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Original Article

Molecular analysis of the determination of developmental fate in the small intestinal epithelium in the chicken embryo

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ABSTRACT Determination of the developmental fate in the small intestinal epithelium of the chicken embryo has not been fully analyzed up to the present. This study was carried out to analyze the determination time of the developmental fate of the small intestinal epithelium under the influence of other mesenchymes. The small intestinal epithelium reassociated and cultivated with the proventricular or gizzard mesenchyme or the dermis expressed chicken intestinal fatty acid binding protein, sucrase and CdxA as occurs during the normal development of the small intestinal epithelium. The presumptive intestinal endoderm taken from an earlier stage embryo and associated and cultivated with the proventricular or gizzard mesenchyme, showed gene expression patterns which were the same as those found in normal development. However, when the dermis was associated, the epithelium expressed sonic hedgehog, but never expressed intestinal epithelial- or stomach epithelial-markers. These results indicate that the determination of the developmental fate in the small intestinal epithelium and acquisition of autodifferentiation potency occur at the early stage of the gut development. Moreover the presumptive intestinal endoderm needs the supportive influence of the gut mesenchyme in order to differentiate fully into the intestinal epithelium.

KEY WORDS: *chicken, small intestinal epithelium, cIFABP, autodifferentiation potency*

Introduction

The digestive organs of vertebrates consist of the epithelium derived from the endoderm and the mesenchyme derived from the splanchnic mesoderm. In chicken embryos, the right and left splanchnopleures fuse in the midline, and form a tube on day 2 to 3 of incubation. At this time, the epithelium is posted inside and the mesenchyme outside. On day 5, the digestive tract becomes divided anteropostriorly into the esophagus, proventriculus (glandular stomach), gizzard (muscular stomach), duodenum, small intestine, cecum, large intestine and allantois. Thereafter both the epithelium and the mesenchyme of each organ gradually differentiate and acquire organ specific characteristics. The esophageal epithelium becomes stratified epithelium with mucus glands. The proventricular epithelium invaginates into the mesenchymal tissue to form the glandular structures and gland epithelial cells begin to express ECPg (embryonic chicken pepsinogen; Hayashi *et al.,* 1988a, b), a zymogen of digestive enzyme pepsin, from day 9 of incubation. The gizzard epithelium forms the simple glands and secretes mucus. The epithelia of these organs situated in the anterior part of the digestive tract commonly express a transcription factor cSox2 (Ishii *et al.,* 1998) and a trefoil factor cSP (chicken spasmolytic polypeptide; Tabata and Yasugi, 1998). The epithelia of the small intestine and large intestine form villous structures and secrete some digestive enzymes such as sucrase.

The importance of the mesenchyme in the development of the proventricular epithelium and gizzard epithelium was repeatedly shown by the experimental studies (Mizuno and Yasugi, 1990; Yasugi, 1994; Yasugi and Fukuda, 2000). For example, when the gizzard epithelium of 6 day embryo was recombined and cultivated with the proventricular mesenchyme of 6 day embryo, the epithelium formed gland structures and expressed *ECPg*, suggesting that the gizzard epithelium differentiated into the proventricular epithelium (Takiguchi *et al.,* 1986; Hayashi *et al.,* 1988b; Urase *et al.,* 1996). The factors responsible for the induction by the proventricular mesenchyme have been identified as extracellular matrices (Koike and Yasugi, 1999) and *BMP-2* (Narita *et al.,* 2000).

Abbreviations used in this paper: cSP, chicken spasmolytic polypeptide; ECPg, embryonic chicken pepsinogen; IFABP, intestinal fatty acid binding protein.

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On the other hand, the small intestinal epithelium of 6 day embryo never produced *ECPg* even under the influence of the proventricular mesenchyme and invariably expressed sucrase, a marker of intestinal epithelial cells (Yasugi *et al.,* 1985; Hayashi *et al.,* 1988b; Urase *et al.,* 1996). It is therefore suggested that the potency to react to the induction of the proventricular mesenchyme is different in the small intestinal epithelium from that in the proventricular or gizzard epithelium of 6 day embryo. Moreover, when the presumptive intestinal endoderm from 1-2 day embryos was recombined and cultivated with the proventricular mesenchyme of 6 day embryo, the epithelium did not express *ECPg* (Yasugi *et al.*, 1991). These results indicate that the small intestinal epithelium loses the responsiveness to mesenchymal induction early in the development. Moreover, the intestinal epithelium taken from 2.5 to 9 day embryos, wrapped with vitelline membrane and cultivated alone, could autodifferentiate into mature intestinal epithelium with PAS-positive goblet cells (Sumiya, 1976).

In these studies mentioned above, the digestive enzyme sucrase and some morphological criteria were used as the differentiation marker of the small intestinal epithelium. Also, the expression of *CdxA*, a homeobox gene specifically expressed in the intestinal epithelium, was examined in the presumptive intestinal endoderm recombined with the proventricular mesenchyme (Ishii *et al.,* 1997). We have also studied the expression of *cSP* (Tabata and Yasugi, 1998) and *cSox2* (Ishii *et al.,* 1998) in the small intestinal epithelium recombined with the proventricular mesenchyme, but the data are still fragmentary. At present many other genes are known to be expressed specifically in the small intestinal epithelium. Among them, a gene encoding an intestinal fatty acid binding protein (IFABP), a low molecular weight cysteine rich protein that is involved in the cellular uptake of fatty acids as well as their transport within the cell to particular organelles (Sweetser *et al.,* 1987), is abundantly expressed in the small intestinal epithelium in zebrafish and *Xenopus*. However, its expression in the small intestinal epithelium associated with heterologous mesenchyme has not been examined. Horb and Slack (2001), using IFABP as a differentiation marker, showed that the endoderm from *Xenopus* neurula and tail-bud-stage possesses considerable ability to respond to the action of mesoderm from various regions.

In this study, we analyzed precisely and comprehensively the determination period of developmental fate of the small intestinal epithelium with epithelial-mesenchymal interactions, by cultivating tissue recombinants of the (presumptive) small intestinal epithelium and the mesenchymes from other digestive organs. Also we examined the ability of autodifferentiation in the small intestinal epithelium or presumptive intestinal endoderm by recombining them with dorsal skin dermis. The results clearly indicate that the developmental fate of the small intestinal epithelium is determined very early in the development, and that the realization of fully differentiated state requires some signals provided by the digestive organ mesenchymes.

Results

Expression patterns of small intestinal epithelial markers during development

To examine the expression pattern of *cIFABP*, we dissected the small intestine of embryos of various developmental stages and examined it with *in situ* hybridization. The expression of *cIFABP* was not detected before day 6 (Fig. 1B), and began to be detected at day 7 of incubation (Fig. 1C), and was maintained during embryonic period (Fig. 1 D,E). The expression of *cIFABP* was seen specifically in the small intestinal epithelium, not in the esophagus, proventriculus and gizzard (data not shown). We also re-investigated the expression of sucrase by the immunohistochemical technique and of *CdxA* by *in situ* hybridization technique. As is reported previously (Matsushita, 1985), sucrase was first expressed in 10 day embryo and was maintained after this stage up to post-hatch stages except that it disappeared just before hatching (Fig. 1 F,G). Expression pattern of *CdxA* in the chicken embryo was previously reported (Ishii *et al.,* 1997). Expression begins in the gut endoderm at stage 10 (Hamburger and Hamilton, 1951), and continues throughout the embryonic stage (Fig. 1 H-J). Thus *cIFABP*, as sucrase and *CdxA*, is a specific marker of the small intestinal epithelium.

Reactivity of the small intestinal epithelium of 6 day embryo to 6 day proventricular mesenchyme or gizzard mesenchyme

We first carried out tissue recombination experiments using digestive organs of 6 day embryo to confirm that the developmental fate of the small intestinal epithelium in the chicken embryo is already determined at day 6 of incubation. When the epithelium and mesenchyme of the same organ were recombined, expres-

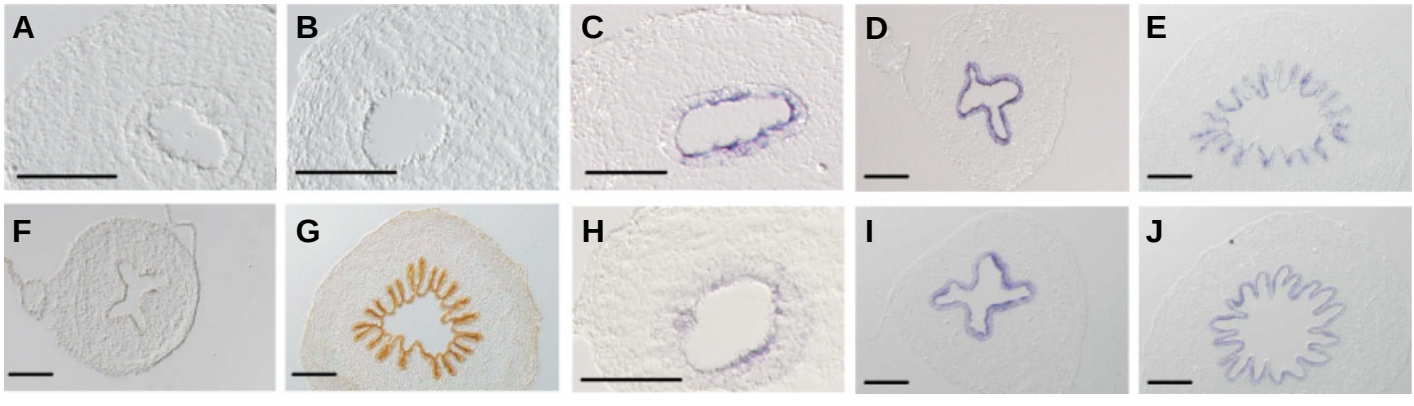

Fig. 1. Expression patterns of *cIFABP* (A-E), sucrase (F,G) and *CdxA* (H-J) in the small intestine *(A-J), of 5 (A), 6 (B,H), 7 (C), 9(D,F) and 12 (E,G,J) day embryos analyzed in transverse sections by* in situ *hybridization (*cIFABP *and* CdxA*) or immunohistochemistry (sucrase). Scale bars, 100 μm.*

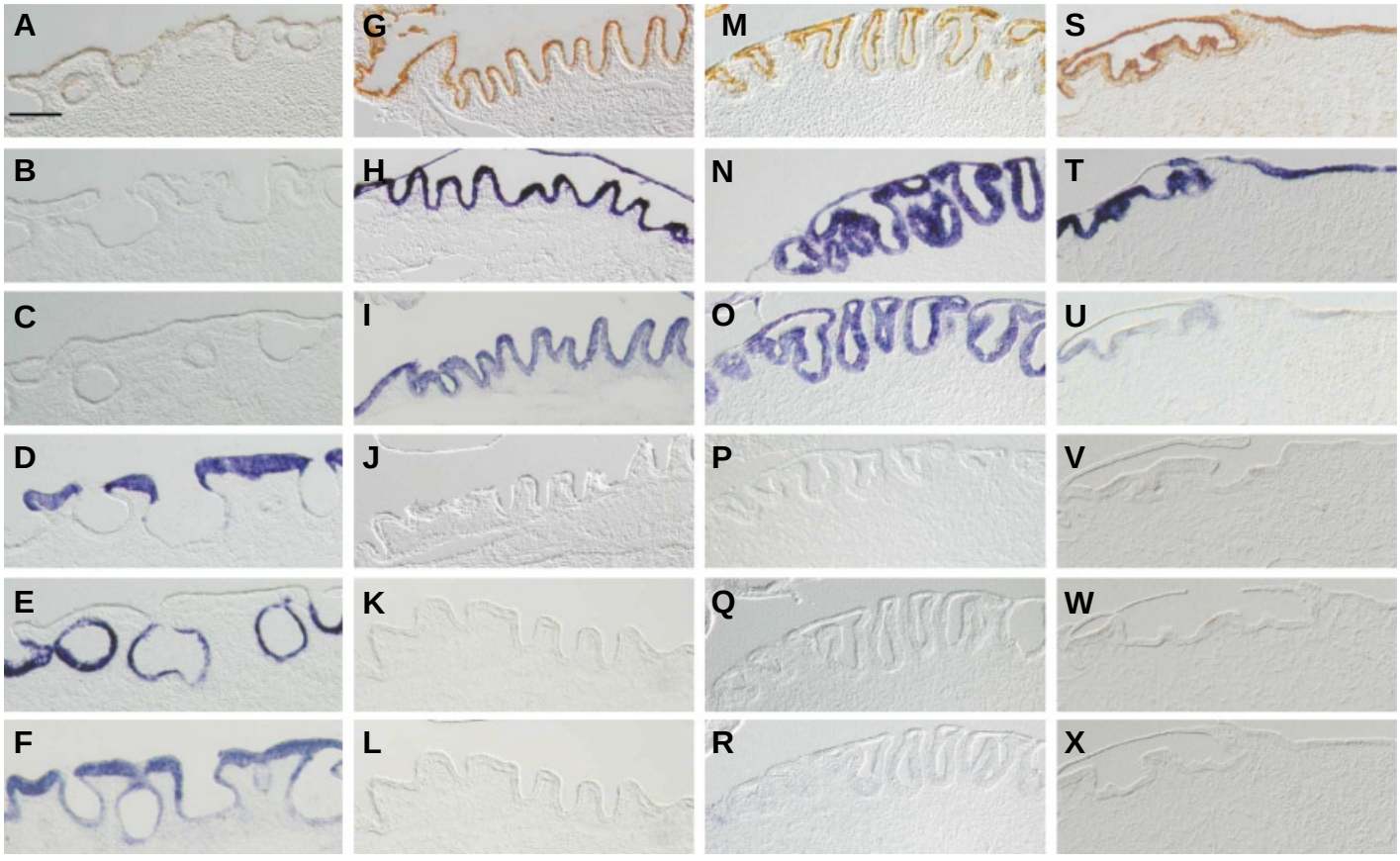

Fig. 2. Expression of sucrase, *cIFABP, CdxA, cSP, ECPg* and *cSox2* in the recombinants of small intestinal epithelium associated with heterologous gut mesenchymes. *The proventricular epithelium of 6 day embryo cultivated with 6 day proventricular mesenchyme (A-F). The small intestinal epithelium of 6 day embryo cultivated with 6 day small intestinal mesenchyme (G-L), proventricular mesenchyme (M-R) and gizzard mesenchyme (S-X). Expression of sucrase* **(A,G,M,S),** cIFABP **(B,H,N,T),** CdxA **(C,I,O,U),** cSP **(D,J,P,V)**, ECPg **(E,K,Q,W)** *and* cSox2 **(F,L,R,X)** *was analyzed by immunohistochemistry or* in situ *hybridization in adjacent sections. Scale bar, 100 μm.*

sions of marker molecules were the same as in normal development (Fig. 2 A-L, Table 1). In brief, when the proventricular epithelium was cultivated with proventricular mesenchyme, the explants expressed *cSP* in luminal epithelium, *ECPg* in glandular epithelium and *cSox2* in both luminal and glandular epithelia (Fig. 2 D-F). When the small intestinal epithelium was cultivated with the small intestinal mesenchyme, the epithelium expressed sucrase, *cIFABP* and *CdxA* (Fig. 2 G-I) and genes specific to stomach epithelium were not expressed (Fig. 2 J-L).

When the small intestinal epithelium was cultured with the proventricular mesenchyme, the epithelium invaginated into the mesenchyme and showed the proventricular gland-like structures. However the epithelium of the explants expressed sucrase, *cIFABP* and *CdxA*, markers of the small intestinal epithelium (Fig. 2 M-O), and never expressed *cSP*, *ECPg* and *cSox2*, markers of stomach epithelium (Fig. 2 P-R). Moreover, the explants composed of the small intestinal epithelium and gizzard mesenchyme expressed markers of small intestinal epithelium (Fig. 2 S-U), but not *cSP* and *cSox2* (Fig. 2 V,X). Thus the intestinal epithelium of 6 day embryo was proved to be determined with its developmental fate and showed sharp contrast to the epithelia of the proventriculus and gizzard that retain reactivity to the inductive influence of the mesenchyme (Takiguchi *et al.,* 1986; Hayashi *et al.,* 1988b; Urase *et al.,* 1996).

TABLE 1

GENE EXPRESSION IN RECOMBINANTS COMPOSED OF EPITHELIUM AND 6 DAY MESENCHYME OF THE GUT

		Number of	Expression [a]					
Epithelium[c]	Mesenchyme[c]	recombinants	sucrase	cIFABP	CdxA	cSP	ECPg	cSox2
PV	PV	24	0	0	0	100	100	100
SI	PV	20	100	100	100	0	0	0
SI	GZ	19	100	100	100	0	0	0
SI	SI	20	100	100	100	0	0	0
PIE	PV	12	0	100 [b]	100 [b]	92 [b]	0	0
PIE	GZ	16	0	0	0	94 [b]	0	0
PIE	SI	12	100	100	100	0	0	0

[a] Percentages of recombinants in which expression was detected.
[b] Strong expression was seen partially in the epithelium.
[c] PV, proventriculus; GZ, gizzard; SI, small intestine; PIE, presumptive intestinal endoderm

Reactivity of the small intestinal epithelium of 6 day embryo to 6 day dermis

Next, we tested the autodifferentiation potency of the small intestinal epithelium of 6 day embryo associated with dermis of the dorsal skin of 6 day embryo, which is supposed to have no activity to induce the epithelium of the digestive organs (Table 2). The

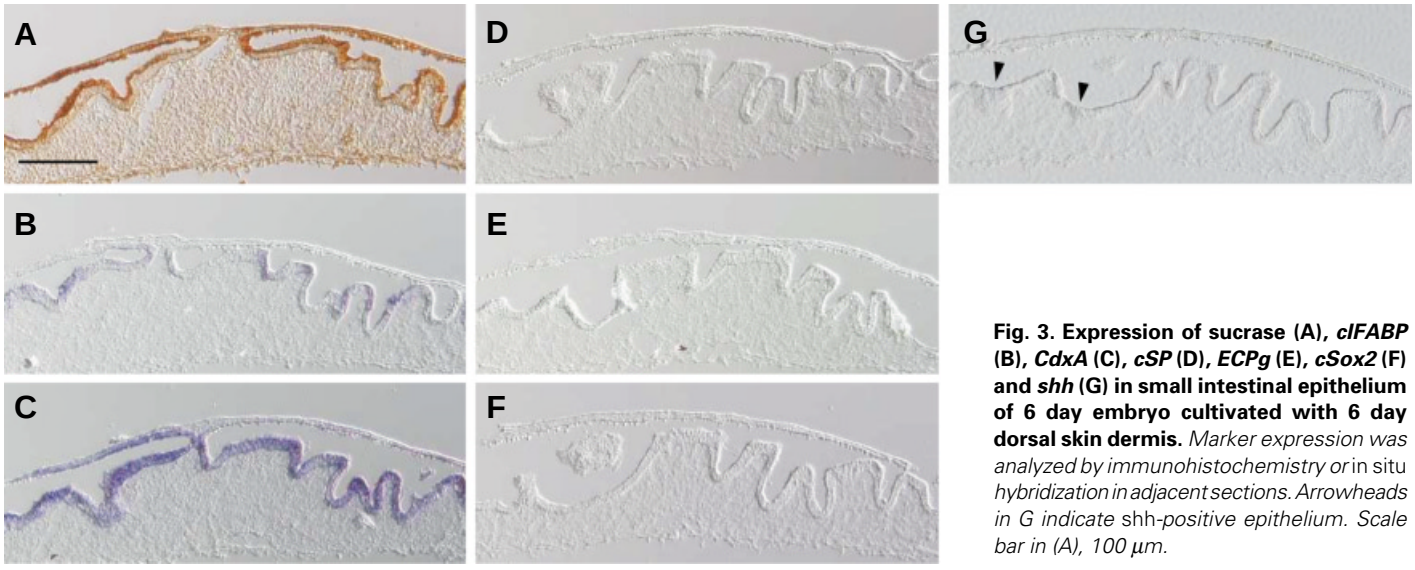

Fig. 3. Expression of sucrase (A), *cIFABP* (B), *CdxA* (C), *cSP* (D), *ECPg* (E), *cSox2* (F) and *shh* (G) in small intestinal epithelium of 6 day embryo cultivated with 6 day dorsal skin dermis. *Marker expression was analyzed by immunohistochemistry or* in situ *hybridization in adjacent sections. Arrowheads in G indicate* shh*-positive epithelium. Scale bar in (A), 100 μm.*

epithelium of explants strongly expressed sucrase, *CdxA* and *cIFABP* (Fig. 3 A-C). Some cells of the epithelium expressed *shh* (Fig. 3G, arrowhead), a pan-endodermal marker. The explants did not show expression of *cSP*, *ECPg* and *cSox2* (Fig. 3 D-F). The results clearly indicated that the small intestinal epithelium can autodifferentiate according to its own developmental fate.

Reactivity of the presumptive intestinal endoderm of 1.5 day embryo to 6 day proventricular mesenchyme or gizzard mesenchyme

The fact that the developmental fate of the small intestinal epithelium is already determined by day 6 of incubation raises the question whether the presumptive intestinal endoderm associated and cultivated with 6 day proventricular mesenchyme or gizzard mesenchyme can differentiate heterologously. When the presumptive intestinal endoderm of 1.5 day embryos was cultivated with small intestinal mesenchyme, the epithelium expressed sucrase, *CdxA* and *cIFABP*, markers of small intestinal epithelium (Fig. 4 A-C), but never expressed *cSP*, *ECPg* and *cSox2* (Fig. 4 D-F). The presumptive intestinal endoderm associated and cultivated with 6 day proventricular mesenchyme invaginated into the mesenchyme and formed the gland-like structures, but still expressed markers specific to the small intestinal epithelium except sucrase (Fig. 4 G-I). Among the markers of stomach epithelium, *cSP* was expressed occasionally in small parts of the epithelium where intestinal markers were not expressed (Fig. 4J), but *ECPg* and *cSox2* could not be detected (Fig. 4 K,L). When the presumptive intestinal endoderm was cultivated with 6 day gizzard mesenchyme, the epithelium expressed *cSP* (Fig. 4P), marker of luminal epithelium of the stomach, but other stomach epithelial markers (Fig. 4 Q,R) and markers of small intestinal epithelium were never expressed (Fig. 4 M-O). These results indicated that the presumptive intestinal endoderm of 1.5 day embryo had different reactivity to the proventricular mesenchyme and gizzard mesenchyme.

TABLE 2

GENE EXPRESSION IN RECOMBINANTS COMPOSED OF EPITHELIUM AND 6 DAY DORSAL DERMIS

	Number of	Expression [a]						
Epithelium[d]	recombinants	sucrase	cIFABP	CdxA	shh	cSP	ECPg	cSox2
SI	16	94	94	94	69 [b]	0	0	0
PIE	6	0	0	0	80 [c]	0	0	0

[a] Percentages of recombinants in which expression was detected.
[b] Expression was seen in very narrow areas of recombinants.
[c] Strong expression was seen partially in the epithelium.
[d] SI, small intestine; PIE, presumptive intestinal endoderm

Reactivity of the presumptive intestinal endoderm of 1.5 day embryo to 6 day dermis

Finally, we tested the autodifferentiation potency of the presumptive intestinal endoderm of 1.5 day embryo associated with 6 day dorsal dermis. The epithelium expressed *shh* (Fig. 5D), a pan-endodermal marker, suggesting it acquired the characteristics of endodermal epithelium, but never expressed other markers of the small intestinal epithelium and stomach epithelium (Fig. 5 A-C, Table 2). The endoderm never formed feather-like structures on the dorsal dermis.

The above results indicate that the developmental fate of the presumptive intestinal endoderm is almost decided before 1.5 day of embryonic development, and the presumptive intestinal endoderm differentiates into the small intestinal epithelium under the existence of the mesenchyme derived from splanchnic mesoderm.

Discussion

It has been repeatedly confirmed that the proventricular or gizzard epithelium associated with the mesenchyme of heterologous organs differentiates heterotypically according to the mesenchymal induction (Takiguchi *et al.,* 1986; Hayashi *et al.,* 1988b; Urase *et al.,* 1996). On the other hand, the small intestinal epithelium of 6 day embryo, or even younger presumptive intestinal endoderm, associated and cultivated with the proventricular mesenchyme, never differentiated into *ECPg*-expressing epithelium (Urase *et al.,* 1993; Yasugi *et al.,* 1985; 1991). These results indicate that the (presumptive) small intestinal epithelium is determined as regards its developmental fate early in the development. In this study we analyzed more precisely and comprehensively the determination

period of developmental fate of the small intestinal epithelium under epithelial-mesenchymal interactions with using the molecular differentiation markers, and investigated the autodifferentiation potency of the small intestinal epithelium. Among these markers, *CdxA* is abundantly expressed in the small intestinal epithelium from early stages of development (Ishii *et al.,* 1997). On the other hand, *cIFABP* and sucrase are expressed when the organ acquires its characteristic features (this study and Matsushita, 1985). Therefore these genes are useful markers of small intestinal epithelium at various stages of the development. It was reported that *IFABP* is expressed specifically in the small intestinal epithelium from early in the development in *Xenopus* (Chalmers and Slack, 1998) and that, during metamorphosis, *IFABP* expression decreases as the tadpole intestinal epithelium is shed. As the more complex adult-like epithelium forms after metamorphic climax, *IFABP* expression is restored (Shi and Par Hayes, 1994; Ishizuya-Oka *et al.,* 1997). In this report we described developmental expression pattern of *cIFABP* for the first time. In chicken embryo, the expression of *cIFABP* begins on day 7 and continues thereafter, solely in the small intestinal epithelium.

Reactivity of the 6 day small intestinal epithelium and 1.5 day presumptive intestinal endoderm to heterologous mesenchymes

We tested whether the small intestinal epithelium, associated and cultivated with the proventricular mesenchyme or gizzard mesenchyme, expresses various intestinal marker genes. The morphological differentiation of the small intestinal epithelium

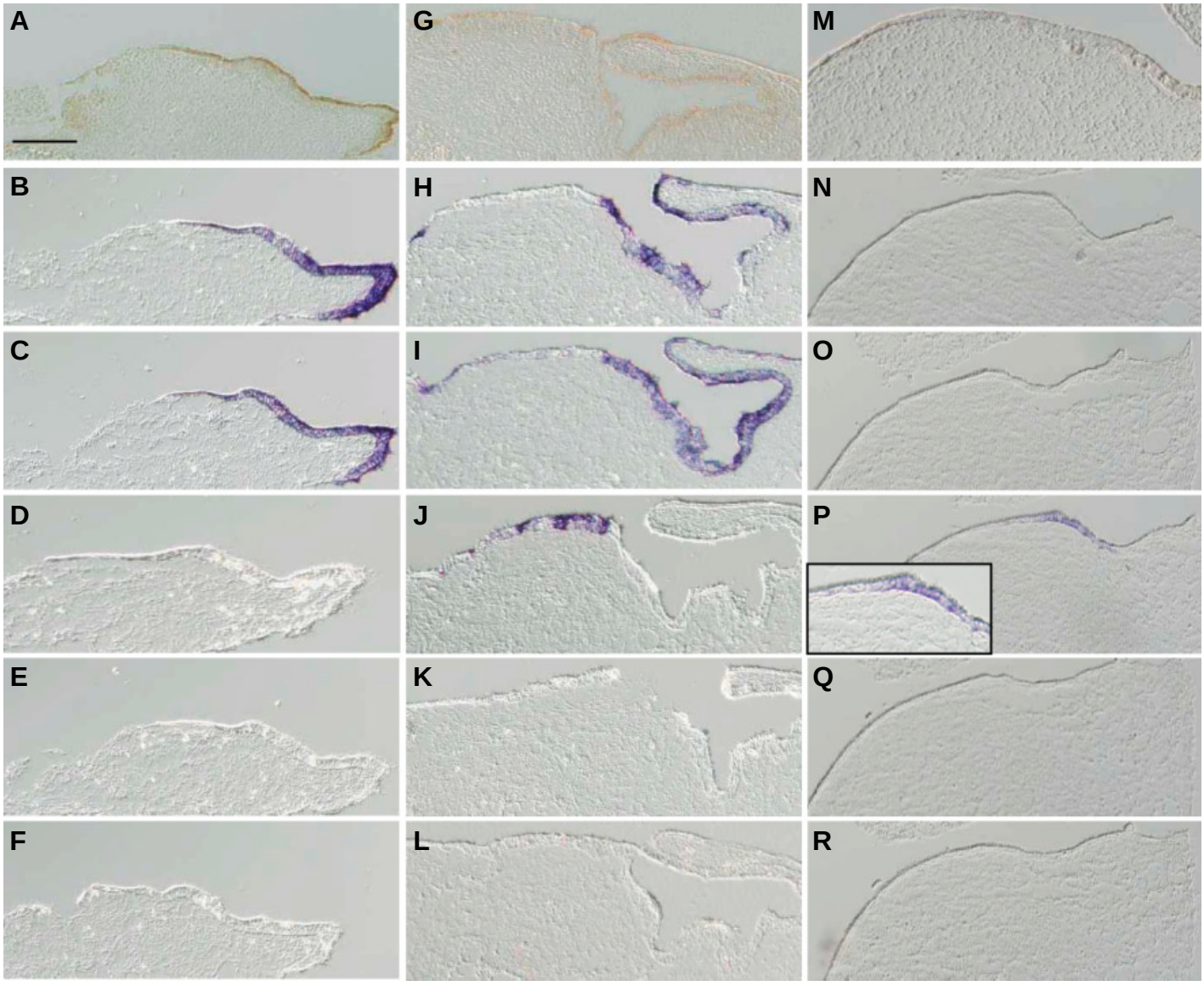

Fig. 4 (Left). Expression of sucrase, *cIFABP, CdxA, cSP, ECPg* and *cSox2* in the recombinants of presumptive intestinal endoderm associated with heterologous gut mesenchymes. *The presumptive intestinal endoderm of 1.5 day embryo cultivated with 6 day small intestinal mesenchyme* **(A-F)**, *proventricular mesenchyme* **(G-L)** *and gizzard mesenchyme* **(M-R)**. *Expression of sucrase (A,G,M),* cIFABP *(B,H,N),* CdxA *(C,I,O),* cSP *(D,J,P),* ECPg *(E,K,Q) and* cSox2 *(F,L,R) was analyzed by immunohistochemistry or* in situ *hybridization in adjacent sections. Inset in P is a higher magnification view of the corresponding section. Scale bar, 100 μm.*

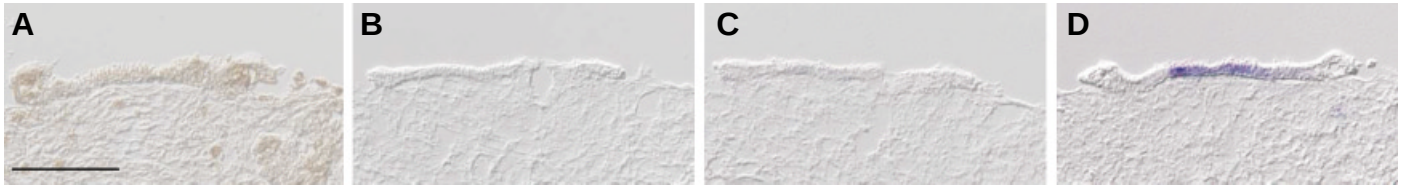

Fig. 5. Expression of sucrase (A), *cIFABP* (B), *CdxA* (C) and *shh* (D) in presumptive intestinal endoderm of 1.5 day embryo cultivated with 6 day dorsal skin dermis. *Marker expression was analyzed by immunohistochemistry or* in situ *hybridization in adjacent sections. Scale bar, 100 μm.*

depended on the nature of the mesenchyme associated: the epithelium formed gland like structures when it was associated with proventricular mesenchyme. However it invariably expressed sucrase, *cIFABP* and *CdxA* and never expressed the stomach epithelial markers, suggesting that the influences of mesenchymal induction can occasionally affect morphological differentiation but not cytodifferentiation.

Presumptive intestinal endoderm of 1.5 day embryo showed different reactivity to the proventricular mesenchyme and gizzard mesenchyme of 6 day embryo. In the association with the proventricular mesenchyme, the endoderm expressed *cIFABP* and *CdxA*, while these genes were not expressed in the association with the gizzard mesenchyme. In both cases *cSP*, marker of stomach epithelium, was induced in some parts of the epithelium. This difference in reactivity may attributable to the difference in the inductive ability of two mesenchymes: the gizzard mesenchyme exerts stronger influence on the heterotypic differentiation of the small intestinal (duodenal) epithelium than the proventricular mesenchyme (Matsushita, 1995b). Thus the presumptive intestinal endoderm, though the main developmental fate is already determined at 1.5 day, could respond to the action of the proventricular and gizzard mesenchymes and express *cSP*, it did not express intestinal markers under the influence of the gizzard mesenchyme.

In both combinations, sucrase was scarcely detected in the presumptive intestinal endoderm. It has been reported that *Cdx2* regulates the expression of sucrase in the mouse (Lorentz *et al.,* 1997) and that *CdxA* binds to the promoter region of sucrase (Sklan *et al*., 2003). It is therefore possible that *CdxA* regulates the expression of sucrase in the chicken. When the presumptive intestinal endoderm was associated with the proventricular mesenchyme, *CdxA* was widely expressed but there was no or only weak expression of sucrase. This may due partly to the cultivation period of the recombinants. We cultivated recombinants for 6 days so that they corresponded to embryos of 7.5 or 8 day of incubation. At this stage sucrase was not yet expressed in the normal course of development.

Autodifferentiation potency of 6 day small intestinal epithelium and 1.5 day presumptive intestinal endoderm

When the small intestinal epithelium associated with dorsal skin dermis which is supposed to have no activity to induce the epithelium of the digestive organs, the epithelium expressed sucrase, *cIFABP* and *CdxA*, but never expressed stomach epithelial markers. From the results, we thought that the small intestinal epithelium has lost reactivity to other heterologous mesenchymes, and it has an autodifferentiation potency. This result is different from the report that, when the proventricular epithelium was associated with the dorsal dermis, it never expressed *ECPg* and the characteristics of the small intestinal epithelium was observed (Urase *et al.,* 1996).

On the other hand, when the presumptive intestinal endoderm of 1.5 day embryo associated with dorsal skin dermis, the epithelium never expressed markers of small intestinal epithelium and stomach epithelium. The fact that expression of shh, a pan-endodermal marker, was maintained in the epithelium indicates that the developmental fate of younger presumptive intestinal endoderm was almost determined but the influences of the mesenchymes of the digestive organs are necessary for the differentiation of the small intestinal epithelium. These results are consistent with the report that the lateral plate mesoderm has instructive influence on the formation of dorsal pancreas (Kumar *et al.,* 2003). Also in mammals, it was demonstrated that presumptive intestinal endoderm is determined according to its origin as for its differentiation fate (Duluc *et al.,* 1994; Ratineau *et al.,* 2003).

Molecular mechanism of regionalization in gut development

Our present results and many reports lead to the conclusion that developmental fate of the posterior gut endoderm is determined early in the development while the anterior gut endoderm seems to retain the reactivity to the heterologous mesenchymes up to day 6 of incubation. The molecular mechanisms underlying the regionalization in gut development has not been clearly understood. We suppose that *CdxA* is involved as one of determination factors. *CdxA* gene is specifically expressed in the endoderm which becomes midgut and hindgut before 1.5 day (Ishii *et al.,* 1997). Moreover, it was reported that mouse *Cdx1* and *Cdx2* are important in anterior-posterior patterning in the intestinal epithelium (Silberg *et al.,* 2000). *Cdx2* defines the stomach-intestine boundary and when the function of *Cdx2* was down-regulated, the proximal part of the intestine formed the gastric-type tissue (Beck *et al.,* 2003). From these reports, *CdxA* is thought to be an important factor involved in the determination of the developmental fate of the small intestinal epithelium in the chicken embryo. We are currently investigating the effects of ectopic expression of *CdxA* gene in the presumptive stomach endoderm of young embryo.

Materials and Methods

Animals

Embryos of White Leghorn chickens *(Gallus gallus domesticus)* were used throughout the experiments.

Isolation of tissue fragments

The presumptive intestinal endoderm was obtained from embryos of 1.5 days of incubation (embryos with 15 to 20 somites, stage 10-12,

Hamburger and Hamilton, 1951). At this stage, the presumptive stomach endoderm locates just under the notochord of level of somite 2-7, and the presumptive intestinal endoderm locates more caudally and laterally (Matsushita, 1995a; 1996; 1999). We used the endoderm of level of somite 10-15 of embryos as a source of presumptive intestinal endoderm. The endodermal fragments were isolated from notochord, somites and lateral plate mesoderm by mild treatment of collagenase type I (Worthington Biochemical Corporation, Lakewood, NJ; Code CLS1, 0.03% in Tyrode's solution for 15 min at 38ºC). Epithelial fragments and the mesenchymes of the proventriculus, gizzard and small intestine (a part just anterior to the yolk sac stalk) of 6 day embryos were obtained by longer (30-60 min) treatment of collagenase. The epithelia were stripped off as a sheet from the underlying mesenchymes and both tissues were washed in Tyrode's solution. The dorsal dermis was isolated from the dorsal skin of 6 day embryos. The skin fragments were rinsed in a calcium- and magnesium-free (CMF) Tyrode's solution, and then treated with double-strength CMF Tyrode's solution containing disodium ethylenediamine tetraacetate (EDTA, 0.25%) at 20ºC for 1 to 3 hours (Mizuno *et al.,* 1990; Urase *et al.,* 1996). The epidermis were stripped off as a sheet from the underlying dermis and washed in Tyrode's solution.

Tissue recombination experiments and in vitro *cultivation technique*

Epithelium or presumptive intestinal endoderm was reassociated with the mesenchyme on the semi-solid agar substratum (Wolff and Haffen, 1952) for 12 h at 37ºC to ensure the coherence of two tissue fragments. The agar substratum was composed of seven parts of agar (2% agar in Gay's solution), 3 parts of Medium 199 with Earle's salts (Gibco, USA) and 3 parts of 13 day chicken digestive tract- and eye-free embryo extract. Recombinants were transferred onto Nuclepore filters (Whattman, USA) of pore size 0.8 μm, rested on stainless-steel grid which was placed within a small culture dish. The medium reached the Nuclepore filter and moistened surface of the explants but not covered them. The culture dishes were incubated for 6 days at 37ºC in 95% air and 5% CO_2. The culture medium was Medium 199 with Earle's salts supplemented with 50% 13 day embryo extract and penicillin (100 U/ml). The culture medium was replaced with flesh medium every second day (Takiguchi *et al.,* 1988).

In situ *hybridization*

Digoxygenin-labeled RNA probes for *in situ* hybridization were prepared from cDNA clones of *ECPg* (Hayashi *et al.,* 1988), *cSP* (Tabata *et al.,* 1998), *cSox2* (Uwanogho *et al.,* 1995), *sonic hedgehog* (*shh*, Nohno *et al.,* 1995), chicken *IFABP* (*cIFABP*, MRC Geneservice, Cambridge) and *CdxA* (Ishii *et al.,* 1997). Organs and explants were fixed with 4% paraformaldehyde in phosphate-buffered-saline (PBS) overnight at 4ºC and embedded in OCT compound (Sakura Finetechnical Co. Ltd., Tokyo Japan). Frozen sections of 10 μm were cut in a cryostat. *In situ* hybridization on frozen sections was performed as previously described (Ishii *et al.,* 1997).

Immunohistochemistry

Adjacent frozen sections were used for *in situ* hybridization and immunohistochemistry. After rehydration with PBS, sections were blocked with 0.5% skim milk in PBS and treated with polyclonal anti-chicken sucrase antibody (Matsushita, 1985) overnight at 4ºC. After three washes with PBS at room temperature, sections were treated with horse radish peroxidase-conjugated anti-rabbit immunoglobulin G for 1 h at 37ºC, and washed with PBS three times. Coloring reaction was done with 0.2 μg/ml diaminobenzidine in 0.1 M Tris-HCl (pH 7.5) and 0.001% H_2O_2.

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