Notch signaling functions as a binary switch for the determination of glandular and luminal fates of endodermal epithelium during chicken stomach development

Yoshimasa Matsuda[1], Yoshio Wakamatsu[2], Jun Kohyama[3], Hideyuki Okano[3], Kimiko Fukuda[1] and Sadao Yasugi[1,*]

[1]Department of Biological Sciences, Graduate School of Science, Tokyo Metropolitan University, 1-1 Minamiohsawa, Hachiohji, Tokyo 192-0397, Japan
[2]Department of Developmental Neurobiology, Graduate School of Medicine, Tohoku University, Sendai, Miyagi 980-8575, Japan
[3]Department of Physiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan
*Author for correspondence (e-mail: yasugi-sadao@c.metro-u.ac.jp)

Development

Summary

During development of the chicken proventriculus (glandular stomach), gut endoderm differentiates into glandular and luminal epithelium. We found that *Delta1*-expressing cells, undifferentiated cells and Notch-activated cells colocalize within the endodermal epithelium during early gland formation. Inhibition of Notch signaling using *Numb* or dominant-negative form of *Su(H)* resulted in a luminal differentiation, while forced activation of Notch signaling promoted the specification of immature glandular cells, but prevented the subsequent differentiation and the invagination of the glands. These results suggest that Delta1-mediated Notch signaling among endodermal cells functions as a binary switch for determination of glandular and luminal fates, and regulates patterned differentiation of glands in the chicken proventriculus.

Key words: Notch, Delta, Su(H), Proventriculus, Stomach, Gland, Chicken

Introduction

The vertebrate digestive tract is a simple tube in the early stages of development, which comprises the endodermal epithelium and surrounding mesenchyme. Distinct digestive organs are specified along the anteroposterior axis and subsequently undergo organ-specific development. In the chicken, the stomach is subdivided into the rostral proventriculus (PV, glandular stomach) and caudal gizzard (muscular stomach). The PV is characterized by the development of compound glands, which secrete digestive enzymes. From stage 29 of Hamburger and Hamilton (Hamburger and Hamilton, 1951) (about day 6 of incubation), the epithelium sequentially begins to invaginate into the surrounding mesenchyme to form simple glands. In this process, homogenous epithelial cells of the PV take either glandular or luminal fate, and luminal cells subsequently express chicken *SP* (chicken spasmolytic polypeptide) (Tabata and Yasugi, 1998) gene, while glandular cells express *Smad8* and later *ECPg* (embryonic chicken pepsinogen) (Hayashi et al., 1988) genes. Gland cells are also characterized by specific downregulation of several genes (Yasugi and Fukuda, 2000).

It is well established that the epithelial-mesenchymal interaction is important for the development of the PV (Yasugi, 1984; Yasugi and Fukuda, 2000). Recently, several secreted molecules have been identified as signaling factors regulating the epithelial development of the PV. Bone morphogenetic protein 2 (BMP2), which is secreted from the surrounding mesenchyme, is an important inducer of gland formation and *ECPg* expression (Narita et al., 2000). *Fgf10*, which is expressed in PV

mesenchyme (Shin et al., 2005), regulates epithelial cell proliferation and differentiation (M. Shin, S. Noji and S. Yasuji, unpublished). Fukuda et al. (Fukuda et al., 2003) have shown that a downregulation of *Sonic hedgehog* (*Shh*) expression in the epithelium is necessary for gland formation. Epidermal growth factor (EGF) signaling can also promote the luminal fate in dispense of gland cell population (Takeda et al., 2002). However, the molecular mechanism(s) that control the binary fate decision and the spatially patterned differentiation of epithelial cells remain to be elucidated.

Notch signaling controls cell fate decision and patterned differentiation in numerous developmental processes (reviewed by Campos-Ortega, 1993; Artavanis-Tsakonas et al., 1999; Kopan, 2002). The Notch transmembrane receptors are activated by cell-surface DSL (Delta, Serrate, Lag2) ligands and mediate direct cell-cell communication. Ligand binding results in a proteolytic cleavage to release the intracellular domain of the Notch proteins (NICD), which subsequently translocates into the nucleus and associates with the DNA-binding CSL (CBF1/Su(H)/Lag1, also known as RBPj) proteins to activate target genes such as the *Hairy/Enhancer of Split* (HES) family of bHLH transcriptional repressors (Beatus and Lendahl, 1998; Hu et al., 2003). Recently, the *Deltex* pathway has also been identified as mediating alternative Notch signaling (Yamamoto et al., 2001; Matsuno et al., 2002; Endo et al., 2003; Hu et al., 2003). Numb is an inhibitory molecule that binds to PEST domain of NICD and disturb nuclear translocation (Wakamatsu et al., 1999; Wakamatsu et al., 2000).

In this study, we have investigated the involvement of Notch signaling in the early gland cell differentiation of the chicken PV. We show that Delta1/Notch1 signaling in the PV epithelium is activated in a scattered fashion prior to epithelial invagination, as the earliest indication of fate segregation in the epithelium. We also demonstrate that an activation of Notch signaling promotes gland-specific gene expression, whereas persistence of Notch signaling prevents progress of epithelial cell differentiation, and that an inhibition of Notch signaling leads to luminal differentiation. Taken together, these results suggest that Notch signaling regulates spatiotemporally patterned differentiation of the glandular epithelium in the developing chicken PV.

Materials and methods

Experimental animals

Embryos of White Leghorn chicken (*Gallus gallus domesticus*) were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Plasmid constructs

FLAG epitope-tagged expression vectors of *CNIC* (constitutively active form of chicken *Notch1*), *CNIC$^{\Delta C89}$* (*CNIC* that lacks C-terminal end) and chicken *Numb* have been described previously (Wakamatsu et al., 1999; Wakamatsu et al., 2000). Throughout our study, *CNIC* and *CNIC$^{\Delta C89}$* were used to activate Notch1. *CNIC$^{\Delta C89}$* was more effective than *CNIC*, although both of them resulted in the same phenotype. *CNIC$^{\Delta C89}$* was used in 3-day cultivation experiment to obtain stable results, while *CNIC* was used in 36-hour culture experiments to compare with rescue experiments. pmiwSV-quail *Delta1* has also been described previously (Maynard et al., 2000; Endo et al., 2002). *Xenopus Su(H)^{DBM}* was kindly provided by Dr C. Kintner (Wettstein et al., 1997) and the insert was subcloned into pmiwSV (Endo et al., 2002). pTP-1*Venus* will be described elsewhere (J. Kohyama, A. Tokunaga, Y. Fujita, H. Miyoshi, T. Nagai, A. Miyawaki, K. Nakao, Y. Matsuzaki and H. Okano, unpublished).

Transfection of plasmids into PV epithelium by electroporation

The apparatus used for electroporation of plasmid DNAs into PV epithelium was set up as follows (Sakamoto et al., 2000). Platinum electrodes were fixed on a glass dish and integrated into a resin chamber (7 mm in height, 8 mm in width and 5 mm in length). A vessel made of 1.5% agarose/Tyrode's solution gel was put into an electrode chamber and filled with 14 µl plasmid DNAs in Tyrode's solution. The outside of the gel vessel was filled with Tyrode's solution. Isolated PV were cut open and placed in the vessel with their epithelial sides facing to the cathode to introduce DNAs into the epithelium. For the optimal transfection, 50 msecond pulses of 30 V and 75 msecond durations were generated 10 times for stage 28 PV and 15 times for stage 29 PV using CUY 21 (BEX). Tissues were immediately washed with Tyrode's solution and cultured for indicated hours. Appropriate concentration of each plasmid DNAs were examined and determined as follows: 200 nM of pEGFP-C1 for 36 hours cultivation and 300 nM for 72 hours culture; 200 nM *CNIC$^{\Delta C89}$* expression vector for 72 hours cultivation; 50 nM *CNIC* expression vector for 36 hours cultivation; 200 nM of pTP-1Venus for reporter assay or 100 nM for cotransfection with *CNIC*; 300 nM expression vector of chicken *Numb* or *Xenopus Su(H)^{DBM}* were used for inhibitory experiments and 400 nM for co-transfection with *CNIC*; and 200 nM pmiwSV-quail *Delta1* for reporter assay.

Organ culture

Explanted PV were laid on a Nuclepore filter (pore size 0.8 µm). This filter was placed on a stainless steel grid, placed into one well of a 24-well culture dish (Falcon, 3047) and cultured at the medium-gas interphase in 5% CO_2 and 95% air at 37°C. The culture medium was 199 with Earle's salt containing an equal volume of embryo extract prepared from stage 38 embryos (Takiguchi et al., 1988).

In situ hybridization

In situ hybridization with digoxigenin-labeled RNA probe was performed on 8-10 µm cryosections as previously described (Ishii et al., 1997). cRNA probes were generated by in vitro transcription from cDNA fragments of *ECPg* (Hayashi et al., 1988), chicken *SP* (Tabata and Yasugi, 1998), chicken *Notch1*, quail *Serrate1*, quail *Delta1*, chicken *Numb* (Wakamatsu et al., 1999; Wakamatsu et al., 2000), chicken *Notch2,* chicken *Serrate2* [gifts from Dr R. Goistuka (Morimura et al., 2001)], chicken *Hairy1*, chicken *Hairy2* [gifts from Dr O. Pourquié, (Palmeirim et al., 1997; Jouve et al., 2000)], chicken *Smad8*, *Shh* [gifts from Dr T. Nohno (Nohno et al., 1995; Ohuchi et al., 1997)]*,* chicken *Gata5* (Sakamoto et al., 2000)*,* chicken *Gata4*, chicken *Gata6* (Laverriere et al., 1994), chicken *Sox2* (Ishii et al., 1998), chicken *Sox21* (Uchikawa et al., 1999), chicken *GK19* (Sato and Yasugi, 1997) and chicken *Fra2* (Matsumoto et al., 1998).

Immunological staining

M2 anti-FLAG (mouse IgG1, Sigma) and goat anti-mouse IgG conjugated with Alexa 546 (Molecular Probes) were purchased from commercial suppliers. Immunological staining on sections was performed as described previously (Wakamatsu et al., 1993). Cryosections (8-12 µm) were prepared on VectaBond-coated slides (Vector). Sections treated with antibodies were also exposed to DAPI (Sigma) to visualize nuclei, and subsequently mounted with VectaShield mounting medium (Vector).

Results

Expression of genes involved in the Notch signaling pathway in developing chicken PV

To investigate whether Notch signaling plays a role in early chicken PV development, we first analyzed the spatiotemporal expression patterns of genes previously identified as Notch signaling components during the stages before secretion of zymogens. Expressions of chicken *SP* and *Smad8* were also investigated to monitor the epithelial cell differentiation. Serial sections of PV for each stage were obtained from the same samples. Sections for each probe were mounted on the same slide glass to compare intensity of gene expressions. In the stage 28 gut, the PV region is apparent with its bulgy shape but is still in the phase prior to PV-specific differentiation, and no obvious difference in morphology or gene expression profiles was detected among the epithelial cells. Expressions of chicken *SP,* marker molecule of luminal epithelial cells, and *Smad8* were not detected (Fig. 1A,D). *Notch1* and *Notch2*, and their potential target genes, *Hairy1* and *Hairy2*, were expressed in the entire epithelium (Fig. 1J,M,P,S). *Delta1*, which encodes a Notch ligand, was expressed in the mesenchyme underlying the epithelium, but not in the epithelium (Fig. 1G).

The PV epithelium begins to invaginate into the mesenchyme at stage 29. This stage is a crucial early phase of gland formation: epithelial invagination is still very small, but luminal surface indentation starts to be observed. Chicken *SP* and *Smad8* expressions were first detected at this stage (Fig. 1B,E). Expression of *Smad8* is restricted to cells in small

Fig. 1. Expression pattern of genes involved in Notch signaling in early gland formation of PV. Transverse serial sections of stage 28, 29 and 30 were analyzed by in situ hybridization. Throughout this paper, all sections that show normal expression patterns are serial and are obtained from the same sample which is staged exactly. Stage 29 PV show the small glands. For stage 30, boxed areas are shown enlarged in the column on the right. (A-C′) Expression of chicken *SP* and epithelial invagination begins at stage 29. At stage 30, chicken *SP*-negative cells are still observed among luminal cells (arrows). (D-F′) Expression of *Smad8* is restricted in invaginating epithelium and begins at stage 29. (G-I′) Expression of *Delta1* in the epithelium begins at stage 29 in a scattered fashion (arrowheads). (J-L′,P-R′) Expression of *Notch1* and *Hairy1* is restricted in uninvaginated epithelium. (M-O′,S-U′) Expression of *Notch2* and *Hairy2* is strong in invaginated epithelium. (V-X′) *Numb* is homogeneously expressed in the epithelium. Serial sections made from different stage PV and hybridized with the same probe were placed on the same slide glass to compare intensity of signals. Broken lines in K and Q underline borders between epithelium and mesenchyme. Scale bar: 100 μm.
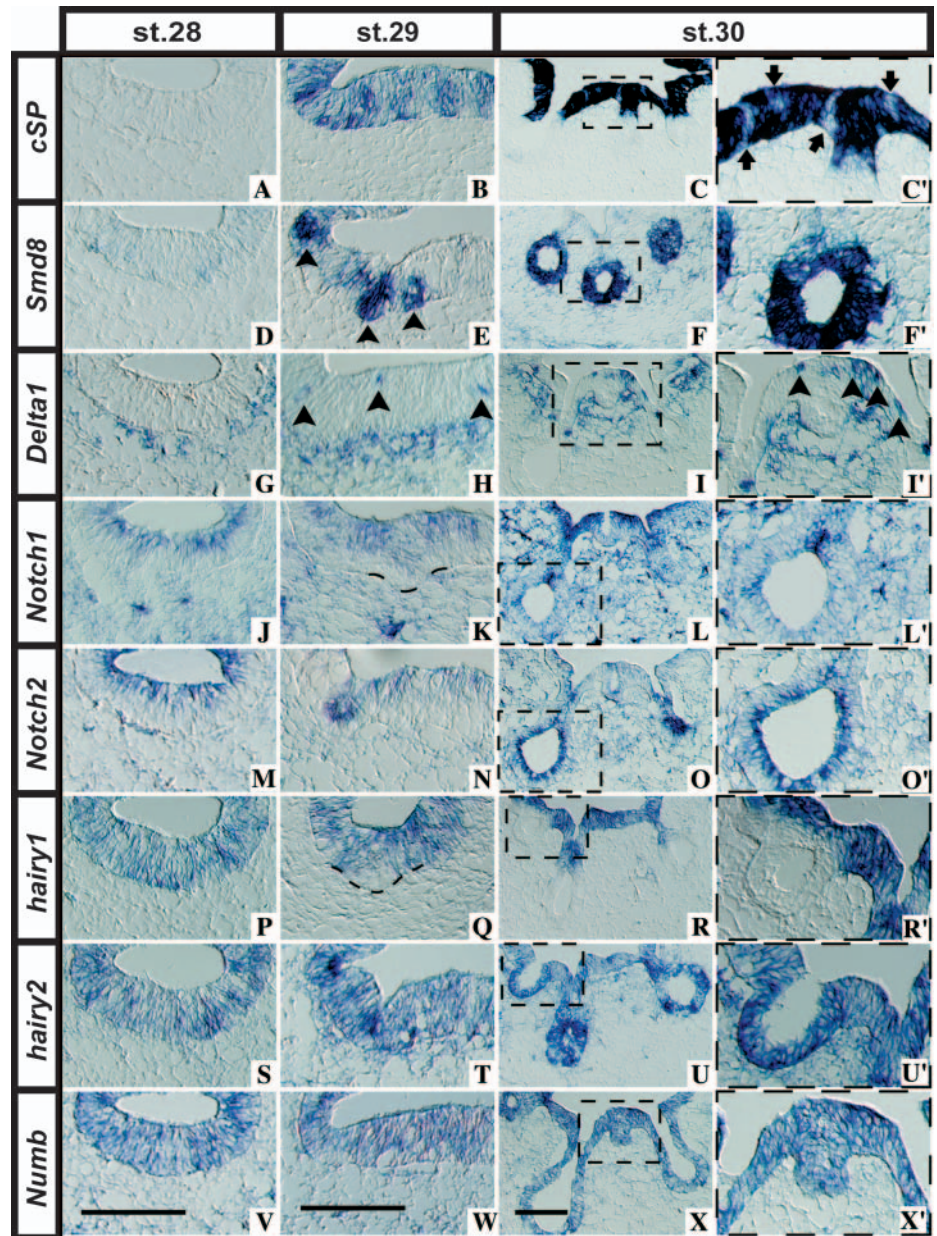
glands and cells just before invagination (arrowheads in Fig. 1). *Delta1* was also first expressed in a subpopulation of uninvaginated epithelial cells in a scattered fashion (Fig. 1H, arrowheads). Mesenchymal expression of *Delta1* was maintained at sites where the overlying epithelium did not invaginate. In the invaginated epithelial cells, the expression of *Notch1* decreased and that of *Hairy1* disappeared (Fig. 1K,Q). By contrast, expression of *Notch2* and *Hairy2* was upregulated in the invaginating epithelium (Fig. 1N,T).

At stage 30, obvious simple glands are formed, although newly formed small glands are also observed. Strong chicken *SP* expression was detected in the luminal epithelium, but chicken *SP*-negative cells were also found in the luminal epithelium (Fig. 1C,C′, arrows), suggesting that undifferentiated cells remained within the uninvaginated epithelium. Expression of *Smad8* was apparently restricted in the invaginated epithelium (Fig. 1F,F′). Expression of *ECPg* was not detected until a further 1.5 days later (data not shown); thus, these stages are prior to secretion-competent gland maturation. The number of *Delta1*-positive cells increased in the uninvaginated epithelium (Fig. 1I,I′). The region where *Hairy1* was expressed coincided with the *Notch1*-positive region and was restricted to the uninvaginated epithelium (Fig. 1L,L′,R,R′), whereas *Hairy2* was expressed in the same pattern as *Notch2* and was restricted to the invaginated glandular epithelium (Fig. 1O,O′,U,U′). *Numb* was expressed in the entire epithelium throughout these stages (Fig. 1V-X,X′). In chicken, Notch ligand genes, such as *Serrate1* and *Serrate2*, have also been identified, but their mRNAs were not detected in the epithelium during these stages (data not shown). Therefore, of all the possible ligand/receptor combinations only *Delta1* and *Notch1* expression overlapped from stage 29, when gland formation and luminal epithelial cell differentiation commence.

From the analysis of expression patterns, we concluded that *Smad8* is a specific marker gene of gland epithelial cells from the early stage of their development, as its expression begins with and persists during the gland formation and is specific to these cells.

Notch signaling is activated in scattered undifferentiated cells fated to the glandular epithelium

We next performed a reporter assay to confirm whether Notch signaling is indeed activated during the normal development of the PV. pTP-1*Venus* was used, a CSL-dependent *Venus* (a *GFP* variant) reporter that carries 12 repeats of CSL-binding sites and that efficiently reflects Notch signaling activity (J. Kohyama, A. Tokunaga, Y. Fujita, H. Miyoshi, T. Nagai, A. Miyawaki, K. Nakao, Y. Matsuzaki and H. Okano, unpublished). First, pTP-1*Venus* was transfected into stage 28 and 29 PV, and the reporter activity was observed after 3 hours of explant culture. As 3 hours cultivation is the shortest time in which to detect reporter-derived proteins, this condition enables us to observe almost 'real-time' activity of Notch signaling. Reporter activity was detected in scattered cells in uninvaginated epithelium of stage 29 PV, but was never detected in invaginated epithelium (Fig. 2A). Reporter activity was not detected in stage 28 PV (data not shown), in which no *Delta1* expression was detected in the epithelium, suggesting that epithelial, not mesenchymal, *Delta1* expression was responsible for the Notch signal activation in the epithelium. This result indicated that activation of Su(H)-mediated Notch signaling begins between stage 28 and 29 in cells scattered in the uninvaginated epithelium. When stage 28 and 29 PV were cultured for 24 or 48 hours after transfection, the reporter activity was detected not only in the uninvaginated epithelium in a scattered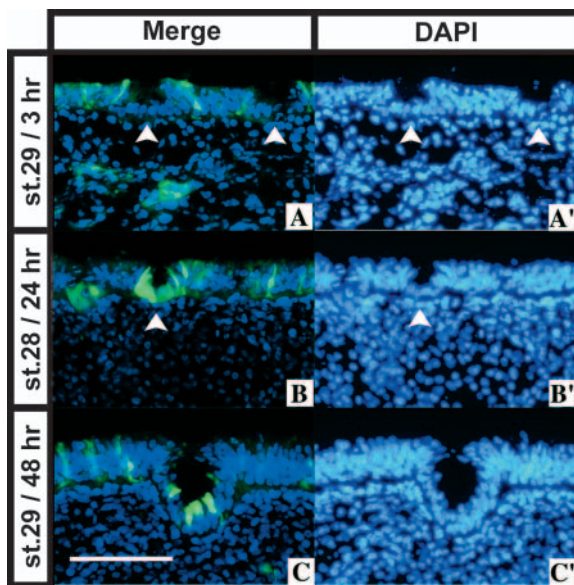 fashion but also strongly in invaginated gland cells (Fig. 2B,C). In this condition, cells in which Notch signaling had once been activated during cultivation could be *Venus* positive. Gland formation in stage 28 PV cultivated for 24 hours is comparable with that in transiently cultivated stage 29 PV (Fig. 2A,B). These results suggest the possibility that cells in which Notch signal was transiently activated took gland cell fate and subsequently invaginated. Consistently, *Venus*-positive cells did not express chicken *SP* mRNA when stage 29 PV was transfected with pTP-1*Venus* and cultured for 3 hours (Fig. 3).

Delta expressed in the epithelial cells activates Notch signaling

As the activation of Notch signaling pathway was observed in a scattered fashion, similar to the expression pattern of *Delta1*, and as only *Delta1* expression was detected in the epithelium among all DSL ligand genes examined, endogenous *Delta1* is probably the ligand responsible for the activation of Notch1. To test the ability of Delta1 as a ligand in the PV epithelium, stage 29 PV was transfected with pTP-1*Venus* and cultured for 3 hours, and the expression of endogenous *Delta1* was compared with the reporter activity of pTP-1*Venus* on the same section (Fig. 4A-C). The *Venus* reporter was activated in cells neighboring the *Delta1*-expressing cells (Fig. 4C′), suggesting that endogenous *Delta1* actually functions as a Notch ligand to activate Notch signaling. Furthermore, when *Delta1* expression vector was co-transfected with pTP-1*Venus* in stage 28 PV and cultured for 36 hours, the reporter activity was not detected (Fig. 4D,E), suggesting that Notch signaling was activated in the neighboring cells but not activated in cells expressing *Delta1*. Consistent with this, when the reporter construct was first electroporated into stage 28 PV, followed by an additional transfection of *Delta1* expression vector 10 hours later, activation of the *Venus* reporter was found around the *Delta1*-transfected cells after 14 hours of additional culture (Fig. 4F-H).
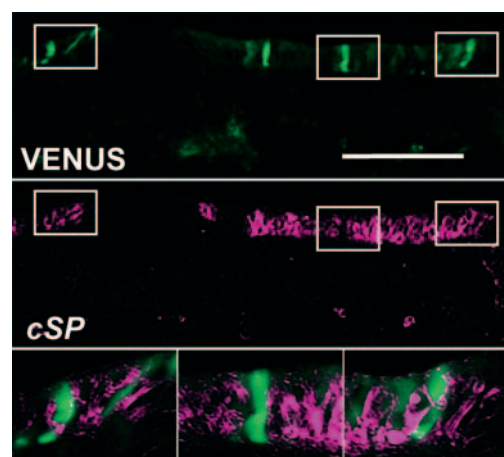

Fig. 2. Reporter activity in different stages and culture length. pTP-1*Venus* reporter construct was transfected in stage 28 or 29 PV and cultured for 3, 24 and 48 hours. (A) After 3 hours of culture, stage 29 PV shows the reporter activity in the uninvaginated epithelium in a scattered fashion. No signals were detected in invaginating epithelial cells (arrowheads). (B) Transfected stage 28 PV were cultured for 24 hours. Gland formation is comparable with that in stage 29 PV after 3 hours of culture (A). A significant number of Venus-positive cells (arrowhead) is observed in invaginating epithelial cells. (C) Stage 29 PV were cultured for 48 hours. Scattered Venus-positive cells are still observed, and obvious gland cells show reporter activity. Scale bar: 100 μm.

Fig. 3. Comparison of reporter activity with expression of chicken *SP*. Stage 29 PV were transfected with pTP-1*Venus* reporter construct and processed for fluorescent in situ hybridization of chicken *SP* after 3 hours of culture. The reporter activity is observed in a scattered fashion. *Venus* expression and chicken *SP* expression do not overlap. Lower three panels are high-magnification views of the boxed areas in the upper and middle panels. Scale bar: 100 μm.

Fig. 4. *Delta1* activates Notch signaling in neighboring epithelial cells. (A-C,C′) pTP-1*Venus* was transfected into stage 29 PV and cultured for 3 hours. *Venus* expression and expression of *Delta1* are adjacent. (C′) A high-magnification view of boxed area in C. Outline of cell shapes is shown on the right. (D,E) Stage 28 PV were co-transfected with pTP-1*Venus* and *Delta1* expression vector, and cultured for 12 hours after transfection. Reporter activity was not detected. (F-H) pTP-1*Venus* was first transfected into stage 28 PV, and *Delta1* expression vector was subsequently transfected 10 hours after the first transfection. After an additional 14 hours of culture, the reporter activity and *Delta1* expression were detected. Exogenous *Delta1* induced activation of the reporter in neighboring cells. Scale bar: 100 μm.
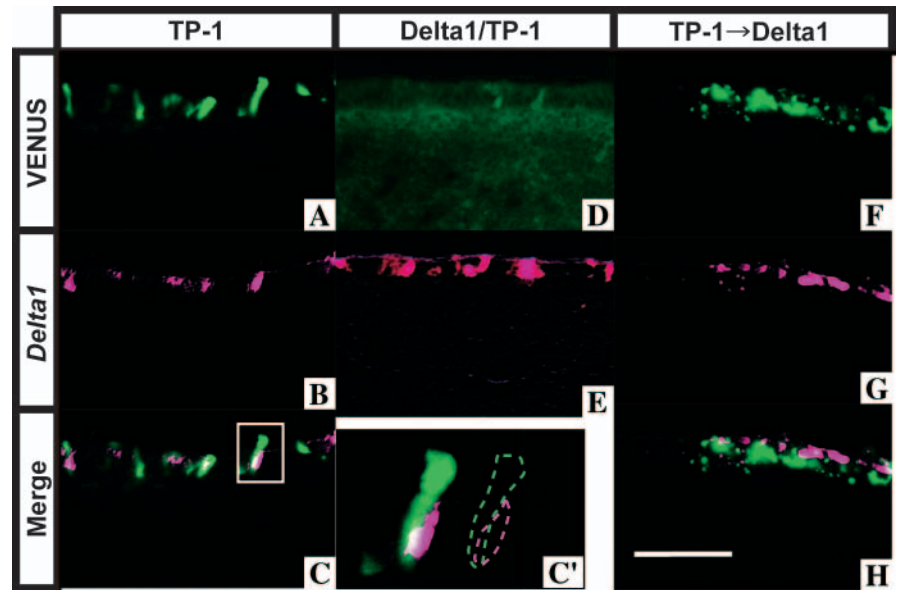
Forced activation of Notch signaling suppresses progress of epithelial development

The results of reporter assay suggested that Notch signaling is activated when the epithelium is specified as glandular and luminal epithelium. We thus examined the function of Notch signaling in the developing PV epithelium. As *Delta1*-overexpression does not activate Notch signaling in *Delta1*-expressing cells (see above), we could not observe any obvious effect on epithelial development (data not shown). Thus, we tried a receptor-mediated strategy to activate Notch pathway. $CNIC^{\Delta C89}$ encodes the intracellular fragment of chicken Notch1 with a C-terminal truncation, including the PEST domain (which is related to protein degeneration and Numb-binding site), and works as a constitutively active form (Wakamatsu et al., 1999; Wakamatsu et al., 2000; Maynard et al., 2000). $CNIC^{\Delta C89}$ was transfected into the epithelium of stage 28 PV, and the transfected explants were cultured for 3 days. In control explants, transfected with an *EGFP* expression vector alone, invagination of epithelium was observed, and differentiation marker genes (chicken *SP* for luminal epithelium and *ECPg* for late glandular epithelium) were expressed in a complementary pattern as in the normal development (Fig. 5B,C). By contrast, $CNIC^{\Delta C89}$ transfection caused severe defects in gland formation. Thus, virtually no epithelial invagination occurred, and the expression of *ECPg* in the glandular epithelium was not

observed (Fig. 5E). In these explants, chicken *SP* expression was also diminished (Fig. 5F), suggesting that the persistent activation of Notch signaling prevents the progress of differentiation.

Notch signaling induces the earliest phase of glandular cell differentiation

To further study the primary response of epithelial cells to Notch signaling, we examined the effect of *CNIC* in shorter time of culture. Thirty-six hours cultivation of stage 28 PV was chosen because epithelium of explanted PV does not express *ECPg* at this point but segregation of epithelial cell fates can be visualized by detecting chicken *SP* or *Smad8* mRNAs. To investigate the identity of Notch-activated cells, expression of various genes normally expressed in the PV epithelium, with different intensity in gland and luminal epithelium, were compared with that in normal PV before and after the beginning of gland formation. Throughout Figs 6 and 7, serial sections of explants were made from the same samples. These sections for each probe were mounted on the same slide glass together with serial sections of normal PV to compare expression intensity. We have always confirmed the efficient activation of Notch signaling by the expression of the co-transfected pTP-1*Venus* reporter in neighboring or next neighboring sections of each panel presented (Fig. 6B, Fig. 7B,K: small insets and not shown;
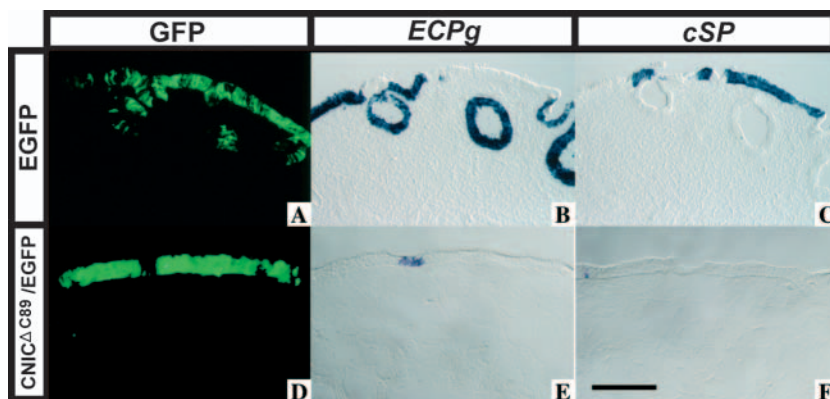

Fig. 5. Forced activation of Notch signaling by $CNIC^{\Delta C89}$ transfection. Stage 28 PV were transfected with indicated expression vectors and cultured for 72 hours. Control explants transfected with *EGFP* form simple glands and express differentiation markers (A-C). Transfection of $CNIC^{\Delta C89}$ inhibits gland formation and expression of differentiation-marker genes (D-F). More than 100 explants and more than 40 sections from each explant were investigated. Successfully transfected epithelial cells never expressed differentiation marker genes. Scale bar: 100 μm.
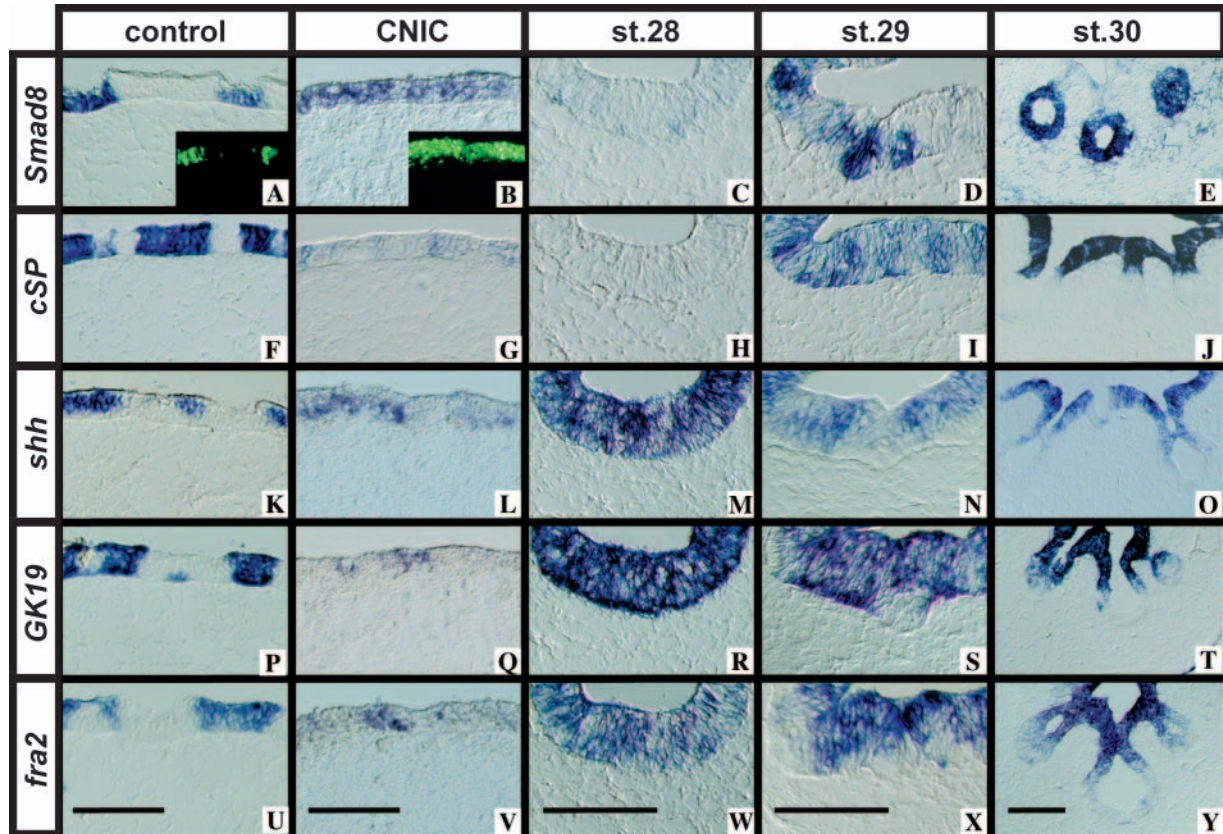
Fig. 6. Induction of glandular cell-specific gene expression by *CNIC*. Stage 28 PV were transfected with TP-1*Venus* reporter (control) or *CNIC* and cultured for 36 hours. Serial sections were made from each explant. Gene expressions were also compared with normally developing PV (right three columns; sections in each column are also serial with Figs 1 and 7). All sections hybridized with the same probe were placed on the same slide glass to compare intensity of signals. Wide and efficient activation of Notch signaling is monitored by co-transfection of *Venus* reporter construct in the neighboring or next neighboring sections (inset in B and not shown). Reporter activity in control explants is also shown (inset in A; neighbor section of A). (B) *CNIC* transfection induces *Smad8* expression. (G,L,Q,V) Expression of chicken *SP* and of genes that are specifically downregulated in gland cells is repressed. More than 12 explants were investigated in three experiments. More than 15 sections made from each explant were investigated for each probe. No obvious difference between explants was observed. Scale bar: 100 µm.

see Discussion). In Fig. 6, genes whose expression is similar to gland cell are listed. *CNIC* transfection inhibited expression of chicken *SP*, *Shh*, *Fra2* and *GK19* genes, all of which are specifically downregulated in the glandular epithelium of normally developing PV (Fig. 6F-Y). As *Shh*, *Fra2* and *GK19* were expressed in the undifferentiated epithelium prior to the gland invagination (Fig. 6M,R,W), and as *CNIC* transfection promoted the expression of *Smad8*, which is specific to the invaginated glandular epithelium in the normal PV (Fig. 6A-E), *CNIC*-transfected cells were similar in character to the early gland cells and were clearly distinct from the undetermined epithelial cells or the luminal epithelial cells. Expression of other genes examined (*Gata4*, *Gata5*, *Gata6*, *Sox2*, *Sox21*, *Hairy1* and *Hairy2*), however, was not obviously identical to gland cells, but it was found that this expression was comparable with those of undetermined epithelial progenitor cells (Fig. 7). Therefore, *CNIC*-transfected cells are probably intermediate between gland cells and undetermined cells. These results suggest that activation of Notch signaling promotes the initial phase of glandular specification but prevents progression of further differentiation and invagination. The distribution of reporter-positive cells,

Delta1-expressing cells and chicken *SP*-negative cells supports this idea.

Su(H)-mediated Notch pathway is necessary for differentiation of glandular epithelial cell

We next tried to rescue the phenotype obtained from the forced expression of *CNIC* by co-transfection of expression vectors such as *Numb* or dominant-negative form of *Suppressor of hairless*, $Su(H)^{DBM}$, into stage 28 PV, and the explants were cultured for 36 hours. Both constructs interfered with the activation of reporter activity of pTP-1*Venus* by *CNIC* (Fig. 8B,D; compare with 8F), and gland formation and the expression of chicken *SP* were nearly recovered (Fig. 8A,C; compare with 8E). These results confirmed that the phenotype obtained by overexpression of *CNIC* was dependent on $Su(H)$ activity. It is also indicated that overexpression of *Numb* or $Su(H)^{DBM}$ is useful to inhibit Notch signaling, while endogenous *Numb* allows endogenous Notch activation, as shown in Fig. 2.

Next, to investigate whether Notch signaling is necessary for glandular cell differentiation, *Numb* and $Su(H)^{DBM}$ were overexpressed in the stage 28 PV and the transfected explants

were cultured for 36 hours. Epithelial invagination was morphologically confirmed (Fig. 9, arrowheads), and luminal epithelium differentiation was monitored by detecting expression of chicken *SP* in neighboring sections (Fig. 9, rightmost column). Transfected cells were identified by immunological detection of FLAG-tag on the transgene-derived Numb protein (Fig. 9G′,I′), or by expression of co-transfected *EGFP* (Fig. 9C′,E′). In the explants with high efficiency of transfection, these transgenes inhibited gland formation and induced luminal differentiation (Fig. 9E-F,I-J). The majority of explants with lower efficiency of transfection, however, often formed some invaginated glands, while transfected cells contributed only to the luminal epithelium and did not participate in the invaginated glands (Fig. 9C-D,G-H). As initial transfection efficiency of these inhibitor constructs and *EGFP* expression vectors were almost comparable when explants were cultivated only for 12 hours (data not shown), cells that escaped from transfection seemed to differentiate only into gland cells. By contrast, overexpression of a dominant-negative form of *Dtx1* (Yamamoto et al., 2001) did not affect overall gland formation, and dominant-negative *Dtx1*-transfected cells could contribute to both glandular and luminal epithelium (data not shown). These results suggest that *Su(H)*-mediated Notch signaling is necessary for the glandular epithelium differentiation.

Discussion

Notch signaling as a binary switch for the fate determination of epithelial cells

In this study, we report the involvement of Notch signaling in the regulation of primary phase of binary fate decision of glandular and luminal epithelium, and subsequent differentiation of gland cells in the chicken PV. The first morphological sign of gland development is an invagination of the epithelium to form simple glands. Prior to such morphological changes, however, specification of luminal and glandular epithelial cells from homogeneous epithelial cells has to occur. In our study, *Delta1*-expressing cells and chicken *SP*-negative cells are scattered in uninvaginated epithelium, like the 'salt-and-pepper' pattern of *Delta*-expressing neuroblasts that later specify the fate of neighboring cells segregating from the neuroectoderm of the *Drosophila* embryo (Kunisch et al., 1994; Campos-Ortega, 1995; Artavanis-Tsakonas et al., 1999). Hence, Notch signal-mediated lateral specification may occur also during the fate determination in the epithelium of developing PV. Consistently, with this idea, cells in which *Su(H)*-mediated Notch signaling is activated are found adjacent to the *Delta1*-expressing cells. As Notch activation leads to the expression of *Smad8*, an early marker for the glandular epithelium, and *Numb* or dominant-negative *Su(H)* transfected cells differentiate only into luminal epithelium, it is conceivable that Notch signaling functions as a binary
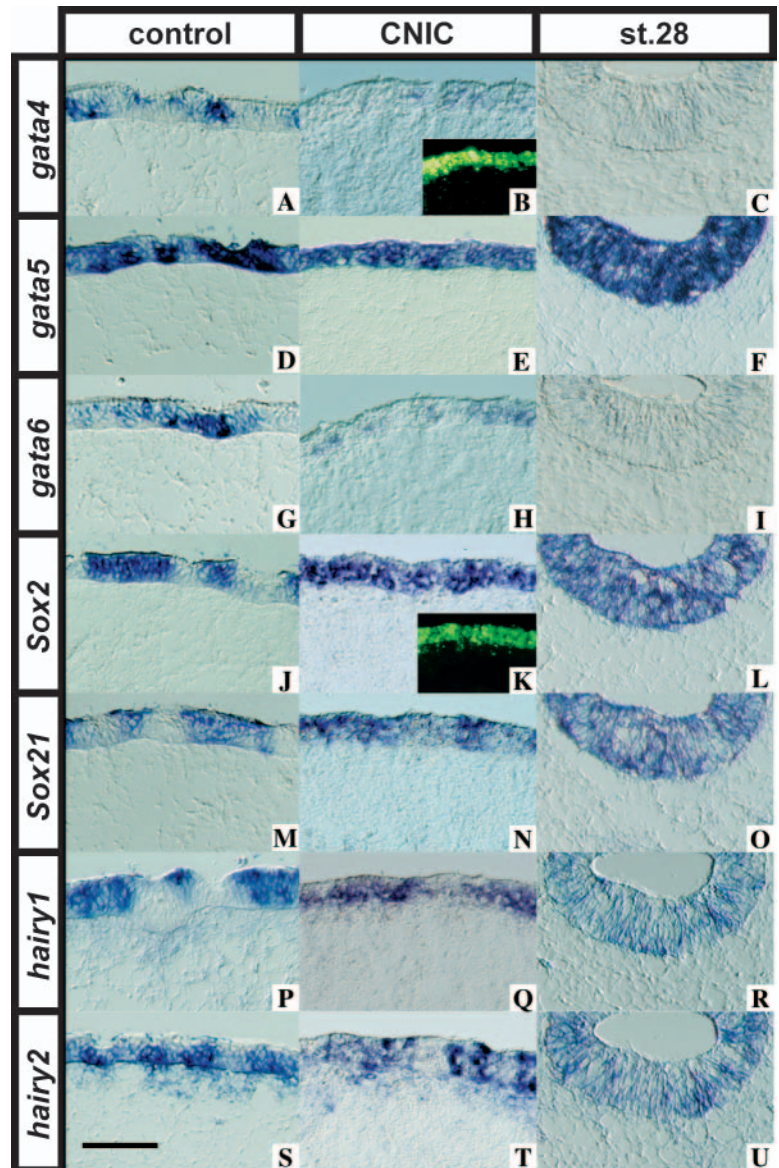

Fig. 7. Expression of genes similar to undetermined progenitor cells in *CNIC*-transfected epithelium. Stage 28 PV were transfected with TP-1*Venus* reporter (control) or *CNIC* and cultured for 36 hours. Serial sections were made from the same sample used in Fig. 6. Activation of Notch signaling is monitored by co-transfection of *Venus* reporter construct in neighboring or next neighboring sections of each panel (insets in B,K; not shown). Sections made from stage 28 of PV and explants, which are hybridized with the same probe, were placed on the same slide glass to compare intensity of signals. Sections of stage 28 PV are also serial with those in Figs 1 and 6. No apparent change was seen in the intensity of gene expression in comparison with stage 28 PV. More than 12 explants were investigated in three experiments. More than 15 sections made from each explant were investigated for each probe. No obvious difference between explants was observed. Scale bar: 100 μm.

switch for the glandular and luminal epithelial fate determination. Because the way in which Notch receptors mediate different signaling cascade has not been clarified, we cannot distinguish functions of Notch1 and Notch2 with only an inhibition experiment. However, *Notch2* is strongly expressed in the invaginated epithelium, whereas reporter-
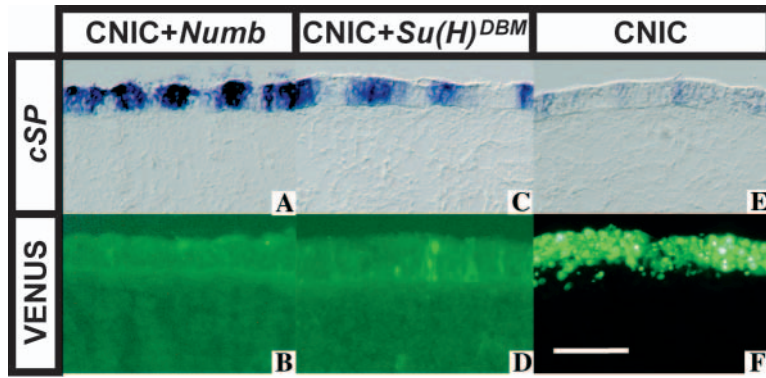
Fig. 8. *Numb* and *Su(H)^DBM* rescued the effect of *CNIC*. Stage 28 PV, co-transfected with pTP-1*Venus* reporter construct, *CNIC* expression vector, and Notch inhibitor construct, were cultured for 36 hours. Both *Numb* and *Su(H)^DBM* reduce excess activation of Notch signaling (compare B and D with F) and reduce well-spaced gland formation (lack of chicken *SP* expression in neighboring sections; compare A and C with E). More than 12 explants were investigated. More than 30 sections from each explant were investigated in three experiments. Scale bar: 100 μm.
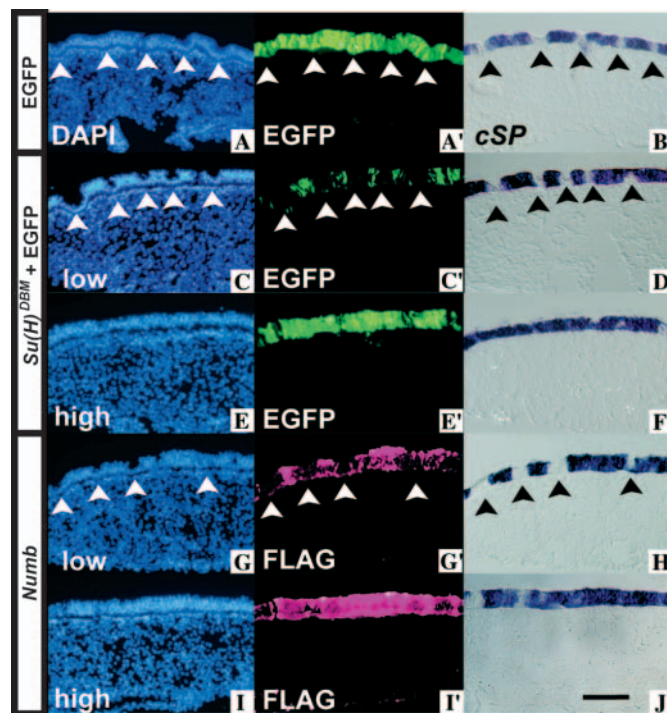

Fig. 9. *Numb* and *Su(H)^DBM* transfected cells preferentially contribute to the luminal epithelium. Efficiently transfected *Numb* (I′) and *Su(H)^DBM* (E′) suppressed gland formation. *Numb*-transfected cells (anti-FLAG immunostaining; G′,I′) and *Su(H)^DBM*-transfected cells (co-transfected EGFP; C′,E′) selectively localize in the luminal epithelium but not the small glands (C′,G′; arrowheads). Expression of *EGFP* in control explants was observed both in the luminal and glandular epithelium (A-B). Chicken *SP* expression in the neighboring sections (rightmost column) indicates the differentiation of luminal epithelium. Gland formation was morphologically confirmed (leftmost column). More than 100 explants for *Numb* and 12 explants for *Su(H)^DBM* were investigated. More than 30 sections from each explant were investigated in at least three experiments. Arrowheads indicate small glands. Scale bar: 100 μm.

positive cells are localized in the uninvaginated epithelium, suggesting a possibility that Notch2 might contribute later steps of gland development. We have also tried to investigate Notch2 function by overexpression of *NICD* derived from chicken *Notch2*, which resulted in severe apoptosis (data not shown). It seems that Notch1 mainly contributes to the initial phase of gland formation in PV.

Use of reporter construct and electroporation as a tool to analyze the function of Notch signaling

We used pTP-1Venus reporter construct to visualize Notch signaling that was introduced into epithelial cells together with CNIC by electroporation method. This made it possible to examine expression of marker genes in cells transfected with CNIC to activate Notch signaling. The reliability of the method depends on the efficiency of transfection. We detected (Fig. 6) expression of marker genes in sections next to ones in which reporter activity was visualized, and found that almost all epithelial cells showed reporter activity. It is therefore logical to conclude that expression patterns of marker genes faithfully reflect the effect of CNIC.

Inhibition of the gland maturation by Notch signaling

In our assay, transfection of *NICD* (*CNIC* and *CNIC^ΔC89*) inhibits the invagination of the glandular epithelium, as well as the expression of late markers of the gland epithelium such as *ECPg*, while the activation of Notch signaling allows the expression of *Smad8*. As BMP2 has previously been shown as an important inducer of gland and *ECPg* expression (Narita et al., 2000), and as Smad8 is a mediator directly regulated by BMP receptor, *Smad8* expression pattern and function closely reflects the early state of glandular epithelium before secretion of zymogen. Consistently, a transient reporter assay revealed that Notch-activated cells were interspersed only in uninvaginated epithelium, but that such cells later contribute to gland epithelium. We therefore conclude that Notch signaling prevents maturation of glandular epithelium, while it promotes initial glandular specification. A similar phenomenon is reported in pancreatic development. In the mouse pancreas, Notch activity is required for the commitment of precursor cells to the exocrine lineage (Apelqvist et al., 1999; Jensen et al., 2000), but also represses the maturation of these cells to express digestive enzymes by preventing the function of Ptf1-P48 complex (Esni et al., 2004). Similarly, in developing dorsal root ganglia of chicken embryo, continuous and strong Notch activation inhibits both neuronal and glial terminal differentiation to maintain progenitor population (Wakamatsu et al., 2000), while transient activation of the signaling promotes glial differentiation (Morrison et al., 2000). Many studies have reported a short time activation and downregulation of Notch signaling, especially in somitogenesis (reviewed by Pourquie, 2003; Aulehla and Herrmann, 2004). Thus, it is reasonable to speculate that Notch signaling initially functions as a fate determination switch, and subsequently acts to control the differentiation of immature gland cell precursors.
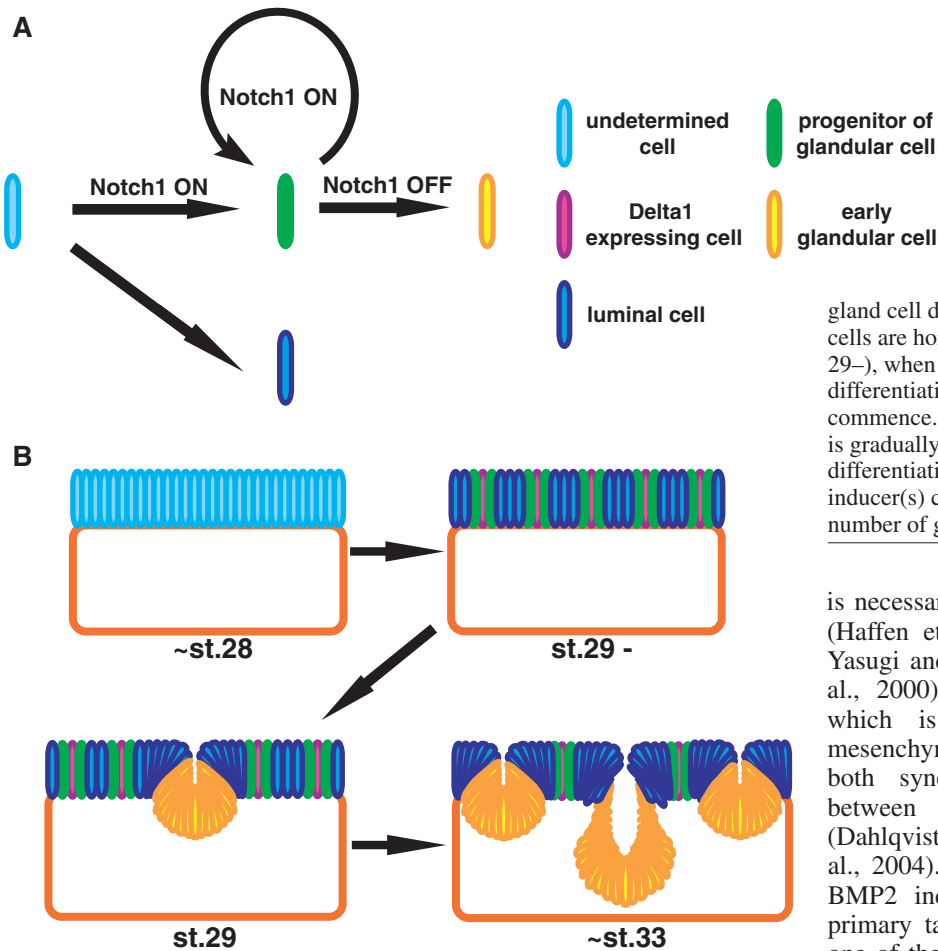
Fig. 10. Models of fate determination of the endodermal epithelium and invagination. (A) Notch1 activation instructs endodermal epithelial cells to immature glandular progenitor fate. Remaining epithelial cell takes luminal fate under the influence of luminal inducers. Until Notch1 signaling is inactivated, progenitor glandular cells remain immature. As Notch signaling stops, immature glandular cells undergo gland cell differentiation. (B) Until stage 28, epithelial cells are homogenous. Slightly before stage 29 (stage 29–), when *Delta1* expression starts, luminal differentiation and specification of glandular progenitors commence. From stage 29 onwards, as Notch1 signaling is gradually inactivated, glandular progenitors undergo differentiation and invaginate into mesenchyme. Luminal inducer(s) compete with Notch pathway and decrease number of gland progenitor.

is necessary for gland formation of PV epithelium (Haffen et al., 1987; Mizuno and Yasugi, 1990; Yasugi and Fukuda, 2000). Narita et al. (Narita et al., 2000) have previously reported that *Bmp2*, which is specifically expressed in the PV mesenchyme, can induce gland formation. Recently, both synergistic and antagonistic relationship between BMP and Notch has been reported (Dahlqvist et al., 2003; de Jong et al., 2004; Itoh et al., 2004). In mouse neuroepithelial development, BMP2 induces expression of *Hes5* and *Hesr1*, primary target genes of Notch signaling; Smad1, one of the mediator of BMP signaling, and NICD form a protein complex, which in turn regulates transcription of the target genes (Takizawa et al., 2003). In BMP signaling, ligand-induced heterotetrameric receptor complex directly phosphorylates Smad proteins such as Smad1, Smad5 and Smad8. Thus, Smad8 would mediate the inductive effect stimulated by BMP2 in gland formation of the PV. In our study, activation of Notch signal derived from CNIC induced expression of *Smad8*. Hence, induction of the downstream component might be one of the interfaces between the BMP and Notch signals that contribute to gland formation.

Shh was shown to induce luminal fate and its downregulation is necessary for glandular differentiation, probably through BMP7, which is expressed in the mesenchyme adjacent to the luminal epithelium (Fukuda et al., 2003). In our study, the activation of Notch signaling downregulates the epithelial expression of *Shh*. Therefore, it is possible that Notch signaling regulates the glandular differentiation partly through the repression of *Shh* signaling.

We also demonstrated that EGF signal induces the luminal fate (Takeda et al., 2002). Antagonistic interaction between Notch and EGF pathways have been described in other systems (de Celis et al., 1997; zur Lage and Jarman, 1999; Culi et al., 2001). For example, *Drosophila ebi*, a target gene of EGF receptor, and a mammalian ortholog of TBL1 associate with Su(H) and SMRTER/SMRT, a nuclear co-repressor protein, and regulate transcription of target genes (Culi et al., 2001). Thus, the recruitment of Su(H) to the repression complex may compete with the NICD/Su(H) complex, which activates target

Notch signaling in the glandular versus luminal fate decision is mediated by Su(H) protein

It has been known that CSL protein mediates Notch signaling by associating with NICD and activates target gene transcription (reviewed by Iso et al., 2003). Recently, however, Notch signaling has also been shown to use an alternative pathway mediated by Deltex/Dtx proteins (Yamamoto et al., 2001; Matsuno et al., 2002; Endo et al., 2003; Hu et al., 2003). In our study, the inhibition of Notch signaling by $Su(H)^{DBM}$ results in a luminal cell fate, while dominant-negative Dtx has no effect, suggesting the 'canonical' $Su(H)$-mediated pathway is predominantly used in this process. Consistently, *Numb* overexpression, which interferes with the nuclear translocation of the Notch intracellular domain (Wakamatsu et al., 1999), also inhibited the glandular differentiation cell-autonomously.

In our study, primary effector molecule(s), which function under Notch signaling, cannot be determined, as *Hairy1* and *Hairy2* were not significantly upregulated by Notch activation. Thus, there is a possibility that Notch1 targets other gene(s), such as unidentified *Hairy* genes and/or *Herp* family genes (reviewed by Iso et al., 2003).

Possible crosstalk of Notch signaling with other signaling in glandular differentiation

It has long been shown that an induction by the mesenchyme

genes. Such antagonistic relations may prevent excess gland formation.

In the normal development, gland formation is a sequential process and the number of glands increases from stage 29 until about stage 33, during the period before *ECPg* expression begins. In our model (Fig. 10A,B), the epithelial cells are homogenous and undifferentiated (Fig. 10, light blue) until stage 28. From stage 29–, slightly before stage 29, *Delta1/Notch1* signaling is activated and commits epithelial cells to keep gland progenitor cells (Fig. 10, green) in a scattered fashion, whereas other epithelial cells differentiate into luminal cells (Fig. 10, dark blue). This function of Notch1 might be completed by luminal effectors such as EGF and/or Shh, which prevent formation of excess glands and act in cooperation with inducers of gland cell differentiation such as BMP2. Notch1 activation is sequentially ceased, and progenitor gland cells begin to invaginate and differentiate into early gland cells (Fig. 10, yellow). Notch1 signaling also keeps progenitor gland cells in the undifferentiated state; these cells can then be released to differentiation as the PV becomes large enough to form additional glands. Thus, Delta1/Notch1 signaling definitively regulates early phase of gland formation before the secretion-competent gland maturation in normal development.

References

Apelqvist, A., Li, H., Sommer, L., Beatus, P., Anderson, D. J., Honjo, T., Hrabe de Angelis, M., Lendahl, U. and Edlund, H. (1999). Notch signalling controls pancreatic cell differentiation. *Nature* **400**, 877-881.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-776.

Aulehla, A. and Herrmann, B. G. (2004). Segmentation in vertebrates: clock and gradient finally joined. *Genes Dev.* **18**, 2060-2067.

Beatus, P. and Lendahl, U. (1998). Notch and neurogenesis. *J. Neurosci. Res.* **54**, 125-136.

Campos-Ortega, J. A. (1993). Mechanisms of early neurogenesis in *Drosophila melanogaster*. *J. Neurobiol.* **24**, 1305-1327.

Campos-Ortega, J. A. (1995). Genetic mechanisms of early neurogenesis in Drosophila melanogaster. *Mol. Neurobiol.* **10**, 75-89.

Culi, J., Martin-Blanco, E. and Modolell, J. (2001). The EGF receptor and N signalling pathways act antagonistically in *Drosophila* mesothorax bristle patterning. *Development* **128**, 299-308.

Dahlqvist, C., Blokzijl, A., Chapman, G., Falk, A., Dannaeus, K., Ibanez, C. F. and Lendahl, U. (2003). Functional Notch signaling is required for BMP4-induced inhibition of myogenic differentiation. *Development* **130**, 6089-6099.

de Celis, J. F., Bray, S. and Garcia-Bellido, A. (1997). Notch signalling regulates veinlet expression and establishes boundaries between veins and interveins in the *Drosophila* wing. *Development* **124**, 1919-1928.

de Jong, D. S., Steegenga, W. T., Hendriks, J. M., van Zoelen, E. J., Olijve, W. and Dechering, K. J. (2004). Regulation of Notch signaling genes during BMP2-induced differentiation of osteoblast precursor cells. *Biochem. Biophys. Res. Commun.* **320**, 100-107.

Endo, Y., Osumi, N. and Wakamatsu, Y. (2002). Bimodal functions of Notch-mediated signaling are involved in neural crest formation during avian ectoderm development. *Development* **129**, 863-873.

Endo, Y., Osumi, N. and Wakamatsu, Y. (2003). Deltex/Dtx mediates NOTCH signaling in regulation of Bmp4 expression in cranial neural crest formation during avian development. *Dev. Growth Differ.* **45**, 241-248.

Esni, F., Ghosh, B., Biankin, A. V., Lin, J. W., Albert, M. A., Yu, X., MacDonald, R. J., Civin, C. I., Real, F. X., Pack, M. A. et al. (2004). Notch inhibits Ptf1 function and acinar cell differentiation in developing mouse and zebrafish pancreas. *Development* **131**, 4213-4224.

Fukuda, K., Kameda, T., Saitoh, K., Iba, H. and Yasugi, S. (2003). Down-regulation of endodermal *Shh* is required for gland formation in chicken stomach. *Mech. Dev.* **120**, 801-809.

Haffen, K., Kedinger, M. and Simon-Assmann, P. (1987). Mesenchyme-dependent differentiation of epithelial progenitor cells in the gut. *J. Pediatr. Gastroenterol. Nutr.* **6**, 14-23.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

Hayashi, K., Yasugi, S. and Mizuno, T. (1988). Isolation and structural analysis of embryonic chicken pepsinogen gene: avian homologue of prochymosin gene. *Biochem. Biophys. Res. Commun.* **152**, 776-782.

Hu, Q. D., Ang, B. T., Karsak, M., Hu, W. P., Cui, X. Y., Duka, T., Takeda, Y., Chia, W., Sankar, N., Ng, Y. K. et al. (2003). F3/contactin acts as a functional ligand for Notch during oligodendrocyte maturation. *Cell* **115**, 163-175.

Ishii, Y., Fukuda, K., Saiga, H., Matsushita, S. and Yasugi, S. (1997). Early specification of intestinal epithelium in the chicken embryo: a study on the localization and regulation of *CdxA* expression. *Dev. Growth Differ.* **39**, 643-653.

Ishii, Y., Rex, M., Scotting, P. J. and Yasugi, S. (1998). Region-specific expression of chicken *Sox2* in the developing gut and lung epithelium: regulation by epithelial-mesenchymal interactions. *Dev. Dyn.* **213**, 464-475.

Iso, T., Kedes, L. and Hamamori, Y. (2003). HES and HERP families: multiple effectors of the Notch signaling pathway. *J. Cell Physiol.* **194**, 237-255.

Itoh, F., Itoh, S., Goumans, M. J., Valdimarsdottir, G., Iso, T., Dotto, G. P., Hamamori, Y., Kedes, L., Kato, M. and ten Dijke, P. (2004). Synergy and antagonism between Notch and BMP receptor signaling pathways in endothelial cells. *EMBO J.* **23**, 541-551.

Jensen, J., Heller, R. S., Funder-Nielsen, T., Pedersen, E. E., Lindsell, C., Weinmaster, G., Madsen, O. D. and Serup, P. (2000). Independent development of pancreatic alpha- and beta-cells from *neurogenin3*-expressing precursors: a role for the notch pathway in repression of premature differentiation. *Diabetes* **49**, 163-176.

Jouve, C., Palmeirim, I., Henrique, D., Beckers, J., Gossler, A., Ish-Horowicz, D. and Pourquié, O. (2000). Notch signalling is required for cyclic expression of the hairy-like gene HES1 in the presomitic mesoderm. *Development* **127**, 1421-1429.

Kopan, R. (2002). Notch: a membrane-bound transcription factor. *J. Cell Sci.* **115**, 1095-1097.

Kunisch, M., Haenlin, M. and Campos-Ortega, J. A. (1994). Lateral inhibition mediated by the Drosophila neurogenic gene delta is enhanced by proneural proteins. *Proc. Natl. Acad. Sci. USA* **91**, 10139-10143.

Laverriere, A. C., MacNeill, C., Mueller, C., Poelmann, R. E., Burch, J. B. and Evans, T. (1994). GATA-4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut. *J. Biol. Chem.* **269**, 23177-23184.

Matsumoto, K., Saitoh, K., Koike, C., Narita, T., Yasugi, S. and Iba, H. (1998). Differential expression of fos and jun family members in the developing chicken gastrointestinal tract. *Oncogene* **16**, 1611-1616.

Matsuno, K., Ito, M., Hori, K., Miyashita, F., Suzuki, S., Kishi, N., Artavanis-Tsakonas, S. and Okano, H. (2002). Involvement of a proline-rich motif and RING-H2 finger of Deltex in the regulation of Notch signaling. *Development* **129**, 1049-1059.

Maynard, T. M., Wakamatsu, Y. and Weston, J. A. (2000). Cell interactions within nascent neural crest cell populations transiently promote death of neurogenic precursors. *Development* **127**, 4561-4572.

Mizuno, T. and Yasugi, S. (1990). Susceptibility of epithelia to directive influences of mesenchymes during organogenesis: uncoupling of morphogenesis and cytodifferentiation. *Cell Differ. Dev.* **31**, 151-159.

Morimura, T., Miyatani, S., Kitamura, D. and Goitsuka, R. (2001). Notch signaling suppresses IgH gene expression in chicken B cells: implication in spatially restricted expression of *Serrate2/Notch1* in the bursa of Fabricius. *J. Immunol.* **166**, 3277-3283.

Morrison, S. J., Perez, S. E., Qiao, Z., Verdi, J. M., Hicks, C., Weinmaster, G. and Anderson, D. J. (2000). Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. *Cell* **101**, 499-510.

Development

Narita, T., Saitoh, K., Kameda, T., Kuroiwa, A., Mizutani, M., Koike, C., Iba, H. and Yasugi, S. (2000). BMPs are necessary for stomach gland formation in the chicken embryo: a study using virally induced BMP-2 and Noggin expression. *Development* **127**, 981-988.

Nohno, T., Kawakami, Y., Ohuchi, H., Fujiwara, A., Yoshioka, H. and Noji, S. (1995). Involvement of the *Sonic hedgehog* gene in chick feather formation. *Biochem. Biophys. Res. Commun.* **206**, 33-39.

Ohuchi, H., Nakagawa, T., Yamamoto, A., Araga, A., Ohata, T., Ishimaru, Y., Yoshioka, H., Kuwana, T., Nohno, T., Yamasaki, M. et al. (1997). The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. *Development* **124**, 2235-2244.

Palmeirim, I., Henrique, D., Ish-Horowicz, D. and Pourquié, O. (1997). Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis. *Cell* **91**, 639-648.

Pourquie, O. (2003). The segmentation clock: converting embryonic time into spatial pattern. *Science* **301**, 328-330.

Sakamoto, N., Fukuda, K., Watanuki, K., Sakai, D., Komano, T., Scotting, P. J. and Yasugi, S. (2000). Role for cGATA-5 in transcriptional regulation of the embryonic chicken pepsinogen gene by epithelial-mesenchymal interactions in the developing chicken stomach. *Dev. Biol.* **223**, 103-113.

Sato, K. and Yasugi, S. (1997). Chicken keratin-19: cloning of cDNA and analysis of expression in the chicken embryonic gut. *Dev. Growth Differ.* **39**, 751-761.

Shin, M., Watanuki, K. and Yasugi, S. (2005). Expression of *Fgf10* and *Fgf* receptors during development of the embryonic chicken stomach. *Gene Expr. Patterns* **5**, 511-516.

Tabata, H. and Yasugi, S. (1998). Tissue interaction regulates expression of a spasmolytic polypeptide gene in chicken stomach epithelium. *Dev. Growth Differ.* **40**, 519-526.

Takeda, J., Tabata, H., Fukuda, K. and Yasugi, S. (2002). Involvement of the signal transduction pathway mediated by epidermal growth factor receptor in the differentiation of chicken glandular stomach. *Dev. Growth Differ.* **44**, 501-508.

Takiguchi, K., Yasugi, S. and Mizuno, T. (1988). Pepsinogen induction in chick stomach epithelia by reaggregated proventricular mesenchymal cells in vitro. *Dev. Growth Differ.* **30**, 241-250.

Takizawa, T., Ochiai, W., Nakashima, K. and Taga, T. (2003). Enhanced gene activation by Notch and BMP signaling cross-talk. *Nucleic Acids Res.* **31**, 5723-5731.

Uchikawa, M., Kamachi, Y. and Kondoh, H. (1999). Two distinct subgroups of Group B Sox genes for transcriptional activators and repressors: their expression during embryonic organogenesis of the chicken. *Mech. Dev.* **84**, 103-120.

Wakamatsu, Y., Watanabe, Y., Shimono, A. and Kondoh, H. (1993). Transition of localization of the N-Myc protein from nucleus to cytoplasm in differentiating neurons. *Neuron* **10**, 1-9.

Wakamatsu, Y., Maynard, T. M., Jones, S. U. and Weston, J. A. (1999). NUMB localizes in the basal cortex of mitotic avian neuroepithelial cells and modulates neuronal differentiation by binding to NOTCH-1. *Neuron* **23**, 71-81.

Wakamatsu, Y., Maynard, T. M. and Weston, J. A. (2000). Fate determination of neural crest cells by NOTCH-mediated lateral inhibition and asymmetrical cell division during gangliogenesis. *Development* **127**, 2811-2821.

Wettstein, D. A., Turner, D. L. and Kintner, C. (1997). The *Xenopus* homolog of *Drosophila* Suppressor of Hairless mediates Notch signaling during primary neurogenesis. *Development* **124**, 693-702.

Yamamoto, N., Yamamoto, S., Inagaki, F., Kawaichi, M., Fukamizu, A., Kishi, N., Matsuno, K., Nakamura, K., Weinmaster, G., Okano, H. et al. (2001). Role of Deltex-1 as a transcriptional regulator downstream of the Notch receptor. *J. Biol. Chem.* **276**, 45031-45040.

Yasugi, S. (1984). Differentiation of allantoic endoderm implanted into the presumptive digestive area in avian embryos. A study with organ-specific antigens. *J. Embryol. Exp. Morphol.* **80**, 137-153.

Yasugi, S. and Fukuda, K. (2000). The mesenchymal factor regulating epithelial morphogenesis and differentiation of the chicken stomach. *Zool. Sci.* **17**, 1-9.

zur Lage, P. and Jarman, A. P. (1999). Antagonism of EGFR and notch signalling in the reiterative recruitment of *Drosophila* adult chordotonal sense organ precursors. *Development* **126**, 3149-3157.