

## Intestinal cytodifferentiation *in vitro* of chick stomach endoderm induced by the duodenal mesenchyme

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### SUMMARY

The inductive action of duodenal mesenchyme on the cytodifferentiation of stomach endoderm in chick embryos was investigated *in vitro* with electron microscopy and immunofluorescence. Morphologically undifferentiated endoderm of the stomach of a 4-day embryo could differentiate only into a mucous secretory epithelium when cultured in the absence of mesenchyme. However, when cultivated in recombination with 6-day duodenal mesenchyme, most cells of 4-day stomach endoderm differentiated into intestinal absorptive cells possessing striated border and sucrase, and goblet cells, but not into stomach-type mucous secretory cells. In contrast, when 4-day stomach endoderm was cultured recombined with mesenchyme of embryonic digestive organs other than intestine, none of the stomach endoderm cells differentiated into intestinal epithelial cells. The competence of stomach endoderm for intestinal cytodifferentiation decreased rapidly with development, but remained until relatively later stages in the gizzard region. The present investigation demonstrates that duodenal mesenchyme can induce stomach endoderm, which has acquired the potency for self-differentiation into stomach-type epithelium, to cytodifferentiate into intestinal epithelium.

### INTRODUCTION

The importance of the region-specific influence of embryonic digestive-tract mesenchyme on the morphogenesis and differentiation of the endodermal epithelium has been amply demonstrated in bird embryos (Sigot, 1963; Le Douarin, 1964; Mizuno & Yasugi, 1973; Gumpel-Pinot, Yasugi & Mizuno, 1978). However, their reports were based on histological observation, mainly light microscopy, and failed to determine whether or not mesenchyme could induce the cytodifferentiation of endodermal cells. In other systems, it has been claimed that induction of morphogenesis is not always associated with induction of cytodifferentiation (Sakakura, Nishizuka & Dawe, 1976; Tyler & Koch, 1977). Therefore, in the present study, we attempted to clarify the inductive influence of mesenchyme on the cytodifferentiation of endoderm in avian embryonic digestive tract using electron microscopy and immunofluorescence.

Previous histological observations during recombination experiments on endoderm and mesenchyme in avian embryonic digestive tract demonstrated

that among the digestive-tract endoderms stomach endoderm is the most responsive to the stimuli of heterologous mesenchymes and that intestinal mesenchyme exerts the strongest inductive action among digestive-tract mesenchymes (Gumpel-Pinot *et al.* 1978; Yasugi & Mizuno, 1978). This evidence attracted our attention in relation to human intestinal metaplasia (Rubin, Ross, Jeffries & Slesinger, 1966; Correa, Cuello & Duque, 1970). Therefore, we have focused on the action of intestinal mesenchyme on the cytodifferentiation of stomach endoderm. Regarding the developmental potency of stomach endoderm, we have demonstrated that stomach endoderm of 4- to 6-day chick embryos possesses the organ-specific potency to self-differentiate into mucous secretory stomach-type cells, but not into intestinal epithelial cells (Ishizuya-Oka, 1983). The purpose of the present investigation is to determine (i) whether stomach endoderm can differentiate into intestinal epithelium with striated border and sucrase when cultured recombined with the intestinal mesenchyme, and in this case, (ii) whether the inductive effect of intestinal mesenchyme is instructive or permissive.

#### MATERIALS AND METHODS

##### *Preparation of tissue fragments*

White Leghorn chicken (*Gallus gallus domesticus*) embryos were used throughout the experiments. Endodermal fragments were obtained from the oesophagus, stomach, and intestinal regions of 4-day embryos and from the proventriculus and gizzard of 4.5- to 10-day embryos as shown in Fig. 1. Mesenchymal fragments were isolated from the oesophagus, proventriculus, gizzard, and duodenum of 6-day embryos.

The tissues were treated with collagenase (Worthington, Code CLS, 0.3 mg/ml

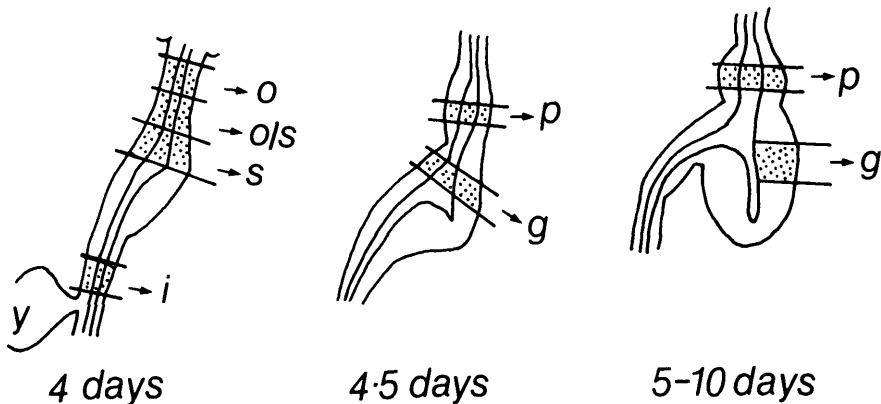


Fig. 1. Diagram showing a part of the digestive tract of the chick embryo. Endodermal fragments isolated from the dotted areas were used for cultivation. o, oesophagus; o/s, junctional area of oesophagus and stomach; s, stomach; i, small intestine; p, proventriculus; g, gizzard; y, yolk sac.

Tyrode's solution) at 37°C for 30 or 40 min. The endoderm and mesenchyme were then separated with fine forceps, rinsed repeatedly in serum-supplemented Tyrode's solution, and then in Tyrode's solution. The endoderm was recombined with a homologous or heterologous mesenchyme.

#### Organ culture

The recombinates were cultured on a Wolff & Haffen (1952) medium for 10 to 15 h to ensure coherence of the two tissues, and then turned upside down with the luminal surface of the endoderm on the medium (Fig. 2), in order to give good culture conditions for endodermal differentiation (Ishizuya, 1981). The culture medium consists of seven parts of 1% agar solution in Gey's solution, three parts of horse serum (Flow Laboratories), three parts of 12-day digestive-tract-free chick embryo extract diluted to 50% in Tyrode's solution, and penicillin G (20 000 i.u./ml). Explants were cultured for 3 weeks with transplantation to fresh media every 7th day.

#### Electron microscopy

Explants were fixed in a modified Karnovsky's fixative (1965) at 4°C for 2 h and postfixed in 1% osmium tetroxide in 0.1M-cacodylate buffer (pH 7.5) at 4°C for 2 h. They were then stained *en bloc* with uranyl acetate, carried through a graded series of alcohols, and embedded in epoxy resin. Alternate ultrathin and 1 µm semithin sections were cut on a Porter-Blum MT2 microtome from the whole explant. For light microscopy, the semithin sections were stained with toluidine blue. For transmission electron microscopy (TEM), the ultrathin sections were further stained with lead citrate and examined with a JEOL 100CX electron microscope.

On the basis of data obtained on the normal development of the intestine *in vivo* (Overton & Shoup, 1964; Ishizuya, 1980) and *in vitro* (Ishizuya, 1981), we

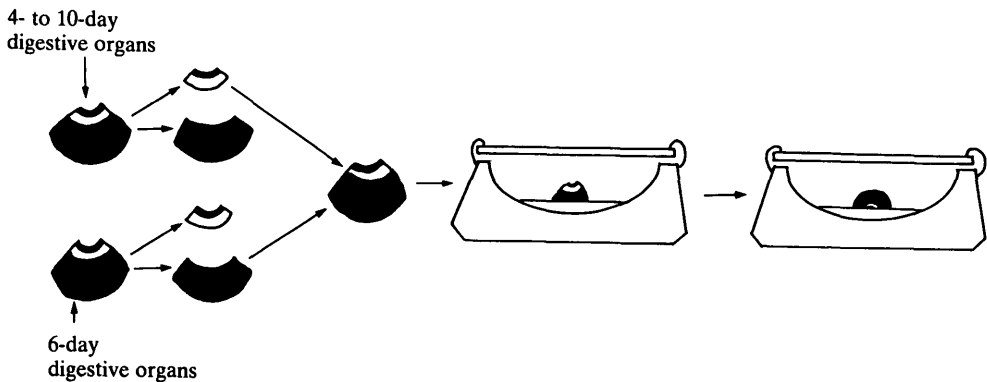


Fig. 2. Diagram showing mode of isolation, recombination, and cultivation. ■, mesenchymal fragment; □, endodermal fragment; —, luminal surface.

designate a regular array of microvilli possessing bundles of core filaments, a uniform diameter at about  $0.08 \mu\text{m}$ , and a density of more than nine microvilli per unit length ( $\mu\text{m}$ ) of the cell surface, as the striated border.

### *Immunofluorescence*

The other explants were fixed in 95 % ethanol at  $4^\circ\text{C}$  for 4 h, embedded in paraffin, and sectioned at  $6 \mu\text{m}$  according to the method of Saint-Marie (1962). The sections were stained by the indirect immunofluorescence method with antisucrease antiserum and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG serum of goat (Miles Laboratories Inc. Indiana, U.S.A.), and observed by fluorescence microscopy. The antisucrease antiserum was kindly donated by Dr Matsushita, and the sucrase used for the immunization of rabbits was purified from the small intestine of adult chicks (Matsushita, 1983).

## RESULTS

### 1. *Heterotypic differentiation of 4-day stomach endoderm cultured recombined with 6-day mesenchyme of various digestive organs*

Morphologically undifferentiated stomach endoderm of 4-day normal embryos (Fig. 3) could differentiate only into mucous secretory epithelium when cultured alone in the absence of mesenchyme (Fig. 4). However, when cultivated recombined with mesenchyme, 4-day stomach endoderm differentiated into a specific type of epithelium according to the origin of the mesenchyme associated (Table 1). Cultivation of endoderm recombined with oesophageal mesenchyme caused differentiation of a stratified cuboidal epithelium (Fig. 5). Stomach endoderm, when cultivated recombined with proventricular or gizzard mesenchyme, differentiated into a mucous secretory epithelium, comparable with stomach epithelium of normal embryos and also with self-differentiated stomach endoderm cultured in the absence of mesenchyme (Figs 6, 7). Endoderm recombined

Table 1. *In vitro* differentiation of 4-day stomach endoderm cultured recombined with 6-day mesenchyme of digestive organs

Origin of mesenchyme associated	Number of explants	Type of differentiation				Number of explants	Antigen of sucrase
		stratified cuboidal	mucous secretory	gland	striated border		
————*	11	0	11	0	0	6	0
Oesophagus	5	5	5	0	0	5	0
Proventriculus	4	0	4	2	0	8	0
Gizzard	4	0	4	0	0	8	0
Duodenum	5	0	5	0	5	5	5

\* When the endoderm was cultured alone in the absence of mesenchyme.

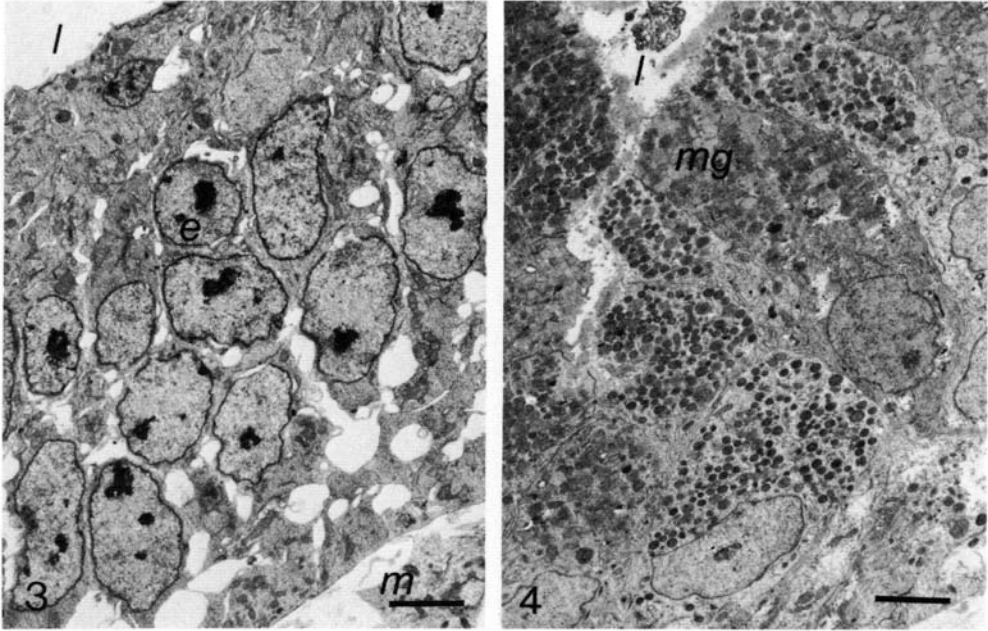


Fig. 3. A cross section of the stomach endoderm of a 4-day normal embryo. e, endoderm; m, mesenchyme; l, lumen. Bar = 5  $\mu$ m.

Fig. 4. A cross section of 4-day stomach endoderm cultured alone in the absence of mesenchyme for 7 days. A mucous secretory epithelium developed. Numerous mucous granules (mg) exist in the apical cytoplasm of the endodermal cells. Bar = 5  $\mu$ m.

with gizzard mesenchyme developed more mucous granules than that with proventricular mesenchyme. In some recombinates of endoderm and proventricular mesenchyme, glands consisting of a simple epithelium were also observed (Fig. 8). These cells forming glands, however, possessed no zymogen granules, which appear in the proventricular glands of 10-day normal embryos. Cultivation of endoderm recombined with duodenal mesenchyme resulted in the differentiation of a simple columnar epithelium comprising goblet cells and absorptive cells, possessing striated border and sucrase, corresponding to the chief cells of the normal intestinal epithelium (Figs 9, 10). The striated border and sucrase could not be detected in any recombinant of stomach endoderm and mesenchyme of digestive organs other than small intestine.

## 2. Competence of 4-day oesophageal and stomach endoderm for cytodifferentiation into intestinal epithelium

4-day endodermal cells in regions of the oesophagus and stomach never differentiated into intestinal epithelial cells when endodermal fragments were cultured alone in the absence of mesenchyme, though they could self-differentiate

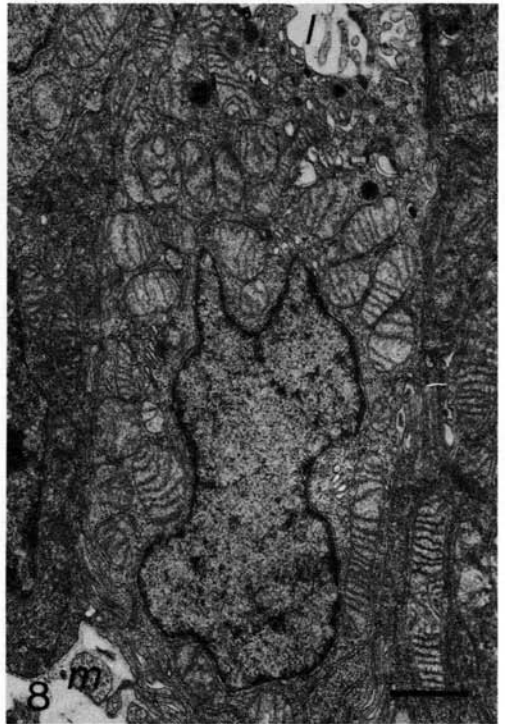
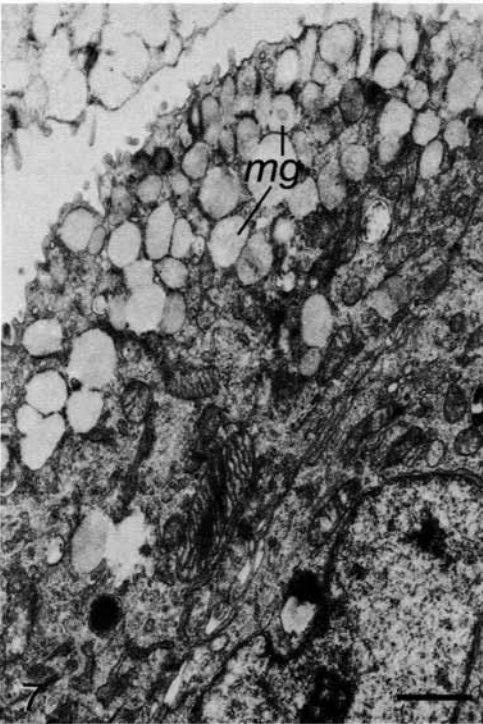
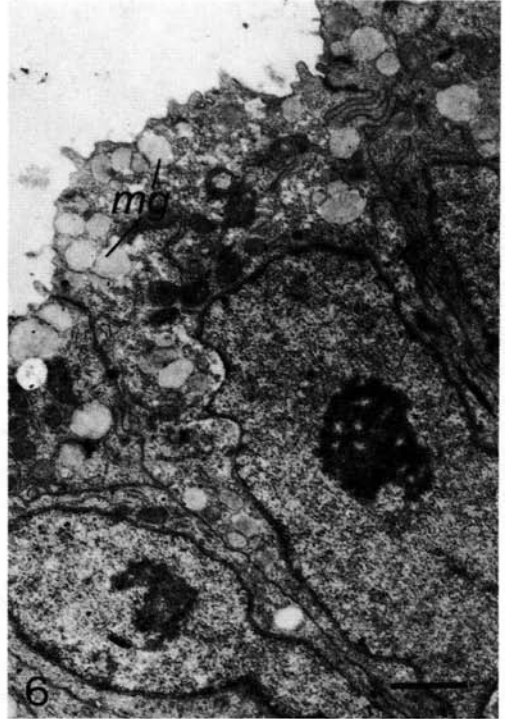
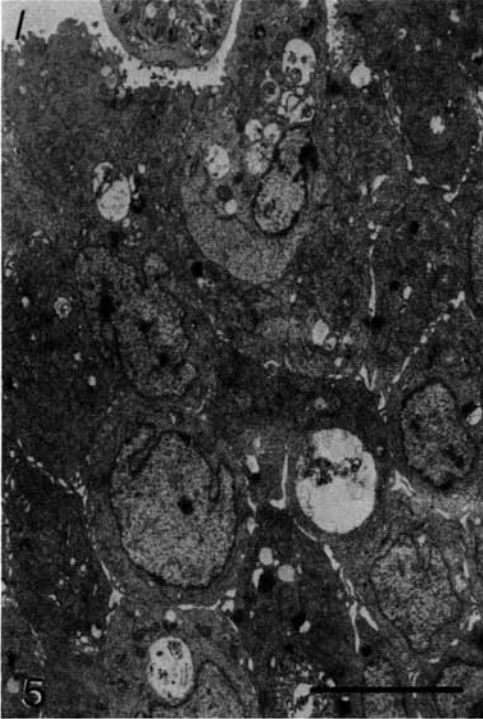


Table 2. *Cytodifferentiation of intestinal epithelium in 4-day digestive-tract endoderm cultured recombined with 6-day duodenal mesenchyme*

Origin of endoderm*	Number of explants	Striated border (range, %)†	Other type of differentiation		Number of explants	Sucrase (range, %)†
			stratified cuboidal	mucous secretory		
o	5	0	5	5	8	1 (4)
o/s	3	3 (32±19)	1	3	5	5 (21±8)
s	5	5 (48±8)	0	5	5	5 (64±19)
i	3	3 (99±0)	0	0	5	5 (93±2)

\* See Fig. 1. o, oesophagus; o/s, junctional area of oesophagus and stomach; s, stomach; i, small intestine.

† Values in parentheses express the proportion of cells possessing a striated border or sucrase to all epithelial cells in an explant (mean ± s.d.). Numbers of cells counted were 500~700 per an explant.

into oesophageal and stomach epithelia. However, when cultured in recombination with duodenal mesenchyme under the same culture conditions, digestive-tract endoderms could cytodifferentiate into intestinal epithelium with a frequency depending on the original region of the endoderm (Table 2). The more anterior the origin of the endodermal fragment, the smaller the number of recombinates expressing intestinal cytodifferentiation and the proportion of intestinalized cells to all the epithelial cells in each recombinant. However, even when the oesophageal endoderm was cultured in recombination with duodenal mesenchyme, the epithelial cells expressing sucrase could be observed in one case out of eight. In each recombinant, differentiated intestinal epithelial cells always appeared in groups, never distributed as a mosaic of single cells. Endodermal cells which were not induced into intestinal epithelial cells mainly differentiated into mucous secretory cells, though stratified cuboidal epithelial cells

Fig. 5. A recombinant of 4-day stomach endoderm and 6-day oesophageal mesenchyme cultured for 3 weeks. A stratified cuboidal epithelium developed. Bar = 5  $\mu$ m.

Fig. 6. A recombinant of 4-day stomach endoderm and 6-day proventricular mesenchyme cultured for 3 weeks. A mucous secretory epithelium developed. Bar = 1  $\mu$ m.

Fig. 7. A recombinant of 4-day stomach endoderm and 6-day gizzard mesenchyme cultured for 3 weeks. A mucous secretory epithelium developed. The mucous granules were more distinct and numerous than those in Fig. 6. Bar = 1  $\mu$ m.

Fig. 8. A recombinant of 4-day stomach endoderm and 6-day proventricular mesenchyme cultured for 3 weeks, showing a cross section of a gland comprising simple cuboidal epithelium surrounded by mesenchyme. Bar = 1  $\mu$ m.

Table 3. *Cytodifferentiation of intestinal epithelium in 4.5- to 10-day proventriculus and gizzard endoderm cultured recombined with 6-day duodenal mesenchyme*

	Origin of endoderm	Number of explants	Striated border (range, %)*	Other type of differentiation		Number of explants	Sucrase (range, %)*
				mucous secretory	gland		
4.5-day	Proventriculus	4	1 (9)	4	1	5	2 (9±3)
	Gizzard	5	2 (32±21)	5	0	6	5 (19±6)
5-day	Proventriculus	5	0	5	3	6	0
	Gizzard	6	2 (13±5)	6	0	5	4 (7±4)
6 to 7-day	Proventriculus	7	0	7	4	12	0
	Gizzard	4	0	4	0	15	0
8 to 10-day	Proventriculus	7	0	7	5	11	0
	Gizzard	5	0	5	0	13	0

\* See Table 2.

also differentiated in the recombinates with the endoderm of the oesophageal region.

### 3. Competence of 4.5- to 10-day stomach endoderm for cytodifferentiation into intestinal epithelium

When cultured alone in the absence of mesenchyme, endodermal cells of 4.5- to 10-day proventriculus and gizzard never differentiated into intestinal

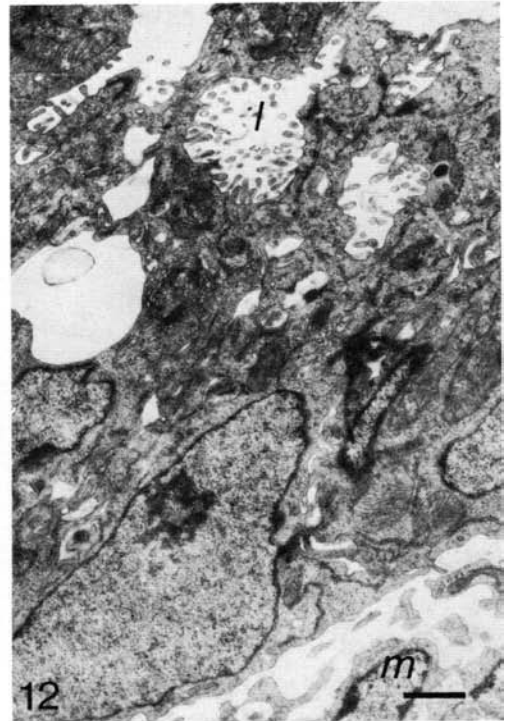
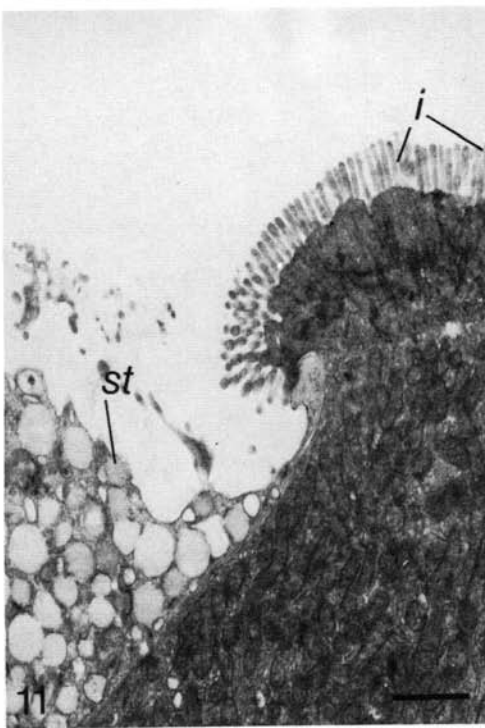
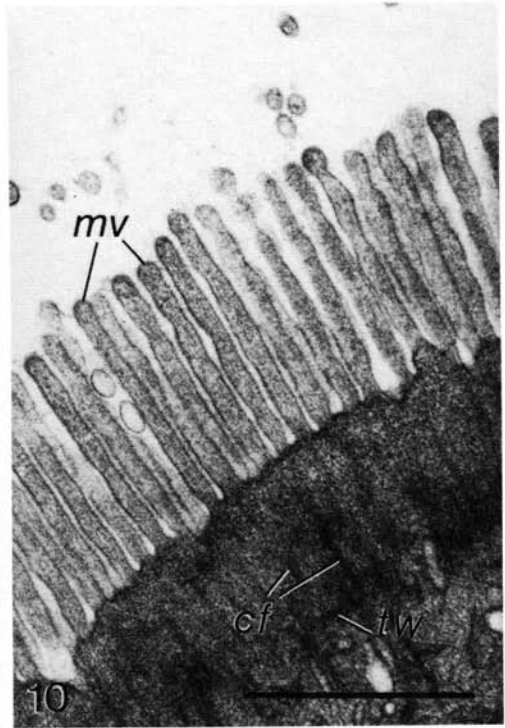
Fig. 9. Sucrase immunofluorescence in 4-day stomach endoderm cultured in recombination with 6-day duodenal mesenchyme for 3 weeks. The surface of the simple columnar epithelium (arrows) was positive. ×900.

Fig. 10. A cross section of 4-day stomach endoderm cultured in recombination with 6-day duodenal mesenchyme for 3 weeks. A typical striated border developed. *mv*, microvilli; *cf*, core filaments; *tw*, terminal web. Bar = 1 μm.

Fig. 11. A cross section of 4.5-day gizzard endoderm cultured in recombination with 6-day duodenal mesenchyme for 3 weeks. Intestinal epithelial cells (*i*) were differentiated in groups between mucous secretory cells (*st*). Bar = 1 μm.

Fig. 12. A recombine of 8-day proventricular endoderm and 6-day duodenal mesenchyme cultured for 3 weeks, showing glandular cells possessing large mitochondria, well-developed Golgi apparatus and rough endoplasmic reticulum, but no zymogen granules. Bar = 1 μm.





epithelial cells. However, when cultivated in recombination with duodenal mesenchyme under the same culture conditions, the endoderm could cytodifferentiate into intestinal epithelium with a frequency depending on the stage and the region of origin of the endoderm (Table 3). With further development of the endoderm, both the number of recombinates expressing intestinal cytodifferentiation and the proportion of intestinal cells to all the epithelial cells in each recombinant decreased. In the presence of duodenal mesenchyme, gizzard endoderm could differentiate into intestinal epithelial cells until later stages (5 days of incubation) than could proventricular endoderm. In each recombinant cultured *in vitro*, differentiated intestinal epithelial cells always constituted a tissue and were never distributed as a mosaic of single cells (Fig. 11). In recombinates

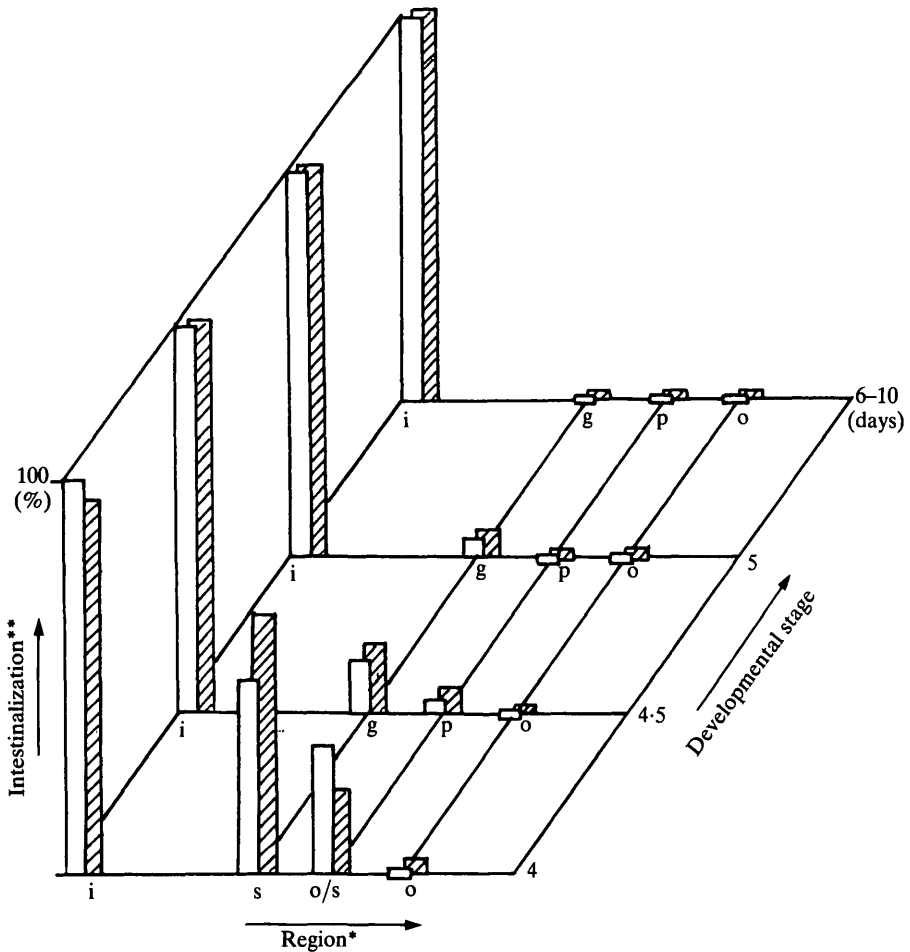


Fig. 13. Developmental changes in the competence of digestive-tract endoderm recombined with 6-day duodenal mesenchyme for cytodifferentiation into intestinal epithelium. Each point represents the mean of at least three explants. \*, o, o/s, s, i, p, g: See Fig. 1; \*\*, See Table 2. ▨, cells possessing striated border; ▩, cells possessing sucrose.

of gizzard endoderm and duodenal mesenchyme, almost all the endodermal cells which were not induced into intestinal epithelial cells differentiated into mucous secretory cells, while in recombinates of proventricular endoderm and duodenal mesenchyme, those cells differentiated into mucous secretory cells and simple cuboidal cells forming glands. The cells of the glands often possessed large mitochondria, well-developed Golgi apparatus and rough endoplasmic reticulum, but no zymogen granules (Fig. 12).

The regional and temporal changes in the competence of endoderm for cyto-differentiation into intestinal epithelium in the present culture conditions are summarized in Fig. 13.

#### DISCUSSION

In a previous paper (Ishizuya-Oka, 1983), we reported that stomach endoderm of 4-day chick embryos already possesses potency for self-differentiation. When cultured alone in the absence of mesenchyme, stomach endoderm can differentiate only into mucous secretory cells, comparable with the chief cells of the surface epithelium of the normal stomach. One of the most important conclusions of the present study is that stomach endoderm possessing such a potency can cytodifferentiate *in vitro* into intestinal epithelium, ultrastructurally as well as functionally, by responding to the inductive action of duodenal mesenchyme. This shows that mesenchymes of digestive tract can alter the cytodifferentiation of the endoderm, although the mesenchyme does not always have such an effect in other organs (Rutter & Weber, 1965; Sakakura *et al.* 1976; Tyler & Koch, 1977). In particular, the intestine-specific enzyme sucrase could be detected in stomach endoderm only when the latter was cultured recombined with duodenal mesenchyme, suggesting that duodenal mesenchyme can control the differentiation processes of stomach endoderm at the genetic level.

To exclude the possibility that the duodenal mesenchyme used in recombination experiments was contaminated with epithelial cells, we repeatedly confirmed by electron microscopy that mesenchymal fragments did not contain endodermal cells and that epithelial cells did not appear when the mesenchymal fragments were cultured alone without the association of endoderm. Therefore, it is unlikely that the cells possessing the potency to differentiate *per se* into intestinal epithelial cells were contaminated in the recombinates. However, at least two possibilities may still remain that endodermal cells possessing the stomach-specific self-differentiation potency differentiated into intestinal epithelial cells, or that a small number of pluripotent cells scattered in the presumptive stomach endoderm proliferated and differentiated into intestinal epithelial cells. Since the proportion of differentiated intestinal cells to all the epithelial cells in cases of the recombinant of 4-day stomach endoderm and 6-day duodenal mesenchyme was too high to be explained by the proliferation of pluripotent cells, the latter possibility seems unlikely.

The other important conclusion of the present study is that the inductive action of duodenal mesenchyme on the cytodifferentiation of stomach endoderm is instructive. Strong evidence for this is that the stomach endoderm could differentiate into intestinal epithelium only when cultured recombined with intestinal mesenchyme, and not when cultured recombined with mesenchyme of any other region of the digestive tract. The observation that cells of the oesophageal endoderm in recombination with duodenal mesenchyme could differentiate into intestinal epithelial cells expressing sucrase (Table 2) is further evidence for instructive induction. In other systems, the inductive action of mesenchyme has been shown to be either instructive (Dhouailly, Rogers & Sengel, 1978; Koller & Fisher, 1980; McAleese & Sawyer, 1982; Cunha *et al.* 1983; Haffen, Lacroix, Kedinger & Simon-Assmann, 1983) or permissive (Lash, 1968; Ronzio & Rutter, 1973; Lawson, 1974; Lehtonen *et al.* 1983). The present study shows that the inductive system of the digestive tract belongs to the former type.

In the present study we regarded sucrase and a striated border as functional and ultrastructural markers for cytodifferentiation of the intestinal epithelium. The reliability of our data is shown by the fact that consistent results were obtained with either marker. Moreover, both markers were always detected on the surface of simple columnar epithelial cells, as was shown in normal intestinal epithelium (Miller & Crane, 1961; Jos, Frézal, Rey & Lamy, 1967). Therefore, the processes involved in the expression of these markers may be coupled, or intestinal mesenchyme may be involved in the induction of each marker. In the present study it was also found that endodermal cells which were not induced into intestinal epithelial cells followed the developmental fates of their regions of origin (Tables 2, 3). This result supports, at the level of cytodifferentiation, the hypothesis proposed by Mizuno (1975) that the development of digestive-tract endoderm is assured by both the self-differentiation potency of the endoderm and the inductive action of the mesenchyme.

We expect this inductive system of the digestive tract to be useful in future studies on tissue interactions, since, (i) *in vitro* analysis of the interaction is possible in this system, (ii) epithelial cells have clear markers for cytodifferentiation, and (iii) the inductive action of the mesenchyme is instructive. Furthermore, this study can be expected to be useful in the analysis of the mechanism of intestinal metaplasia of the stomach.

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