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[REVIEW]

The Mesenchymal Factors Regulating Epithelial Morphogenesis and Differentiation of the Chicken Stomach

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ABSTRACT—It is now well established that epithelial-mesenchymal interactions are essential for the formation of many organs in the development of the animals. Chicken digestive organs provide a valuable model system for analysis of the mechanisms underlying the epithelial-mesenchymal interactions. Here we will present our recent data indicating that the mesenchymal factors necessary for the epithelial differentiation in the chicken stomach are composed of several components such as growth factors and extracellular matrices. The possible involvement of bone morphogenetic protein-2 will be discussed.

Introduction

The notion that cell-to-cell or, more accurately, tissue-to-tissue interactions are requisites for the formation of organ systems having normal morphology and function was developed by Spemann (1901) in his study on the development of the amphibian eye. His notion or paradigm of induction persists for about one century and is still a leading principle in the field of developmental biology, although the stress is now on the elucidation of molecular nature of the interactions.

Since the time of Spemann, many organ systems, such as skin (Peterson and Grainger, 1985), kidney (Bard *et al.*, 1996), tooth (Peters and Balling, 1999), limb (Tickle and Eichele, 1994), urogenital sinus (Takeda *et al.*, 1990), salivary gland (Nogawa and Mizuno, 1981), mammary gland (Streuli *et al.*, 1991), lung (Minoo and King, 1994), pancreas (Hebrok *et al.*, 1998), liver (Fukuda-Taira, 1981; Hentsch *et al.*, 1996) and heart (Schultheiss *et al.*, 1995), only to mention organs especially well studied, have been analyzed and found to involve complex interactions between tissues composing the organs or between neighboring tissues.

These studies have revealed that we can distinguish two types of interactions: instructive induction and permissive induction. The former is characterized by the experiments in which the developmental fate of the reactive tissue (effector tissue) was changed by the influence of the affective tissue (inducer) and directed toward the fate of the inducing tissue. A typical example was provided by the

experiment of Rawles (1963) showing that the dermis of scale induces scale development in the associated presumptive feather epithelium. On the other hand, the idea of permissive induction came from the fact that the differentiation of pancreatic epithelium is determined rather early in the development but its realization depends on the specific influence of the pancreatic mesenchyme (Wessells and Cohen, 1967).

However, now it is said that the two types of interactions cannot be separated so clearly. It is apparent from many studies that not only inductive influence of the inducer tissue but also the reactivity of effector tissue is important for the determination of the latter. Rather, the developmental fate of a tissue is determined gradually by reciprocal tissue interactions which include complex and strictly regulated molecular cascades the understanding of which is one of the main aims of the developmental biology today.

In this review article, we will present some data indicating that the differentiation of a tissue is determined by the inductive influence of another tissue and reactivity of the responding tissue, and refer to the biological and molecular natures of the interactions, based on the results obtained with the experiments on the differentiation of the digestive organs in the chicken embryos.

Development and gene expression patterns of the chicken digestive organs

Vertebrate digestive tract is composed of endodermal epithelium and mesodermal mesenchyme. At the early stages of avian and mammalian development, both tissues are not a tube but is extending as flat sheets. Meanwhile, from embryonic day (E) 1.5 to 3, sheets of left and right

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sides of the embryonic body form a fold and fuse at the ventral side of the body. The formation of tube first begins at the rostral end of the gut and proceeds backward, while it commences a little later from the caudal end of the gut and proceeds forward. Both rostral and caudal tubes meet at the middle part of the embryo and thus the primitive gut tube is formed. At first the dorsal side of the endoderm is not covered with mesenchyme, but soon mesenchymal tissue surrounds entire epithelium.

From E3 to E4 we cannot explicitly distinguish each digestive organ, but liver and pancreas have already bulged out from the main gut tube. Also lung bud (trachea) sepa-

rates from the pharynx, upper part of the gut. From E5, digestive organs such as esophagus, stomach, small intestine and large intestine can be seen macroscopically, but the internal structure of the organs has not yet specified: these organs consist of undifferentiated inner epithelial and outer mesenchymal tissue.

It is to be noted that bird has two stomachs: the proventriculus and gizzard. The proventriculus is a glandular stomach in which epithelium forms compound glands and gland epithelial cells later synthesize and secrete pepsinogen (embryonic chicken pepsinogen ECPg; Hayashi *et al.*, 1988a, b; Fig. 1), a zymogen of digestive enzyme pepsin.

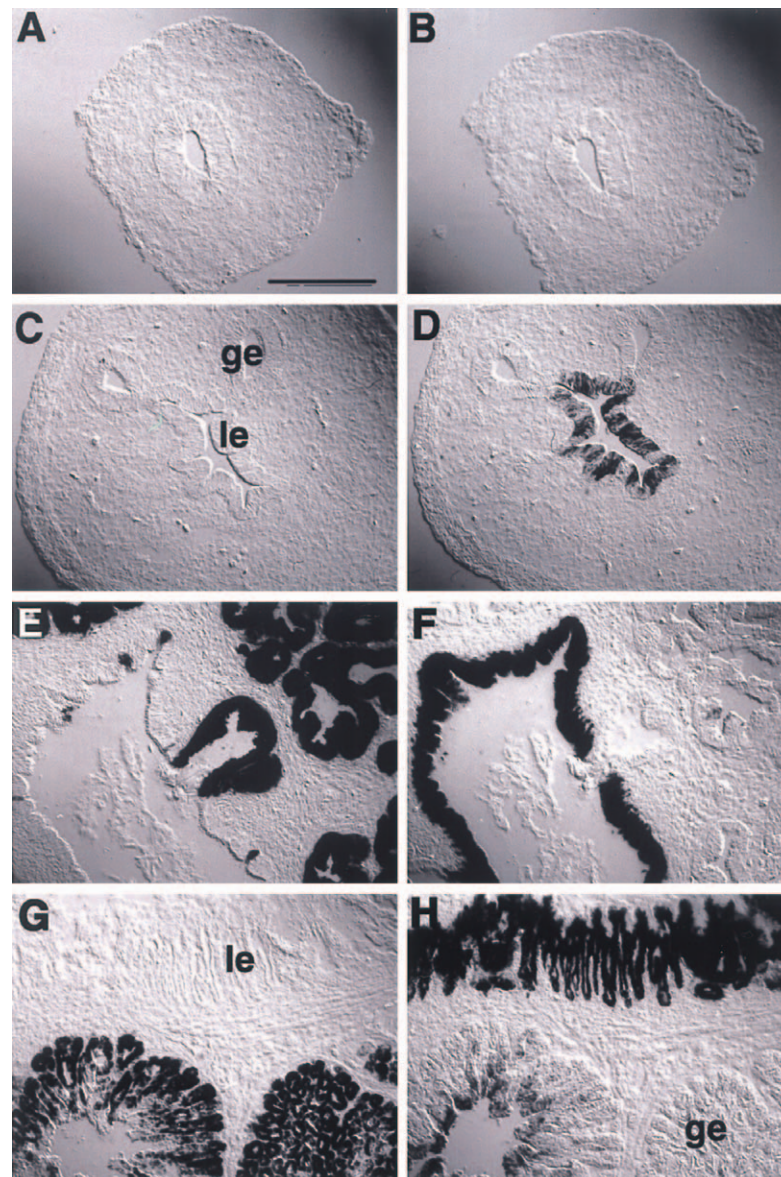


Fig. 1. Expression of *ECPg* (A, C, E, G) and *cSP* (B, D, F, H) genes during the development of the proventriculus in chicken embryo examined by in situ hybridization. (A, B) E6. The epithelium does not yet form glands. No *ECPg* and *cSP* expression (C, D) E8. Glands are formed but gland epithelial cells (gl) do not express *ECPg* gene. Luminal cells (le) begin to express *cSP*. (E, F) E13. (G, H) E16. Compound glands develop well and gland epithelial cells (gl) express *ECPg* while luminal epithelial cells (le) are positive to *cSP* but never express *ECPg*. Note that expression of both genes is complementary. Bar: 200 μ m. From Tabata and Yasugi (1998).

The gizzard does not develop compound glands. The epithelium of the gizzard actively secretes mucous substances and there develop massive smooth muscle layers in the mesenchyme.

We are analyzing expression patterns of many genes known to be important in organogenesis, with special attention to the expression in epithelial cells (Fig. 2). A gene expressed at the earliest stage is *CdxA*, a homologue of *caudal* gene of *Drosophila* and encoding a transcription factor containing homeodomain (Frumkin *et al.*, 1991). It is expressed in the presumptive intestinal epithelium (Ishii *et al.*, 1997) and later it becomes expressed in the entire epithelium of intestine. *cSox2* gene which encodes HMG-box-containing transcription factor, is expressed in the epithelium destined to differentiate into epithelium of esophagus, proventriculus or gizzard, i.e. the anterior

organs (Ishii *et al.*, 1998), *cSox2* is expressed continuously in the epithelium of anterior organs and its caudal limit coincides with the boundary of gizzard-intestine. This boundary also marks the anterior end of *CdxA* expression. Thus the expressions of *cSox2* and *CdxA* respectively characterize anterior organs and intestine. Soon after the gut tube is formed epithelial cells of ventral and lateral sides express a gene encoding a morphogen sonic hedgehog (*shh*), and its expression soon extends to the dorsal side of the tube when mesenchymal cells underlie the epithelium (Narita *et al.*, 1998; Roberts *et al.*, 1998). In later development of the gut, *shh* is expressed in almost all epithelial cells but ceased to be expressed in cells of epithelia which bulge from the main duct, such as pancreatic duct, yolk stalk and proventricular glands.

Expression of transcription factor genes other than

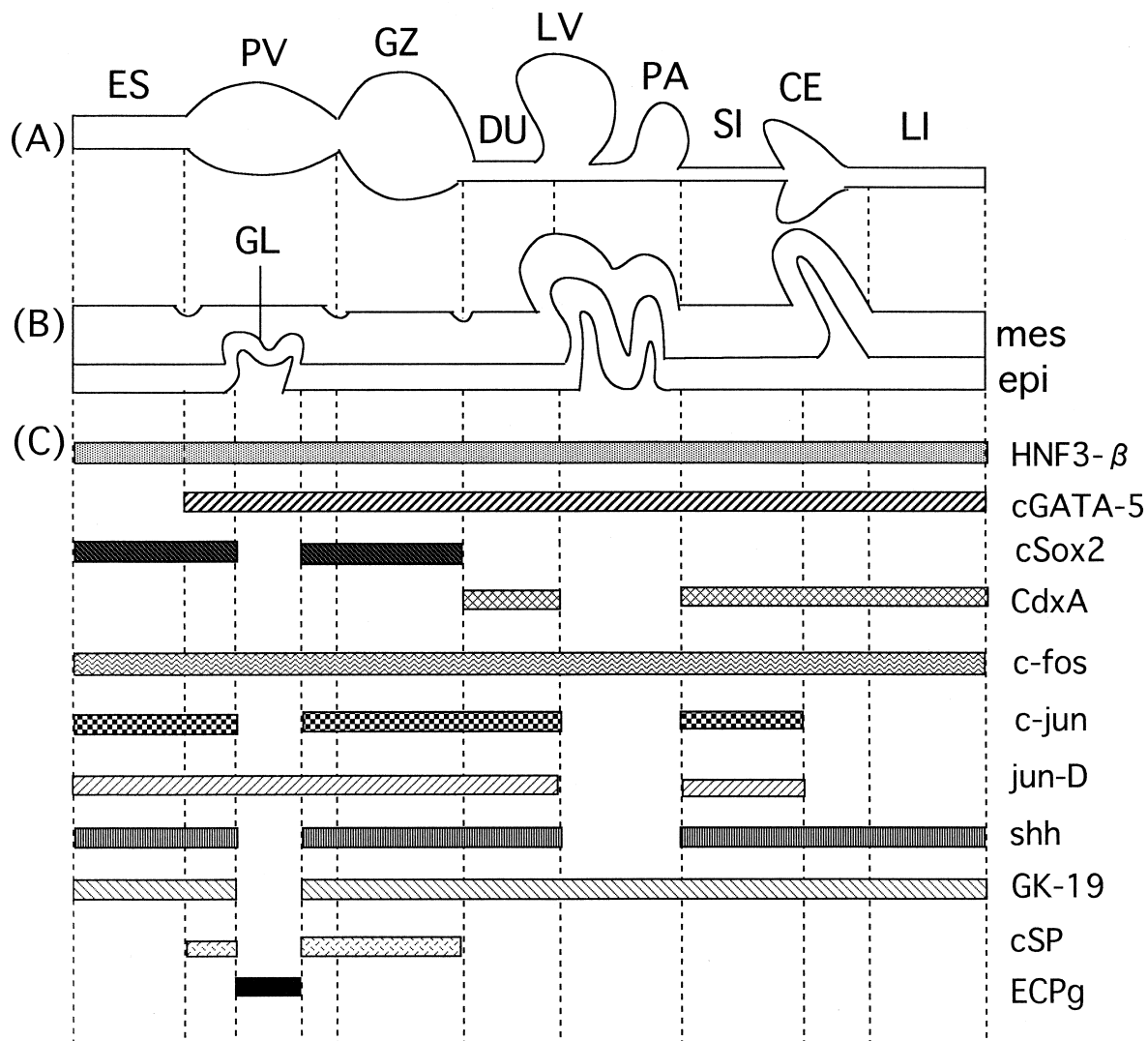


Fig. 2. Expression patterns of genes in epithelial cells of the gut of chicken embryo (E9). Compiled from: Sakamoto *et al.* (1998), Ishii *et al.* (1997, 1998), Matsumoto *et al.* (1998), Narita *et al.* (1998), Sato and Yasugi (1998), Tabata and Yasugi (1998) and Fukuda *et al.* (1994). (A) Schematic figure of the gut. ES, esophagus; PV, proventriculus; GZ, gizzard; DU, duodenum; LV, liver; PA, pancreas; SI, small intestine; CE, cecum; LI, large intestine. (B) Sagittal section of the gut. GL, proventricular glands; mes, mesenchyme; epi, epithelium. (C) Expression patterns of genes along the antero-posterior axis of the gut. Names of genes are indicated on the right side.

cSox2 and *CdxA* has been also studied. Among them are *HNF3-β* and *GATA5*. The former is expressed throughout the gut while the latter is not expressed or only weakly expressed in the esophagus. *GATA5* seemed to be upregulated in epithelial cells of proventricular glands (Sakamoto *et al.*, unpublished data).

We also surveyed the expression of other genes of interest in view of the morphogenesis and cytodifferentiation. Oncogenes belonging to *fos* and *jun* family such as *c-fos*, *fra-2*, *c-jun* and *junD* were shown to be expressed in characteristic manners. Among them, *fra-2* was expressed in the luminal epithelium but not in gland epithelium, just as *cSox2* and *shh*. We argued that the expression of *fra-2* and *junD* is controlled by *shh* and *Indian hedgehog* which are expressed in similar pattern as *fra-2* and *junD*, but from earlier developmental stages (Matsumoto *et al.*, 1998).

We cloned a new gene of which expression is restricted to the luminal epithelium in the proventriculus. The gene, designated chicken spasmodic polypeptide (*cSP*), encodes a mucous-cell-associated protein containing a trefoil structure (Tabata and Yasugi, 1998). It is expressed from E8 in the proventricular epithelium and, after the formation of glands, only in the luminal epithelium, showing sharp contrast with the expression of ECPg (Figs. 1 and 2). Thus *cSP* expression is a very specific marker of luminal epithelium of the proventriculus. It is also expressed in the epithelium of esophagus and gizzard. Another gene expressed in the

epithelium of the gut cloned in our laboratory is that encoding keratine-19 (*GK-19*, Sato and Yasugi, 1997). Keratins are intermediate filaments of many epithelial cells and *GK-19* is expressed in the epidermis, endodermal epithelium and lung, but not in the liver and heart. Interestingly enough, it is expressed in the notochord and floor plate just as *shh* (Echelard *et al.*, 1993) and HNF-3β (Sasaki and Hogan, 1993). In the proventriculus, the expression of *GK-19* gene is again downregulated in gland epithelial cells for a while after the onset of gland formation. The same is true for a keratine detected by an antibody PKK1 (Takiguchi-Hayashi *et al.*, 1996).

These results demonstrated that gland epithelial cells of the proventriculus are very unique population of epithelial cells from the view point of gene expression. With these gene expressions as very sensitive and specific markers of epithelial cell differentiation, we performed experiments to reveal epithelial-mesenchymal interactions in the process of stomach formation.

Epithelial-mesenchymal interactions in the gut formation

Our "classical" experiments about the effect of mesenchyme on the epithelial differentiation judged by the morphological criteria and ECPg expression were summarized in Yasugi (1995) and in Uruse *et al.* (1996). In brief, the mesenchyme exerts inductive influence on the morpho-

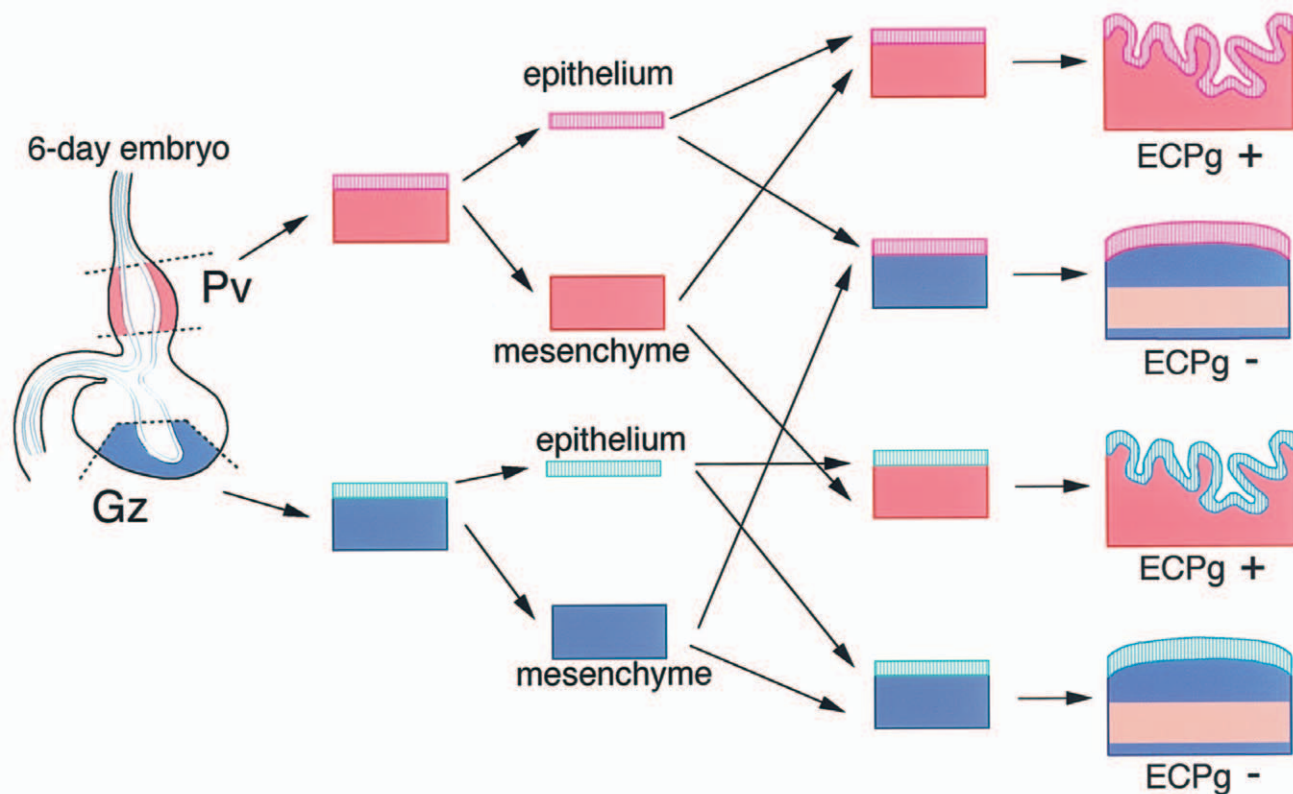


Fig. 3. Tissue recombination experiments in which epithelia and mesenchymes of proventriculus (Pr) and gizzard (Gz) were isolated and recombined in various combinations. The recombinants were cultured *in vitro* and examined the gland formation and expression of ECPg. Proventricular mesenchyme exerts inductive influence while gizzard mesenchyme inhibits the differentiation toward proventricular epithelium.

logical differentiation of the epithelium: the proventricular mesenchyme, for example, induced gland formation in epithelia derived from esophagus, gizzard and intestine. On the other hand, when the epithelial differentiation was assessed by ECPg expression, somewhat different results were obtained. The proventricular mesenchyme could induce ECPg expression in the epithelia of esophagus and gizzard, but not in the intestinal epithelium. The gizzard mesenchyme completely inhibited the ECPg expression even in the proventricular epithelium (Fig. 3). Moreover a tissue recombination experiment demonstrated that the lung mesenchyme had the same or even stronger inductive effect on ECPg expression in the heterotypic epithelia (Urase *et al.*, 1996).

From these results we have presented a hypothesis to explain the expression of *ECPg* gene solely in gland epithelial cells of the proventriculus as follows (Yasugi, 1995): 1) All epithelial cells of anterior digestive organs (esophagus, proventriculus and gizzard) have a potency to express ECPg (and to form glands) under the appropriate conditions provided by the mesenchyme of the proventriculus or lung, 2) the mesenchymes of esophagus and gizzard have an inhibitory effect against gland formation and ECPg expression, 3) intestinal epithelium has no potency to express ECPg from the early developmental stage (Yasugi *et al.*, 1991). As a results of interaction between mesenchymal inductive influence and epithelial reactivity, only proventricular epithelium becomes capable of expressing ECPg in the normal course of development.

The effect of mesenchyme on expression of genes other than the *ECPg* was also investigated. As mentioned above, *cSox2* is expressed in the epithelium of anterior organs and *CdxA* in the intestinal epithelium. When young (E4) stomach epithelium was associated with intestinal mesenchyme and cultivated *in vitro*, *CdxA* expression was induced in some parts of epithelium where expression of *cSox2* weakened. At the same time, sucrase activity, a marker of intestinal epithelium (Matsushita, 1983) was observed in epithelial cells with *CdxA* expression (Ishii *et al.*, 1997, 1998). Likewise expression of *shh* and *cSP* was shown to be regulated by the mesenchymal influences (Narita *et al.*, 1998; Tabata *et al.*, 1998). Thus we can say that region-specific expression of genes characterizing each organ is ultimately determined by the mesenchyme.

The nature of mesenchymal influences

We have tried to elucidate the molecular nature of the mesenchymal factors that induce or inhibit gland formation of the proventricular epithelium. First attempt was to examine if the factors could pass through the filter of appropriate pore size. In many organ systems the inducing factors have been shown to reach the effector tissue across the filter (Saxén *et al.*, 1976).

The proventricular or gizzard epithelium was placed on the Nuclepore filter and the proventricular mesenchyme was attached to the opposite side, and the combination was

cultivated *in vitro* for several days. We found that epithelial cells expressed ECPg if the pore size of the filter was larger than 0.45 μm . In this case, inspection by scanning electron microscopy of filter surface to which only the mesenchyme was attached revealed that many cell processes of the mesenchymal cells penetrated the filter, so that epithelial cells might touch directly with these cell processes. Thus we hypothesized that the direct contact between epithelial cells and mesenchymal cells is necessary for the induction of ECPg expression in epithelial cells (Takiguchi-Hayashi and Yasugi, 1990).

On the other hand, when mesenchymal cells of the proventriculus and gizzard were mixed in various proportions and the cell aggregate was recombined with the gizzard epithelium, even 5% of gizzard mesenchymal cells completely inhibited the gland formation and ECPg expression in the epithelium, suggesting that inhibitory effects of the gizzard mesenchyme are mediated by some water-soluble, far-reaching substances (Urase *et al.*, 1993).

Substantial progress was made about the nature of inducing substances of the proventricular mesenchyme by the experiments using the technique of cultivation of epithelium covered with some extracellular matrices. Cultivation of epithelium or epithelial cells of the embryonic chicken gut is difficult and we could not detect ECPg in the epithelial cells cultivated *in vitro* (Tabata and Yasugi, 1996). Then we devised a system in which the epithelium was placed on the filter and clotted with Matrigel (Kleinman *et al.*, 1986) or collagen solution. In this method, the epithelium grew healthy. Nevertheless, the proventricular epithelium could not express *ECPg* gene if it was cultivated alone. To induce ECPg expression in the epithelium, it was necessary to cultivate mesenchyme on the opposite side of the filter, which has very narrow pores not permitting the pass through of cell processes. In this case ECPg-expressing cell clusters scattered in the epithelium. It is to be noted that the lung mesenchyme has much stronger effect on ECPg induction than the proventricular mesenchyme (Koike and Yasugi, 1999, Fig 4). Moreover we demonstrated that mesenchyme secretes inducing substances onto culture gel made of agar. The gel on which the mesenchyme of the lung or proventriculus was precultured could evoke *ECPg* gene expression in the epithelium cultivated on it after the removal of the mesenchyme (Koike and Yasugi, 1999). From these results we can say that the mesenchymal effects can be separated into at least two factors: one is provided by appropriate extracellular substances and another by some soluble substances.

That organogenesis *in vitro* requires some extracellular substances and growth factors is demonstrated by studies of Nogawa and Takahashi (1991) and Taub *et al.* (1990).

In seeking a candidate of the soluble factors, we noticed that BMP (bone morphogenetic protein)-2 is expressed solely in the proventricular mesenchyme around the time of gland formation (E5 to E7). Other BMPs such as BMP-4 or -7 are expressed also in the gut mesenchyme but in rather

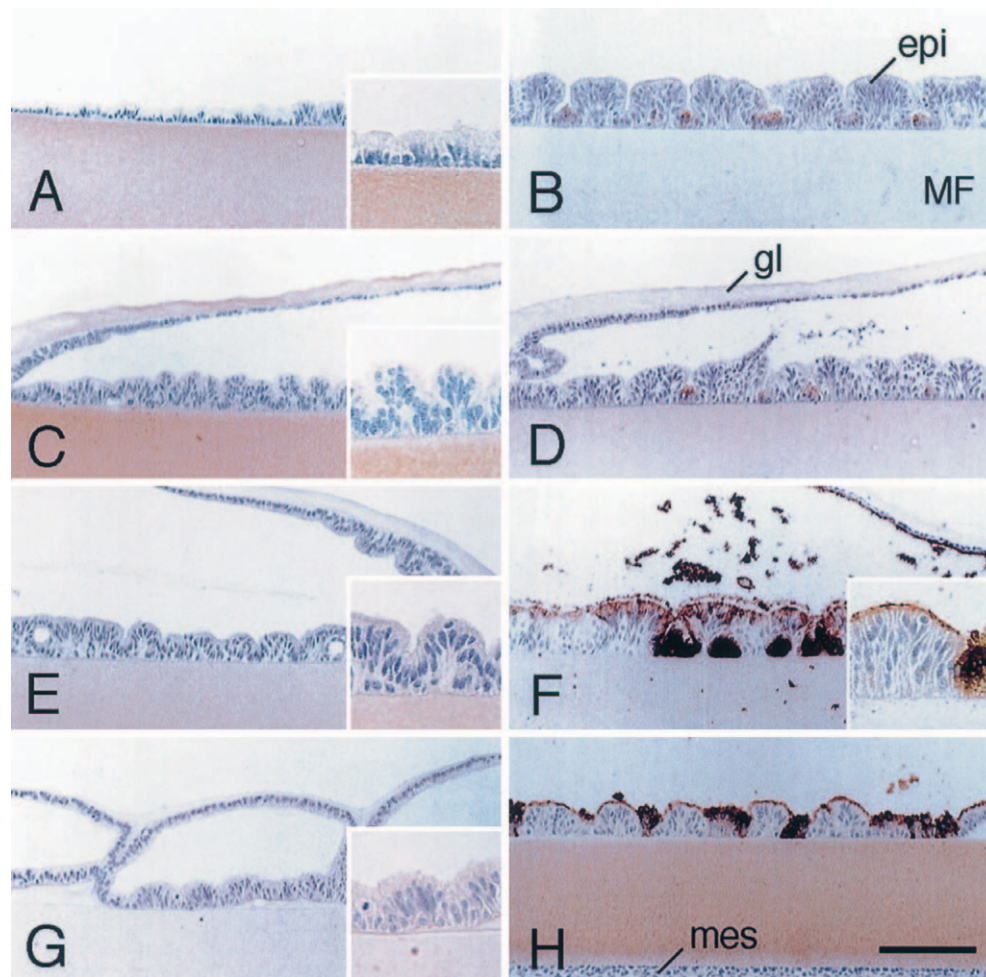


Fig. 4. ECPg expression in the explants of the proventricular epithelium (epi) cultured under various gel (gl) conditions. The epithelium was cultured on the Millipore filter (MF) without gel (A, B), or covered with collagen gel (C, D), collagen plus Matrigel (E, F) or Matrigel (G, H). (A, C, E, G) Cultivation without mesenchyme. (B, D, F, G) Cultivation with the proventricular mesenchyme (mes) on the opposite side of the filter. ECPg was detected with immunohistochemical staining and appears as brown. Insets in A, C, E, F and G are higher magnification views of each section. Note that active ECPg production is seen only when the epithelium was cultured clotted in the appropriate gel and with the mesenchyme. Bar: 100 μ m for A to H, and 50 μ m for insets. From Koike and Yasugi (1999).

ubiquitous manner. BMP-2 is expressed abundantly also in the lung mesenchyme. We therefore supposed that BMP-2 has gland-inducing ability in the proventriculus and tested the effect of overexpression of the gene in the proventricular or gizzard mesenchyme, using retroviral vectors. The gizzard epithelium cultivated combined with the proventricular mesenchyme with overexpressed BMP-2 made much more glands and almost all epithelial cells expressed ECPg, showing that BMP-2 has stimulatory effect on the differentiation of epithelium toward proventriculus. When we overexpressed the gene in the gizzard mesenchyme and cultivated it with proventricular epithelium, the latter never formed glands nor expressed ECPg, suggesting that the inhibitory effect of the gizzard mesenchyme cancels BMP-2 effect or that some cofactors necessary for BMP-2 action are lacking in the gizzard mesenchyme.

Noggin, a specific antagonist of the BMP signaling, showed a strong inhibitory effect on the gland formation and

ECPg expression. So we can say that BMPs are necessary for gland formation and, among BMPs, BMP-2 is by far the important factor for the proventricular epithelial differentiation (Narita *et al.*, 2000). The morphogenesis of mouse lung rudiment is also dependent on the expression of BMPs (Bellusci *et al.*, 1996).

Regulation of *ECPg* gene expression by the mesenchyme

Whatever the molecular nature of the mesenchymal signals that elicit proventricular glands, epithelial cells of glands soon begin to express *ECPg* gene from E8 or 9. We have been interested in the mechanisms of regulation of gene expression by the mesenchymal influence.

We cloned *ECPg* gene from the genomic library and found that it is composed of 9 exons as in other pepsinogen genes of the vertebrates (Hayashi *et al.*, 1988b). We then analyzed the regulatory segment of the 5' upstream of the

gene. Various segments of the promoter region were connected to a reporter luciferase gene and introduced into epithelial cells of the proventriculus and gizzard by lipofection. Epithelial cells were then mixed with mesenchymal cells and cultivated *in vitro*. Cells soon sorted themselves out and, in the combination of proventricular or gizzard epithelial cells and proventricular mesenchymal cells, epithelium formed glands and expressed *ECPg*. The expression of luciferase gene was confined to epithelial cells of the glands. Measurement of luciferase activity definitively showed that stretch of 1 kb just upstream to ORF of *ECPg* gene is necessary and enough for the right expression of luciferase in the epithelium combined with proventricular mesenchyme. So we concluded that mesenchymal signals act on the *ECPg* gene expression via the 1 kb stretch of 5' upstream of the gene (Fukuda *et al.*, 1994).

There are four binding sites of GATA transcription factor and one site of Sox factor in the 1 kb stretch (Sakamoto *et al.*, 1998). *cGATA5* is expressed in the proventricular epithelium when the *ECPg* gene expression begins and *cSox2* expression in the proventricular epithelium decreases soon after the onset of gland formation (Ishii *et al.*, 1998). These data suggest that *ECPg* expression in glandular epithelial cells of the proventriculus is regulated by the mesenchymal factors which affect the proportion of *cSox2* protein and *cGATA5* protein and these transcription factors in turn control *ECPg* expression via its promoter region.

Perspective

The elucidation of molecular mechanisms of epithelial-mesenchymal interactions in organogenesis is one of the most important and urgent problems in developmental biology both for the understanding of basic concepts of the developing systems and for the application of our knowledge to the construction of artificial organs by tissue engineering. The gut is one of the targets of the tissue engineering for therapeutic use (Choi *et al.*, 1999; Yasugi and Fukuda, 2000).

Our study has revealed the involvement of mesenchymal factors in the differentiation of epithelium and some candidates of factors have been mentioned. However, there is still a great gap to be filled up between the action of these factors and epithelial cell differentiation. We do not know whether BMPs act directly on epithelial cells, how BMPs interact with extracellular matrix, what intracellular signaling cascades of epithelial cells convey the information of mesenchymal factors to nucleus in which *ECPg* gene is ultimately transcribed. These problems will be solved by the use of dominant negative receptor molecules of BMPs and by the detection of some genes known to be regulated by BMP signaling, such as *Msx* gene (Takahashi *et al.*, 1996).

Another important question is to find out a master key gene necessary for the formation and differentiation of proventricular glands. As is mentioned above, gene expression pattern in gland epithelial cells changes drastically at about E6. Many genes coordinately cease to be expressed

or are downregulated. This means that expression of these genes is controlled by one or small number of genes of which expression is induced by the mesenchymal factors. Recently some key genes have been reported in the development of pancreas (Jonsson *et al.*, 1994), limb (Sekine *et al.*, 1999) and so on. The comparison of regulatory elements of genes downregulated in the proventricular gland cells to look for common sequences to which the same or similar transcription factors can bind will greatly advance the study of search for the master key gene in the development of the gut in chicken embryo.

Finally, the comparison of the developmental process and molecules involved between avian and mammalian digestive organs will be necessary to understand the common process of gut formation in vertebrates. It has been shown that several growth factors are expressed in the murine or rat gut (Matsubara *et al.*, 1996; Murphy, 1998) during development. Also *Cdx1* and *Cdx2*, homologues of *CdxA*, are expressed in the intestine and have been demonstrated to be important for the differentiation of intestine (Duprey *et al.*, 1988; Subramanian *et al.*, 1998; Beck *et al.*, 1999). Moreover, the importance of mesenchymal influence on the epithelial differentiation has been repeatedly stressed (Tsukada *et al.*, 1998). However, we do not know whether these molecules have the same functions in the development of avian and mammalian gut. These problems must be answered in future studies executed both on avian and mammalian guts.

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