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ORIGINAL ARTICLE

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Molecular and cellular analysis of embryonic avian tongue development

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Abstract Signalling cascades first described in *Drosophila* have been found to regulate patterning and outgrowth in a number of structures in higher vertebrates. We sought to determine whether the evolutionarily conserved genes were important during the development of the tongue. In situ hybridisation was used to determine the temporo-spatial expression of a panel of conserved genes. Histological examination and incorporation of BrdU were used to determine the mechanism by which the tongue develops. We show that evolutionarily conserved genes were expressed in distinct dynamic patterns during tongue development. *Sonic Hedgehog* (*Shh)* and *Patched* (*Ptc*) were found only in the dorsal tongue epithelium. *Shh* expression was only observed in the suprabasal layers, whereas *Ptc* was observed in both basal and suprabasal layers. Cell division in the epithelium was concentrated in regions devoid of *Shh*. Expression of *bone morphogenetic protein-7 (BMP)* was identical to that of *Shh*. *Shh* and *Ptc* expression were never detected in the mesenchyme. Ectopic expression of *Noggin* (a potent antagonist of the BMPs) caused severe abnormalities in tongue morphology, including swelling of the mesenchymal component and a thickening of the epithelial layer. Data from this study suggests that the epithelium and mesenchyme express quite different genes during development. However BMP activity acts to inhibit growth in both tissues.

Keywords Tongue · Chick · Embryonic · *Sonic hedgehog* · *Patched* · BCC

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Introduction

The transition from aquatic to terrestrial life required major adaptations to the feeding apparatus. Most fish rely on being able to create a vacuum in water by rapidly retracting the hyobranchial apparatus resulting in prey being sucked into the mouth. Their tongue consists of a simple fleshy fold called the fish tongue that is sufficient for guiding food into the gullet. Once animals emerged from water this method of feeding became obsolete and primitive terrestrial animals developed extendable muscular structures capable of capturing and retrieving prey.

The avian tongue forms in the floor of the mouth from swellings that are situated at the level of the first three visceral arches (Hamilton et al. 1965). At the mandibular arch level, three thickenings can be found: an unpaired median tongue bud (*tuberculum linguale impar*) and two paired distal lingual buds (*tubercula linguales laterales*, lateral lingual swellings), which appear on the inner aspect of the mandibular prominence. Another swelling (*pars copularis*, copula, hypobranchial eminence), is unpaired and located at the level of the second and third visceral arches including their lower ends. Between the *pars copularis* and the median tongue bud a small pit develops known as the *foramen caecum*. It is a vestige of the invagination from the floor of the pharynx which gives rise to the thyroid primordium. The *pars copularis* extends caudally to the primordium of the epiglottis. The main part of the tongue is formed by the lateral lingual swellings and is covered by ectoderm. They grow rapidly and fuse with the *tuberculum impar* forming the anterior part of the tongue that shows a dorsal and an inferior surface merging into each other at the apex.

The *tuberculum impar* itself is thought to form only the centre of the anterior part of the tongue. The early tongue consists of an epithelium (mainly of ectodermal origin) covering a mesenchyme of neural crest origin. The musculature of the tongue originates from the dermomyotomes of the occipital somites. Very little is

known about how the tongue develops in terms of the growth characteristics or the genes that regulate development. One of the most remarkable findings in development biology has been the discovery that vertebrates and invertebrates deploy almost identical signalling cascades for organogenesis (Relaix and Buckingham 1999). In this study we determined whether genes conserved during evolution participated during the early development of the tongue. Furthermore by performing histological and cytogenetic studies we sought to understand how the chick tongue grows during early embryogenesis. Our results show that a number of developmentally important genes are expressed during avian tongue development. Interestingly, the relationships between these genes differ to those found in other developmental systems such as the embryonic limb. This study provides evidence that development of the epithelium and mesenchyme are controlled by differing genes. These results are discussed in the context of gaining a better understanding of human diseases caused by deregulated epidermal development.

Materials and methods

Molecular analysis

Whole mount in-situ hybridisation. Embryos were dissected into PBS and the tongue region excised and placed immediately in 4% paraformaldehyde made in PBS with 0.1% Triton-X100. Samples were then processed for in-situ hybridisation according to Neito et al. (1996). Probes used in this study were: Shh (1 kb fragment provided by Dr J. Dodd, New York), Ptc (1 kb fragment provided by Professor C. Tabin, Harvard), BMP-2, -4 and –7 (0.8 kb, 0.95 kb and 1.1 kb respectively provided by Dr A. Graham, London), Follistatin (1.1 kb fragment cloned by KP), EphA4 (0.4 kb fragment cloned by KP), FGF-4, -8 and –10 (0.5 kb, 0.8 kb and 0.8 kb respectively provided by Professor G. Martin, San Francisco). A minimum of three samples were examined at each stage. Twenty-µm cryosections were performed on in-situ hybridised samples.

Histology

Examination of tongue epithelial morphology was performed by semi-thin histology. Embryos were fixed in 4% paraformaldehyde. After rinsing and dehydration, the embryos were embedded in Epon (Serva) and sectioned at 0.7 µm. Sections were stained in Methylene Blue (Serva) and Azur II (Fluka).

Proliferative activity was determined by the BrdU method. Chick embryos were incubated in ovo with 0.2 ml of a 40 mM 5-bromo-2'-deoxyuridine (BrdU) solution in PBS (Sigma) for 20–30 min. After rinsing, the embryos were fixed in 3% glacial acetic acid in 97% methanol and embedded in paraffin. The sections were stained with anti-BrdU antibody (Dako).

Retroviral expression of *noggin*

Noggin-expressing virus was prepared according to Capdevila and Johnson (1998). RCAS-Noggin was injected at differing depths into the tongue-forming primordia of Hamburger and Hamilton (1951; HH) stage 22–24 embryos (E4) using a micro-injector. Embryos were harvested at 7–8 days of development and the lower jaw and tongue were fixed in 4% PFA. Control embryos were injected with RCAS virus encoding alkaline phosphatase (AP). A minimum of three embryos injected at each stage were examined at E7–8 days for each procedure.

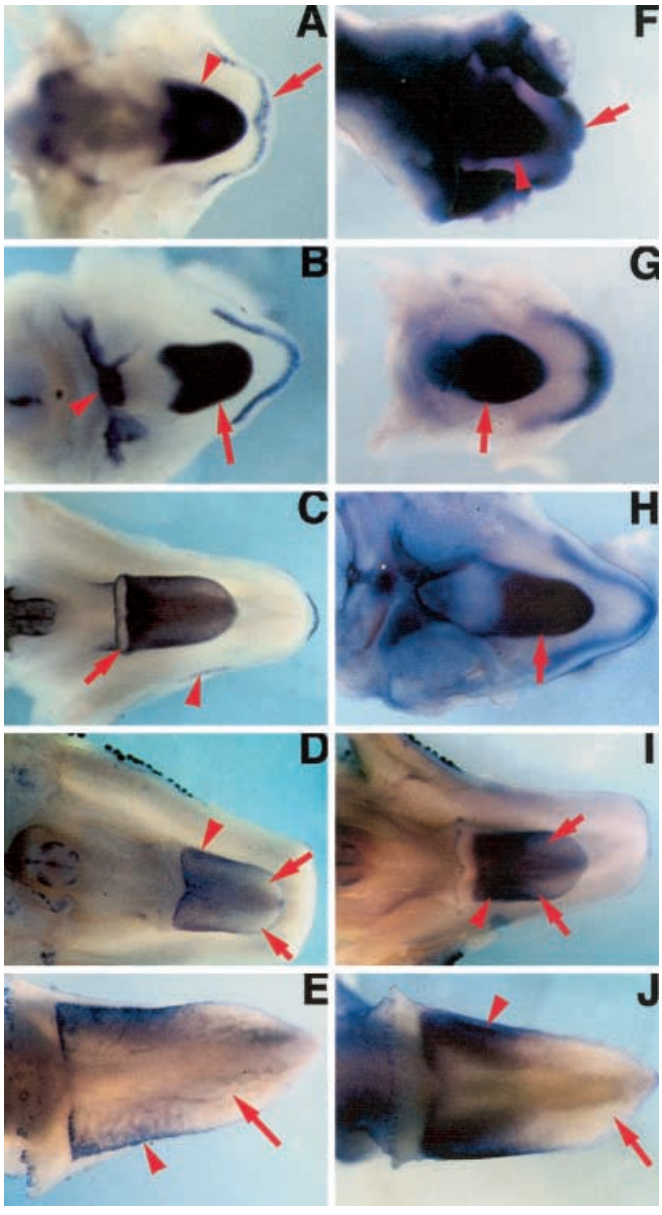

Fig. 1A–J Whole-mount expression of *Shh* (**A–E**) and *Ptc* (**F–J**) during avian tongue development. **A** At stage 26 (4.5–5 days), expression was uniform over the dorsal aspect of the tongue (*arrowhead*). A punctate pattern of expression of *Shh* was also detected at along the entire dorsal margin of the mandible (*arrow*). **B** At day 6, uniform expression was seen in the tongue (*arrow*). Expression also detected in the larynx (*arrowhead*). **C** At day 8, distal regions showed slightly weaker expression compared to proximal areas. Expression was downregulated in the papillae-forming region (*arrow*). Expression in the mandible was downregulated in the lateral aspects (*arrowhead*), but was still high in the distal-most regions. **D** At day 11, expression was maintained at the lateral margins of the tongue (*arrowhead*) and only at low levels in distal regions (*arrow*). Expression over the larynx was also downregulated and transcripts were no longer detectable in the mandible. **E** At day 13, distal regions no longer expressed *Shh* (*arrow*) and only at low levels in the lateral region (*arrowhead*). **F** At stage 26 (4.5–5 days), high levels of *Ptc* expression were detected in the elevated tongue primordium (*arrowhead*) and in the mandible (*arrow*). **G** At day 5.5, continuous *Ptc* expression extended from the distal tip of the tongue to the larynx (*arrow*). **H** At day 7.5, strong uniform expression persisted in the tongue (*arrow*). **I** At 11 days, expression was downregulated in the distal lateral area (*arrow*) compared to proximal regions (*arrowhead*). **J** At day 13, expression was still maintained at high levels in the proximal lateral (*arrowheads*) but not in distal regions (*arrow*)

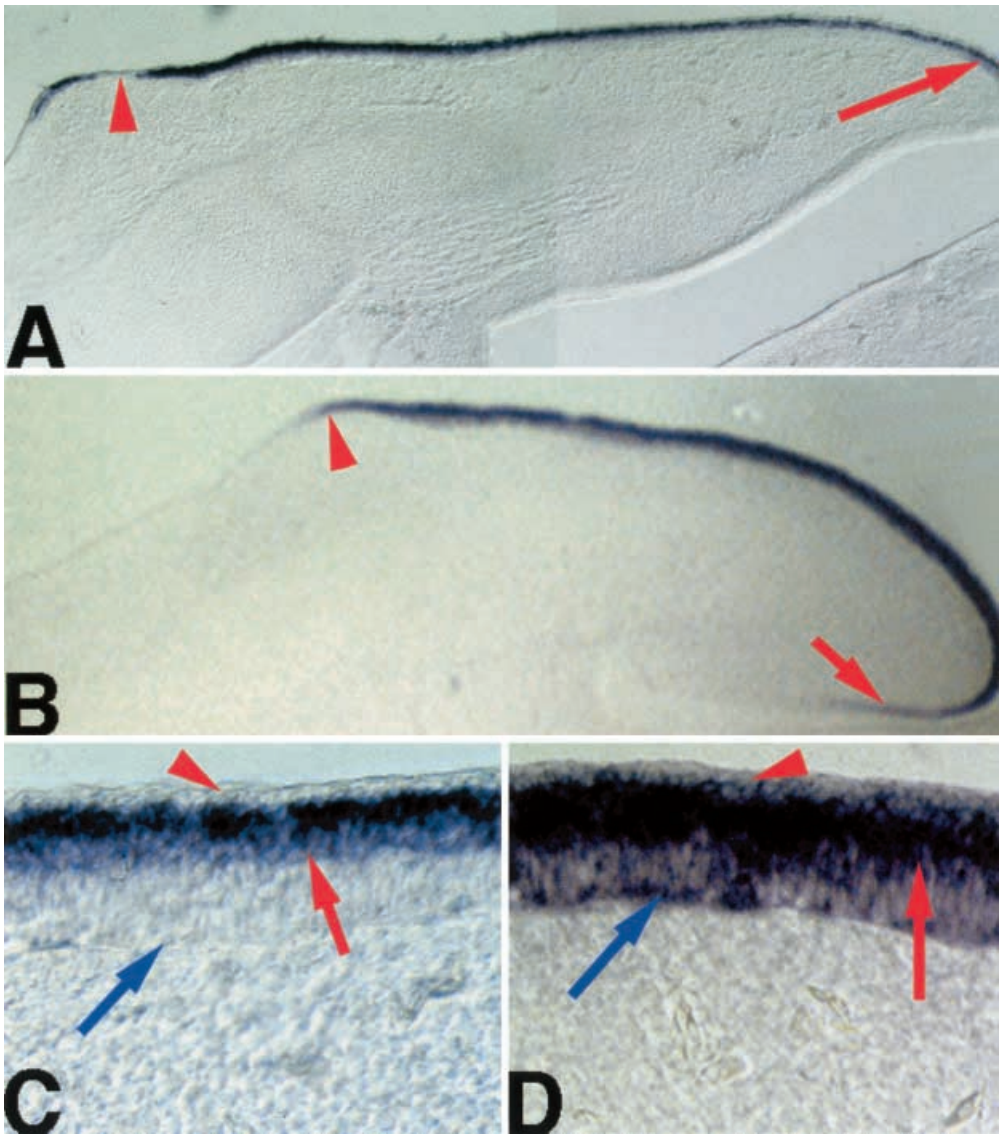

Fig. 2A–D Expression of *Shh* and *Ptc* in dorsal epithelium. **A** Sagittal sections of day-8 tongue showing *Shh* expression is confined to dorsal epithelial (*arrow*). Uniform expression was found throughout the dorsal layer except in the papillae-forming region (*arrowhead*). **B** Sagittal section of day-7.5 tongue also showing expression of *Ptc* in epithelial layer. Expression extended into the ventral aspect (*arrow*) with no downregulation in the papillae forming region (*arrowhead*). **C** Higher magnification of **A** showing *Shh* expression in dorsal suprabasal epidermis (*red arrow*) but not in the periderm (*arrowhead*) or basal layer (*blue arrow*). **D** Higher magnification of **B** showing *Ptc* expression in both basal (*blue arrow*) and suprabasal layers (*red arrow*). Periderm does not express *Ptc* (*arrowhead*). Note the sharp boundary of expression between the epithelium and underlying mesenchyme

Results

Expression of patterning genes during avian tongue development

Embryonic limb development has been used as a model to study early outgrowth and patterning. A number of key genes have been identified that regulate these processes and interestingly these same genes are employed during the development of other tissues. We determined the expression of these genes during chick tongue development.

Expression of *Sonic Hedgehog (Shh)*

Shh is one of the three vertebrate homologues of the *Drosophila* gene Hedgehog (Riddle et al. 1993). Shh has been shown to pattern a variety of tissues during vertebrate embryogenesis. In the limb it is expressed in the posterior distal mesenchyme in a domain known as the zone of polarising activity (ZPA) from which it not only patterns the digits but also indirectly participates in outgrowth. Recent studies suggest that cells can secrete Shh (McMahon 2000).

Strong expression of *Shh* was detected from the earliest development of the tongue primordium. A broad expression domain of *Shh* was detected as early as HH-stage 21 (3.5 days) when the tongue-forming primordium becomes elevated from the mandible (data not shown). This domain became more apparent by HH-stage 26 (4.5–5 days) when the tip of the tongue was detached from the floor of the mouth cavity. Very strong expression of *Shh* was detected in the dorsal surface of the tongue (Fig. 1A). At day 6, expression was also found uniformly on the dorsal surface of the tongue (Fig. 1B). Expression of *Shh* was upregulated at the dorsal margin of the mandible. *Shh* was also detected further back in the oral cavity close to the larynx. At day 8, expression of *Shh* in the tongue had become regionalised so that there was a weaker signal in the distal aspect of the tongue compared to more proximal medial regions

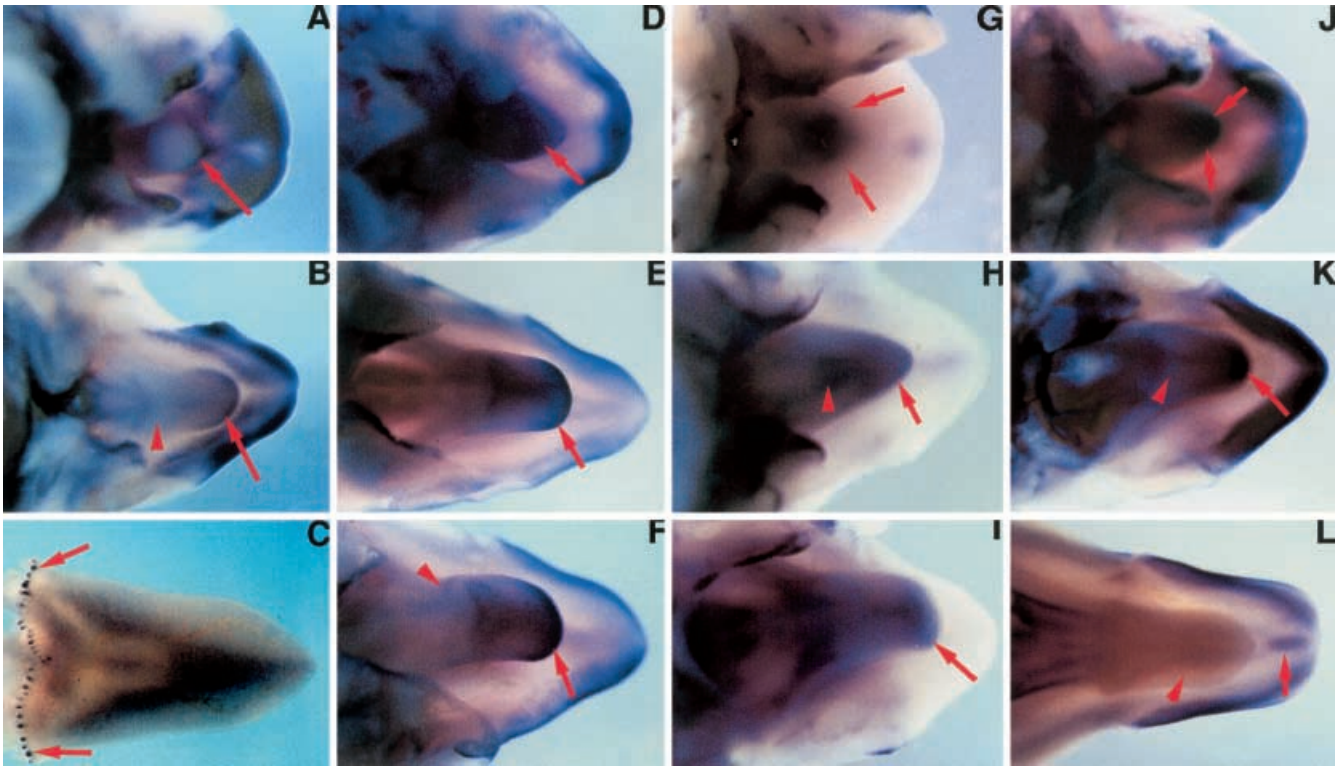

Fig. 3A–L Whole-mount expression of *BMP-4*, *BMP-7*, *Follistatin* and *EphA4* during chick tongue development. Expression of *Bmp-4* (**A–C**) was weak at day 6 in the tongue (**A**). **B** At day 7.5, expression was confined to the distal tip (*arrow*) with no expression in proximal regions (*arrowhead*). **C** At day 13, expression was found in the papillae (*arrows*). Expression of *BMP-7* (**D–F**) was found throughout the tongue at day 6.5 (**D**; *arrow*). **E** At day 8, strong expression was present especially at the distal tip (*arrows*). **F** At day 9, *BMP-7* was found in the distal portion (*arrow*) with a sharp boundary demarcating the proximal region (*arrowhead*) with significantly lower levels of transcription. Expression of *Follistatin* (**G–I**) was detected at day 6 (**G**) in the distal portion of the tongue but did not extend to the extreme tip (*arrows*). **H** At day 7.5, very low patchy expression was evident in the mandible. In the tongue, expression in the distal portion (*arrow*) was similar to the proximal region (*arrowhead*). **I** At day 9, expression still extended throughout the tongue. Expression of *EphA4* (**J–L**) was detected at high levels at day 6.5 (**J**). Note high levels of expression in the distal mandible. **K** At day 7.5, expression was highest in the distal portion of the tongue (*arrow*), although transcripts were detected in more proximal regions (*arrowhead*). **L** At day 11, transcripts could not be detected in the tongue (*arrowhead*) although expression was still present in the mandible

(Fig. 1C). Histological examination revealed that the gene was localised to the epithelium immediately under the periderm but was not expressed basally (Fig. 2A, C). Expression in the mouth was downregulated over the larynx. By day 11, expression of *Shh* was only detected in the proximal-lateral aspects of the tongue and to a lower level in the central groove (Fig. 1D). By day 13, transcripts were only detected at the lateral edges of the tongue (Fig. 1E). Expression also was detected in the distal tips of the papillae.

Expression of *Patched*

Ptc, a seven trans-membrane protein, is the receptor for Shh. On binding of Shh, the repressive action of Ptc on the signalling component Smoothened (Smo) is alleviated. Smo signalling leads to the expression of a multitude of genes including *Ptc*. Therefore the expression of *Ptc* has been used as an indicator of Shh activity (Goodrich et al. 1996).

Expression of *Ptc* was more widespread than that of *Shh* during the formation of the tongue. At HH-stage 26 (4.5–5 day), *Ptc* transcripts were detected throughout the oral cavity (Fig. 1F). At 5.5 days, expression of the gene was detected in a domain in the tongue extending proximally to the larynx (Fig. 1G). At 7.5 days of development, *Ptc* expression was found uniformly over the tongue (Fig. 1H). Sagittal sections revealed transcripts to be located predominantly in the suprabasal but also the basal layer (Fig. 2B, D). At 11 days, strong expression was detected laterally but less distally (Fig1I). Unlike *Shh* expression, *Ptc* transcripts were relatively abundant in the lateral regions of the tongue at day 13 (Fig. 1 J).

Expression of *BMP-4 and 7*

Members of the BMP gene family encode secreted proteins related to the *Drosophila* gene *Decapentaplegic* (Dpp; Kingsley 1994). During vertebrate limb development, *BMP* expression is induced by Shh (Riddle et al. 1993). However, the BMPs antagonise the action of Shh in a variety of tissues (Monsoro-Burq et al. 1996, Amthor et al. 1999). High concentrations of BMPs can

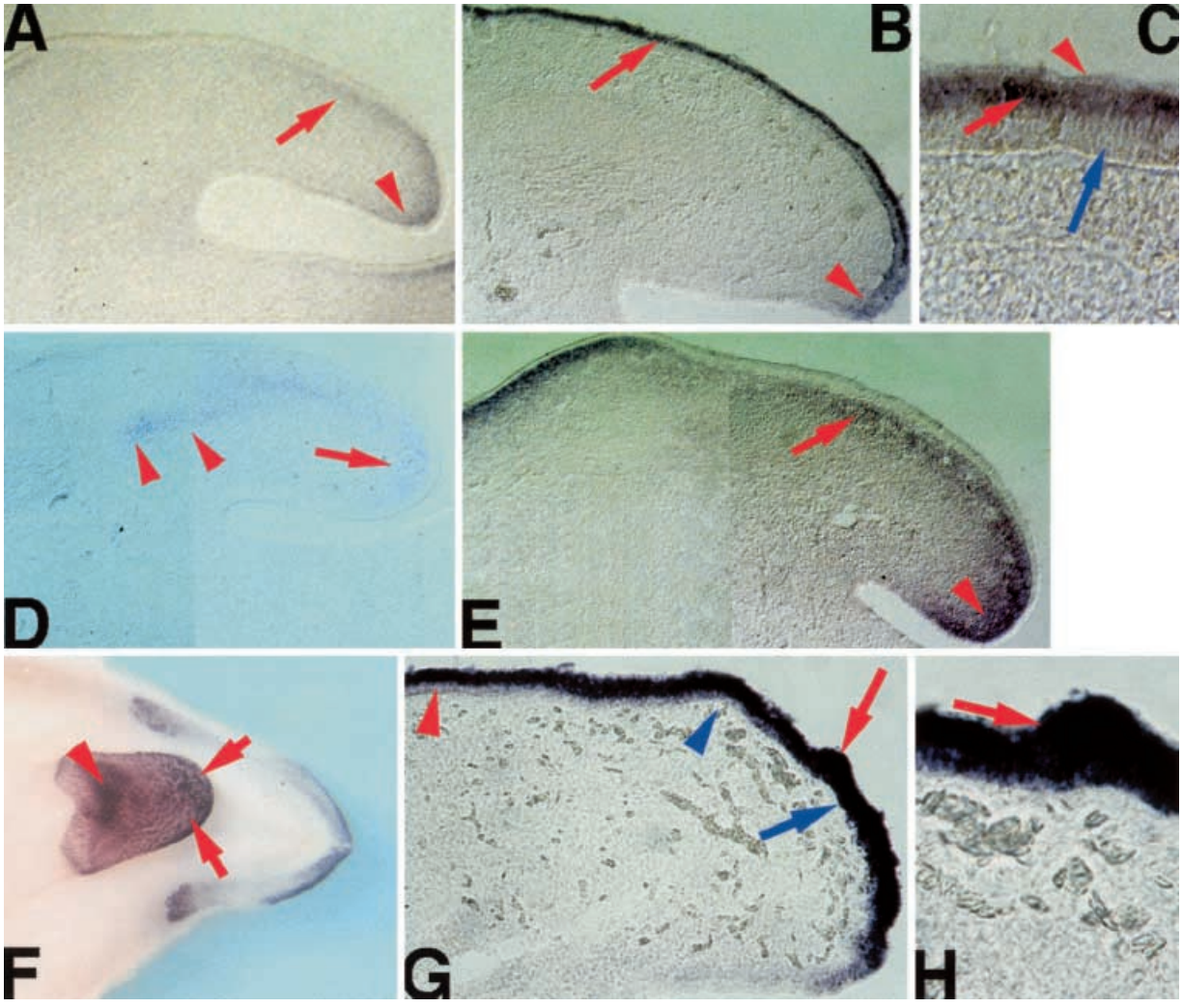

Fig. 4A–H Tissue localisation of *BMP-4*, *BMP-7*, *Follistatin* and *EphA4* during chick tongue development and the effect of the BMP antagonist Noggin on outgrowth. **A** Sagittal section of 7.75-day tongue showed *BMP-4* expression immediately under the length of the epithelium (*arrow*). Expression extended into ventral regions (*arrowhead*). **B** Sagittal section of day-8 tongue showed high levels of *BMP-7* expression in dorsal suprabasal epithelium (*arrow*). A sharp boundary of expression was present between dorsal and ventral regions (*arrowhead*). **C** High magnification of **B** shows expression of *BMP-7* in the suprabasal layer of the epithelium (*red arrow*) and not in the basal (*blue arrow*) or peridermal (*red arrowhead*) cells. **D** Sagittal section at day 7.5 showing *Follistatin* expression with elevated levels in the distal mesenchyme (*arrow*) and in deep proximal regions coinciding with muscle (*arrowheads*). **E** Sagittal section of day 6.5 tongue showing *EphA4* expression located in the sub-epithelial layer (*arrow*) with elevated levels in the distal tip (*arrowhead*). **F** *Shh* expression in a day-7 tongue after injection of Noggin-encoding virus. The tongue has bent towards the left. Uniform expression was detected in proximal regions (*arrowhead*) but in distal regions the expression was punctate (*arrows*). **G** Sagittal section of Noggin-injected tongue (from **F**) showing normal stratified layer expression of *Shh* in proximal regions (*red arrowhead*) compared to expression throughout the epithelial layer in distal regions (*blue arrow*). Local thickenings were detected in the epithelium (*red arrow*). Mesenchymal tissue was also thicker in the distal region (*blue arrowhead*; compare with Fig. 2B). **H** High magnification of **G** showing expression of *Shh* throughout the thickness of the epithelium (*arrow*, compare with Fig. 2C)

induce apoptosis (Yokouchi et al. 1996). At day 6, *BMP-4* was expressed at the distal tip of the tongue. Strong expression was detected in the surrounding mandibular tissue (Fig. 3A). At 7.5 days, *BMP-4* transcripts were localised to the distal tip of the tongue (Fig. 3B) and sagittal sections revealed expression confined to the mesenchyme immediately under the epithelium (Fig. 4A). Strong expression was also detected in the mandible. Expression of the gene was subsequently downregulated in the tongue and was no longer detectable by day 10 although it was expressed in the mandible (data not shown). Expression of *BMP-4* was re-initiated along the entire length of the papillae at day 13 (Fig. 3C).

At day 6.5, *BMP-7* was expressed in the mandible and the tongue (Fig. 3D). This expression was maintained at day 8.0 and sagittal sections revealed that the gene was expressed only in the suprabasal layer of the epithelium. (Fig. 4B, C). Therefore *BMP-7* expression in the epithelium resembled the *Shh* profile. Strong expression was maintained at day 9, being particularly strong at the distal tip. At the proximal end a sharp boundary of expression was detected (Fig. 3F) immediately distal to the region to the prospective papillae forming domain.

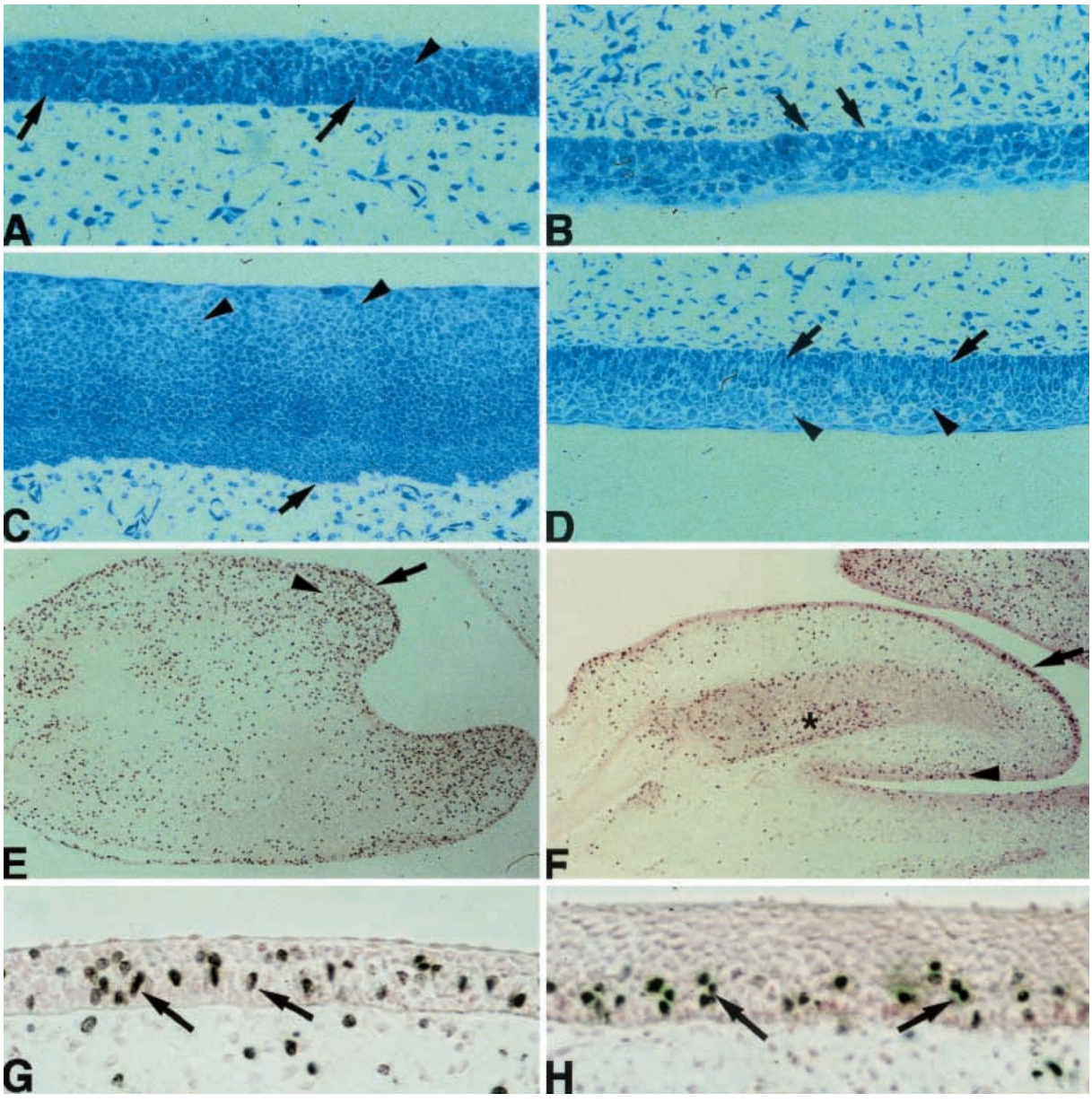

Fig. 5A–H Histological examination and localisation of cell proliferation during tongue development. **A** Dorsal aspect of day-8 tongue showing a columnar basal layer (*arrows*) and two to four layers of polygonal suprabasal cells (*arrowhead*). **B** Ventral aspect of day 8 tongue did not have a columnar basal layer (*arrows*) and the epithelium was 3–5 cells deep. Flattened peridermal cells are on the surface. **C** Dorsal aspect of a day-12 tongue showed a basal columnar layer (*arrow*) under a 25–30 cell suprabasal layer. Note that cells in the suprabasal layers enlarge as they move towards the dorsal surface (*arrowheads*). **D** Ventral aspect of a day-12 tongue showed a more prominent columnar basal layer (*arrows*). However, the suprabasal layer was still only a few cells thick. Note that cells in more suprabasal layers in the ventral epithelium were larger than similarly situated cells in the dorsal layer (*arrowheads*). **E** BrdU incorporation in day-6 tongue showed even distribution of labelled nuclei throughout the mesenchyme (*arrowhead*). Epidermal labelling is particularly high at distal tip (*arrow*). **F** At day 8, more BrdU incorporation was seen in dorsal epithelium (*arrow*) compared to ventral surface (*arrowhead*). BrdU-labelled nuclei in the mesenchyme were less abundant than before. Labelled nuclei were present in muscle (*asterisk*). **G** High power view of a day 8 dorsal epithelium showing BrdU in the basal layer (*arrows*). **H** Higher power view of a day-12 dorsal epithelium showing localisation of labelled nuclei in the basal layer (*arrows*)

Expression of *Follistatin*

Follistatin is a 36–40 kD-secreted glycoprotein and an inhibitor of BMP activity (Patel 1998). At day 6, *Follistatin* expression was found in the distal portion of the tongue (Fig. 3G). At 7.5 days, *Follistatin* was expressed throughout the tongue with slightly higher levels located to the distal portion (Fig. 3H). Sagittal sections revealed medial expression to be localised to tongue muscle whereas the distal domain was under the epithelium. The sub-epidermal expression was localised to the dorsal surface (Fig. 4D). By day 9, expres-

sion was detected in the developing tongue muscle and at a lower level at the extreme distal tip of the tongue (Fig. 3I).

Expression of *EphA4*

EphA4 is a receptor tyrosine kinase. During limb and branchial arch outgrowth its expression coincides with regions of high mesenchymal proliferation including the progress zone (Patel et al. 1996). *EphA4* was detected in the entire developing tongue at day 6.5, with highest levels of transcripts being located at the distal aspect. Strong expression of the gene was also detected in the mandible (Fig. 3 J). Sagittal sections revealed high mesenchymal expression immediately under the epithelium (Fig. 4E). At 7.5 days, high expression was found in the distal tongue and mandible (Fig. 3 K). At 11 days, expression of *EphA4* was no longer detectable in the tongue although strong expression was found in the lateral and medial aspects of the mandible (Fig. 3L).

We were unable to detect the expression of *FGF-4*, *–8* or *–10*, *Noggin* and *BMP-2* at any stages of tongue development (data not shown).

Cellular organisation and division in the embryonic avian tongue

The sharp boundary of *Shh* expression at day 8 on the dorsal epithelium (Fig. 2C) may reflect differing cellular characteristics within this tissue. We determined the cellular organisation using semi-thin histology and indeed the tongue epithelium displayed two major layers of equal thickness. At day 8, the epithelium consisted of a basal layer of columnar cells under a layer of polygonal cells; two to four cells in depth. These were covered by a very thin periderm. (Fig. 5A). The ventral surface, of approximately similar depth, lacked the tall columnar cells and consisted of more rounded cells (Fig. 5B). Six days later, the dorsal surface had a columnar layer at the basement membrane but was now overlain by over 25–30 suprabasal polygonal cells (Fig. 5C). However, in the same time period the ventral surface had expanded only by one or two cell diameters (Fig. 5D).

We determined whether the growth of the tongue through cell division was regionalised, using the incorporation of BrdU as an indicator of proliferative activity. There were high levels of BrdU incorporation in both the mesenchyme and epithelium at day 6. BrdU incorporation in the tongue mesenchyme was uniform, whereas in the mandible there was a concentration of labelled nuclei in the distal tip (Fig. 5E). At day 8, BrdU incorporation in tongue mesenchyme was very low, with most labelling coinciding with the position of muscle. Dorsal epithelium displayed more labelled nuclei than ventral, while the distal dorsal tip epithelium showed high levels of proliferative activity (Fig. 5F, G). At day 12, the basal localisation of BrdU could be seen (Fig. 5H) BrdU incorporation was less frequent suprabasally. The suprabasal layers of the epithelium are condensed due to fixing and processing artefacts.

Determining the role of the BMPs during tongue development

Results from the expression studies and the location of cell proliferation especially in the epithelium, revealed an intriguing molecular and cellular organisation. We found that cell division took place predominantly in the dorsal epithelium, where it was localised to the basal layer. *Shh* expression was excluded from the basal layer but was nevertheless active in these cells as shown by the expression of *Ptc*. *BMP-7* co-localised with *Shh* in the suprabasal layer. *BMP-4* was localised to the mesenchyme. Previous studies have shown that BMPs can antagonise the action of Shh during vertebrate embryogenesis (Monsoro-Burq et al. 1996, Amthor et al. 1999). We investigated the function of the BMP during tongue development by ectopically expressing Noggin, a potent antagonist of the BMPs (Zimmerman et al. 1996). Introduction of Noggin at E4 had a dramatic effect on tongue morphology at E7–8 causing it to swell and grow at an angle in over 60% of samples (n=11; Fig. 4F). The dorsal surface was uneven and sections revealed that both the mesenchymal and epithelial components were affected by ectopic Noggin (Fig. 4G). The proximal region appeared quite normal whereas the mesenchyme was thicker distally. In the epidermal layer, a single columnar basal layer was present proximally, but more distally the thickness of the epithelium was greatly increased. *Shh* expression, which is normally confined to the suprabasal layer, could be detected throughout the epithelium. *Shh* expression in the basal layer was confined to regions of thickened epithelium (Fig. 4G, H). Injection of control virus encoding alkaline phosphatase resulted in normal tongue development judged both morphologically and after in situ hybridisation with *Shh* in all samples examined (n=10; data not shown).

Discussion

The avian tongue is made up of numerous tissues including muscle, bone, mesenchyme and a cornified epithelium. The differentiation of these tissues must be co-ordinated to ensure correct development of the tongue. Unlike the mammalian tongue the dorsal surface of chicken tongue does not contain taste buds, which are found in the roof and the floor of the mouth (Ganchrow and Ganchrow 1987, Ganchrow et al. 1991). A few taste buds have also been found on the ventral surface of the tongue of chickens. In this study we have determined the expression of several genes that regulate outgrowth and patterning. We have used these markers to determine how the early chick tongue develops.

Our results suggest that growth of the tongue occurred throughout the structure and so argue against the

presence of a distally situated growth zone as seen in limbs. The role of the progress zone as proposed by Summerbell et al. (1973) was to impart positional value related to the duration a cell spends in this region. Cells leaving the progress zone early have positional information that allow them to develop into more proximal structures than cells leaving later. This model is extremely attractive when an organism has to generate heterogeneous elements from a single cell type along an axis. Our results suggest that the tongue does not develop in a proximal to distal direction using the genes employed during limb development. BrdU incorporation showed that cell proliferation was prevalent fairly uniformly in the mesenchyme of the tongue. BrdU incorporation was predominantly in the basal epithelium, but rarely extended suprabasally. Interestingly, we noted that the dorsal tongue epithelium had higher labelling compared to the ventral epithelium.

We did not find mesenchymal expression of *Shh, Ptc,* and *Bmp-7* in the tongue. Furthermore expression of *Bmp-4,* and *Follistatin* was found in a sub-epithelial layer along almost the entire length of the tongue. The only gene we found concentrated in the mesenchyme at the distal tip was *EphA4*. We were not able to detect the expression of *FGF4, FGF8, FGF10* or *Bmp-2* during any stage of tongue development. Members of the FGF family are key components to the progress zone during chick limb development as they directly maintain mesenchymal cell proliferation from the most distal structure in the limb, the apical ectodermal ridge (AER), which is absent in tongue (Niswander et al. 1993). This again emphasises that tongue and limb develop using differing genes.

The most striking features of the development of the tongue were the expressions of *Shh* and *Ptc*. Both these genes were only localised to the dorsal epidermis. The expression of both genes was highest up to day 8, after which expression decreased, which coincides with the onset of cornification. During normal development *Shh* was found only in the dorsal suprabasal layers of the tongue epithelium, whereas the expression of *Ptc* extended to the basal layer. Although we were unable to detect the expression of *Shh* in both the distal ventral regions of the tongue and the papillae primordia, *Ptc* expression in these regions suggests that there was Shh activity in these regions at some stage of development. The sharp boundary of *Ptc* expression in the epidermis suggests that the influence of Shh during tongue development is confined to the epithelium. This could be established by either the basement membrane acting as barrier preventing the diffusion of Shh into the mesenchyme, or that the mesenchyme is not competent to respond to Shh signalling.

We showed that ectopic Noggin expression, an antagonist of BMPs, caused tongue enlargement especially in the distal mesenchyme (in a region expressing *BMP-4*) and resulted in thickening of the epithelium. The simplest explanation is that BMP signalling inhibits growth of both the mesenchyme and epithelium. However with regard to the epithelial thickening following injection of Noggin, we should consider an alternative mechanism based on the ability of BMPs to antagonise Shh activity. The epithelial thickening on the dorsal surface following Noggin injections shows similarities in gene expression to those seen in human tongue basal cell carcinomas (BCC). Shh is overexpressed in many BCCs and results in suprabasal cell division in stratifying epithelia (Fan and Khavari 1999). The level of *Shh* expression itself may be the driving force, since no Shh mutations in BCC have been seen (Reifenberger et al. 1998). Over-expression or mutation of downstream proteins in the Shh pathway can also lead to BCC (Oro et al. 1997; Oro and Scott 1998). Therefore the suprabasal layer is capable of undergoing cell division and Shh has the ability to drive this process. We suggest that during normal development the ability of Shh to mediate cell division in the suprabasal layer could be inhibited by the action of BMPs. Results from this study suggest that BMP-mediated signalling could play a role in the development of BCC. Future work will test this model in the human situation.

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