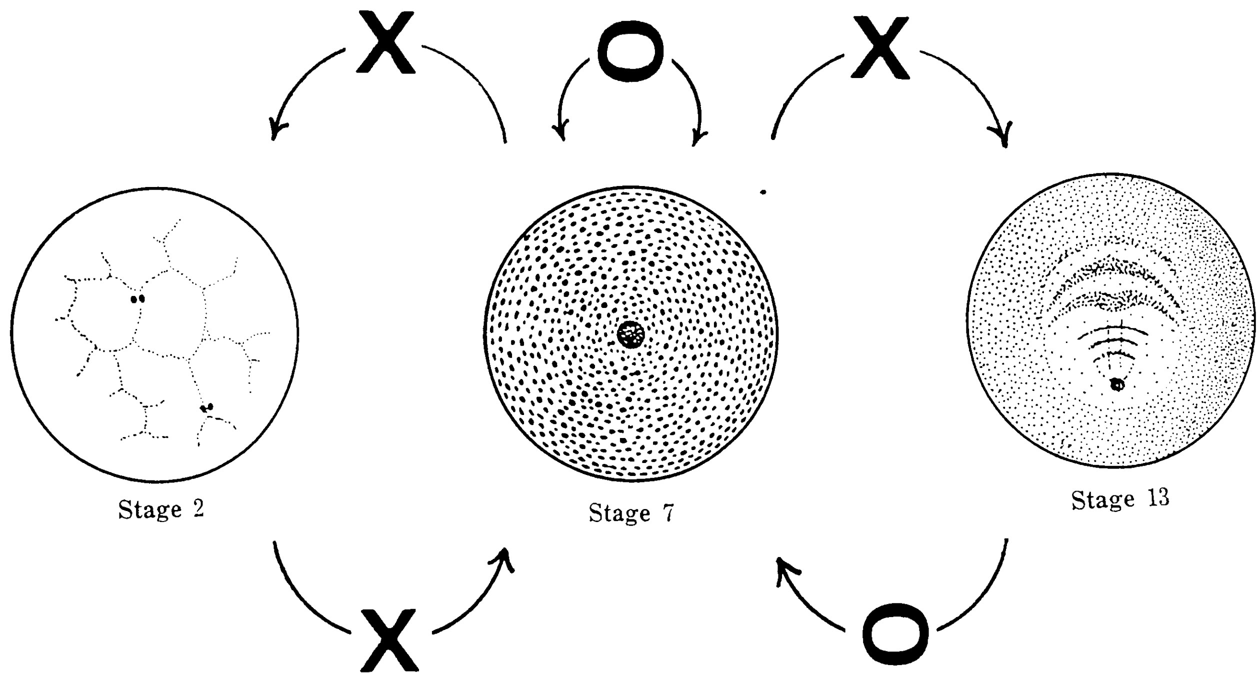

Fig. 3. Summary of results

4) and blastula stages (Stage 5 and Stage 6) into embryos at Stage 6 and Stage 7 resulted in the formation of secondary embryos, but the rate of formation was very low (Table 5). The characteristics of the secondary embryos did not differ from those of secondary embryos induced after grafting center cells of early gastrulae into embryos at the early gastrula stage (Stage 7).

Grafts of center cells of stages later than early gastrulae into embryos at the early gastrula stage

When the center cells (growth zones) of embryos at stages later than early gastrulae were grafted into embryos at the late blastula stage (Stage 6) and early gastrula stage (Stage 7), secondary embryos were induced at a high rate (Table 5). Cells other than center cells did not induce secondary embryos after similar grafting. For example, when the posterior cumulus was grafted into embryos at Stage 6 or Stage 7, 18 of 92 treated embryos developed and none of them had secondary embryos.

The homogenates of whole tissues of larvae induced secondary embryos at a high rate after grafting into embryos at Stage 6 or Stage 7. Several tissues of adult horseshoe crabs were also able to induce secondary embryos, including cartilage (endoskeleton), midgut gland (liver) and ovary. Injection of blood did not induce secondary embryos.

The characteristics of the secondary embryos induced after grafts of tissues of adults, larvae and embryos at stages later than Stage 7 did not differ from those of secondary embryos induced after grafting center cells of early gastrulae into embryos at the early gastrula stage (Stage 7).

The above-mentioned results are summarized in Fig. 3.

DISCUSSION

Secondary embryos were formed at a high rate after grafts of center cells of embryos at the early gastrula stage (Stage 7) into embryos at the late blastula stage (Stage 6) and Stage 7 (Table 1) [6, 7]. The rate of induction of secondary embryos in embryos receiving grafts at Stage 6 was as high as that in grafted Stage 7 embryos. This must mean that the cells of the embryos at the late blastula stage (Stage 6) have the capacity to respond to the signal for embryonic induction.

Secondary embryos rarely developed after grafting center cells of Stage 7 embryos into the embryos at other stages, except Stage 6. This indicates that only embryos at Stages 6 and 7 have the capacity to respond to the signal for embryonic

induction.

When small holes were opened, without grafting, on the chorions of embryos immediately after fertilization, secondary embryos sometimes developed in the treated embryos. I interpret this phenomenon as follows. The chorions of embryos immediately after fertilization are still very soft. The distribution of substances in these eggs is thought not to be stable, and the distribution of cells is determined later, according to the distribution of substances. The shock of opening a hole seems to deragne the normal distribution of substances and cells, and treated embryos may sometimes develop secondary embryos.

When the center cells were grafted into embryos at the stages of late cleavage and early blastula (Stages 3 to 5), secondary embryos were sometimes induced, although at a low rate. I interpret these results as follows. One possibility is that embryos at Stages 3 to 5 have a weak capacity to respond to the signal for embryonic induction. Another posibility is that the grafted center cells or the substance for induction are retained in treated embryos until the latter develop to Stages 6 and 7.

The cortical cytoplasms (surface layers) of unfertilized eggs and embryos at early cleavage stages (Stages 1 and 2) did not induce secondary embryons in embryos at Stage 6 or Stage 7. The cortical cytoplasms of such eggs may not yet possess the induction substance for embryogenesis.

On the other hand, the surface cells of embryos at late cleavage and blastula stages (Stages 3 to 6) induced secondary embryos in embryos at Stage 6 and Stage 7, although the rate of formation of secondary embryos was low. The finding may mean that the surface cells of embryos at Stages 3 to 6 contain the substance for embryonic induction.

When center cells of embryos at the early gastrula stage (Stage 7) were grafted into embryos at the late gastrula stage and after, secondary embryos were never induced. But the center cells (growth zone) of embryos at the late gastrula, or later, stages did induce secondary embryos after grafting into embryos at Stage 6 or Stage 7. This must indicate that the center cells of embryos at stages later than the early gastrula stage (Stage 7) still contain the substance for embryonic induction.

The homogenates of whole tissues of larvae and some tissues of adults induced secondary embryos after injections into embryos at early gastrula. This indicate that the substance for embryonic induction is contained in tissues of larvae and adults.

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