Specification of pharyngeal endoderm is dependent on early signals from axial mesoderm

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SUMMARY

The development of taste buds is an autonomous property of the pharyngeal endoderm, and this inherent capacity is acquired by the time gastrulation is complete. These results are surprising, given the general view that taste bud development is nerve dependent, and occurs at the end of embryogenesis. The pharyngeal endoderm sits at the dorsal lip of the blastopore at the onset of gastrulation, and because this taste bud-bearing endoderm is specified to make taste buds by the end of gastrulation, signals that this tissue encounters during gastrulation might be responsible for its specification. To test this idea, tissue contacts during gastrulation were manipulated systematically in axolotl embryos, and the subsequent ability of the pharyngeal endoderm to generate taste buds was assessed. Disruption of both putative planar and vertical signals from neurectoderm failed to prevent the differentiation of taste buds in endoderm. However, manipulations of contact between presumptive pharyngeal endoderm and axial mesoderm during gastrulation indicate that signals from axial mesoderm (the notochord and prechordal mesoderm) specify the pharyngeal endoderm, conferring upon the endoderm the ability to autonomously differentiate taste buds. These findings further emphasize that despite the late differentiation of taste buds, the tissue-intrinsic mechanisms that generate these chemoreceptive organs are set in motion very early in embryonic development.

Key words: Anterior endoderm, Gastrulation, Specification, Notochord, Prechordal mesoderm, Axolotl

INTRODUCTION

For more than a century, vertebrate taste buds have been singled out as the prime example of neural induction of sensory organs during development. This view has held that late in embryogenesis, sensory nerve fibers contacted the oral and pharyngeal epithelia, and induced a subset of epithelial cells to give rise to multicellular taste buds (Guth, 1957; Hosley et al., 1987; Torrey, 1940). The implications of this idea are that innervation dictates the timing of taste bud development and the position of these receptor organs. Recently, however, this scenario has been called into question. In axolotls, a species of aquatic salamander, morphological differentiation of taste buds is completely independent of developing nerves (Barlow et al., 1996), and in mouse embryos, early patterning of the tongue epithelium also occurs independently of nerve contact (Farbman and Mbiene, 1991; Hall et al., 1999; Nosrat et al., 2001). In addition, taste bud development in amphibians is independent of mesenchymal cell contact; isolated pharyngeal endoderm, destined to give rise to taste buds, will do so, even when removed from embryos shortly after gastrulation (Barlow and Northcutt, 1997). These findings imply that the ability to develop taste receptor organs is an inherent characteristic of the pharyngeal epithelium, which is acquired by the end of gastrulation, long before the receptor organs differentiate.

In this new context, we proposed a series of hypothetical embryonic events that would give rise first to a specified pharyngeal endoderm field and subsequently to a distributed array of taste buds within that tissue (Barlow and Northcutt, 1998; Northcutt and Barlow, 1998). Because pharyngeal endoderm was specified by the end of gastrulation, we reasoned that signals during gastrulation might be responsible. Unlike the rest of the endodermal axis, the cells destined to give rise to the pharyngeal endoderm and taste buds are suprablastoporal and sit at the dorsal lip of the blastopore at the onset of gastrulation (Barlow and Northcutt, 1995; Pasteels, 1942; Vogt, 1929). During gastrulation, these cells involute first, shear past the inner surface of the presumptive neurectoderm and reach the anterior end of the embryo. In part, signals from the involuting endoderm and mesoderm induce the overlying ectoderm to become neural (Harland, 2000). Additionally, planar signals from the organizer are transmitted within the epithelium, which also neuralize the ectoderm (Doniach, 1992; Nieuwkoop, 1997; Ruiz i Altaba, 1992). Interestingly, a smattering of published reports has suggested that signaling during gastrulation is reciprocal (Nieuwkoop, 1997). For example, in *Xenopus*, gene expression is disrupted

in axial mesoderm that had gastrulated in the absence of overlying ectoderm (Poznanski and Keller, 1997), suggesting that normal expression patterns require reciprocal, vertical signals. Given that the pharyngeal endoderm involutes as the anterior companion of axial mesoderm, reciprocal signals might also be important for the acquisition by the endoderm of the ability to generate taste buds autonomously.

Alternatively, signals from the axial mesoderm to presumptive pharyngeal endoderm during gastrulation might be responsible. At the onset of gastrulation in axolotls, the axial mesoderm, including presumptive prechordal mesoderm and notochord, is immediately animal to the pharyngeal endoderm domain (Cleine and Slack, 1985; Pasteels, 1942; Vogt, 1929). This location puts these two tissues in close proximity throughout gastrulation (Brun and Garson, 1984; Lundmark, 1986), which would facilitate signaling between them. Axial mesoderm, and notochord in particular, is important in patterning the endoderm of the gut (Hebrok et al., 1998; Kim et al., 1997; Wells and Melton, 1999; Wells and Melton, 2000). However, these studies have focused on the gut posterior to the pharynx, and the impact of notochordal signaling on gut patterning has been shown to occur later, after gastrulation (Hebrok et al., 1998; Kim et al., 1997; Wells and Melton, 2000). Thus, this study focused on the effect of putative signals from axial mesoderm on specification of the most anterior, pharyngeal endoderm of the gut axis at a much earlier stage of development, during gastrulation.

I have performed a series of experimental manipulations of putative signals during gastrulation, and assessed the ability of presumptive pharyngeal endoderm to subsequently make taste buds. Systematic disruption of presumed vertical and planar signals from neurectoderm during gastrulation did not prevent taste bud development in pharyngeal endoderm. However, interruption of contact of pharyngeal endoderm with axial mesoderm resulted in the loss of taste bud-generating capability. My results indicate that the pharyngeal endoderm is specified during gastrulation, and that this occurs via receipt of signals from axial mesoderm. Finally, while the ability to differentiate taste buds is acquired through signals from the notochord during gastrulation, the induction of pharyngeal endoderm as a distinct embryonic tissue probably precedes its acquisition of differentiative capabilities, and may occur coincident with the early induction of dorsal mesoderm. In summary, these results indicate that tissue-autonomous mechanisms responsible for the genesis of taste buds are set in motion very early in embryonic development, in contrast to the late differentiation of these sensory organs.

MATERIALS AND METHODS

Embryos

Axolotl embryos were acquired from the Indiana University Axolotl Colony between stages 4 and 8 (Bordzilovskaya et al., 1989) and maintained in 20% Holtfreter's solution (HF) at 20°C.

Exogastrulae

Stage 9 blastulae, before dorsal lip formation (at stage 10), were treated fungicidally with 0.02% formalin in 20% Holtfreter's (HF) solution for 10 minutes before manual removal from their egg jellies in sterile 100% HF with antibiotics (HFA: 400 μg/ml each of penicillin and streptomycin, 25 μg/ml gentamycin). Dejellied embryos were transferred to 100% HFA with 10% Ficoll and devitellinized with fine forceps. Demembranated embryos were transferred to 200% HFA in dishes lined with 1% agarose. Under this high salt condition, embryos exogastrulate (Holtfreter, 1933). Isolates were created at progressive stages of gastrulation by slicing off the exogastrulating endomesodermal region of the embryo at the juncture of the ectoderm and endomesoderm at stages 10.5 (stage 10 + 6 hours), 11.5 (+14 hours) and 13 (+24 hours). Exogastrulae and isolates were maintained in 200% HFA until stage 41 (~11-12 days), until intact controls had developed taste buds (Barlow and Northcutt, 1995).

Keller sandwiches

As above, stage 9 embryos were selected, dejellied, then transferred to DFA solution (Sater et al., 1993) and devitellinized with fine forceps. At stage 10, when the dorsal lip forms (pigmented apices of bottle cells visible, Fig. 1A), Keller sandwiches were constructed according to the method of Keller for Xenopus laevis (Keller, 1991) with slight modification due to species differences. The dorsal involuting marginal zone and presumptive neurectoderm was removed from each of two embryos (Fig. 1B,D). The vegetal endoderm, identifiable because of the large size and loosely adherent nature of these cells, was swept from the internal surface of the explant with an eyebrow tool, revealing the forming bottle cells at the dorsal lip. Unlike Xenopus, the majority of mesoderm is superficial in axolotls, and an extensive and obvious deep layer of axial mesoderm is not present (Brun and Garson, 1984; Lundmark, 1986; Smith and Malacinski, 1983; Vogt, 1929). The two explants were placed together with identical orientation and their internal surfaces apposed, and were maintained in DFA until stage 41 (Fig. 1D, part a).

To test for planar signals from neurectoderm during gastrulation, Keller sandwich isolates were generated by slicing off the converging and extending endomesoderm at progressive stages of gastrulation (stage 10 + 30 minutes; +2 hours – stage 10.1; +6 hours – stage 10.5: Fig. 1D, part b1; +14 hours – stage 11.5; +24 hours – stage 13: Fig. 1D, part b2). By stage 10.5, the presumptive endomesoderm and neurectoderm could be distinguished by differences in pigmentation and morphogenesis. Keller sandwiches did not show obvious morphogenesis within 2 hours of construction; in these cases, the vegetal third of each sandwich was kept. Isolates were raised in DFA until stage 41.

Dorsal lip explants

To test if pharyngeal endoderm was specified by signaling from axial mesoderm during gastrulation, a series of dorsal lip explants were constructed (Fig. 1C,E, parts a-c). Single explants made at stage 10 were trimmed to include only the presumptive axial mesoderm (notochord and prechordal mesoderm) plus pharyngeal endoderm, based on: (1) the fate maps of Vogt (Vogt, 1929) and Pasteels (Pasteels, 1942); and (2) an average gastrula diameter of 2 mm (Bordzilovskaya et al., 1989). The width of the dorsal lip explants circumscribes a 60° arc centered on the dorsal midline, which based on a circumference of 6.3 mm, translates to 1.05 mm. The height of each explant covers a 65° arc, extending animal from the blastopore lip (=1.14 mm) (Pasteels, 1942). Dorsal lip explants were then subdivided into presumptive mesoderm (most animal 40°=0.7×1 mm) and endoderm (immediately suprablastoporal 25°=0.34×1 mm; Fig. 1E, part a).

In another series of experiments, dorsal lip explant size was doubled by trimming Keller sandwiches (two apposed explants) to the dimensions of the dorsal lip explant as above (Fig. 1E, part b). These dorsal lip sandwich explants were divided into endodermal and mesodermal portions per the fate map and measurements described above.

A third series of dorsal lip explants was used to test further if direct contact between presumptive axial mesoderm and pharyngeal endoderm was necessary for specification of the endoderm. Dorsal lip explants were constructed as above, and divided into specific domains, again according to the fate map: most animal axial mesoderm,

Fig. 1. Diagrams of experimental manipulations of axolotl gastrulae. (A) A tilted dorsal vegetal view of an intact axolotl embryo at stage 10 reveals the state of the bottle cell apices (arrows) at the time of microsurgical manipulation. (B) Lateral view of the axolotl early gastrula fate map according to Pasteels (Pasteels, 1942). Dorsal is right. An, animal; bp, blastopore; ec, ectoderm; en, endoderm; lp, lateral plate mesoderm; nc, notochord; ne, neurectoderm; pcm, prechordal mesoderm; pe, pharyngeal endoderm; s, somitic mesoderm. (C) Dorsal view of the axolotl early gastrula fate map (Pasteels, 1942). Colors indicate tissues as in B. The broken black lines divide the spherical embryo at 30° intervals, both in latitude and longitude. The broken white line indicates the region of the explants shown in Ea, Eb and Ec, extending from 45° (the level of the blastopore is bp) to 110° (the animal extent of the axial mesoderm), and 30° bilaterally

from the midline. (D) Keller sandwiches were constructed from paired dorsal marginal zones and allowed to develop intact until the hatchling larval stage (a). Keller endomesodermal isolates were generated (b) by removing the converging and extending endomesoderm (pink and blue regions below broken line) at early (b1) and later (b2) stages of gastrulation. (Ea) Single dorsal lip explants were trimmed to the size of the presumptive axial mesoderm (pink) and pharyngeal endoderm (blue) according to published fate maps. The predicted endodermal and mesodermal regions were then separated microsurgically (see Materials and Methods section for details). (Eb) Keller sandwiches were constructed, as in D, trimmed to the size of the future axial mesoderm and pharyngeal endoderm, and subdivided into suprablastoporal endoderm (blue) and more animal axial mesoderm (pink). (c) Fused dorsal lip explants were created by first trimming Keller sandwiches, and then subdividing them into even thirds. The most animal axial mesoderm and suprablastoporal endoderm were either raised separately (c1) or allowed to fuse (c2). The intermediate portions, containing the imprecise border between endoderm and mesoderm, were also raised separately (c1,c2).
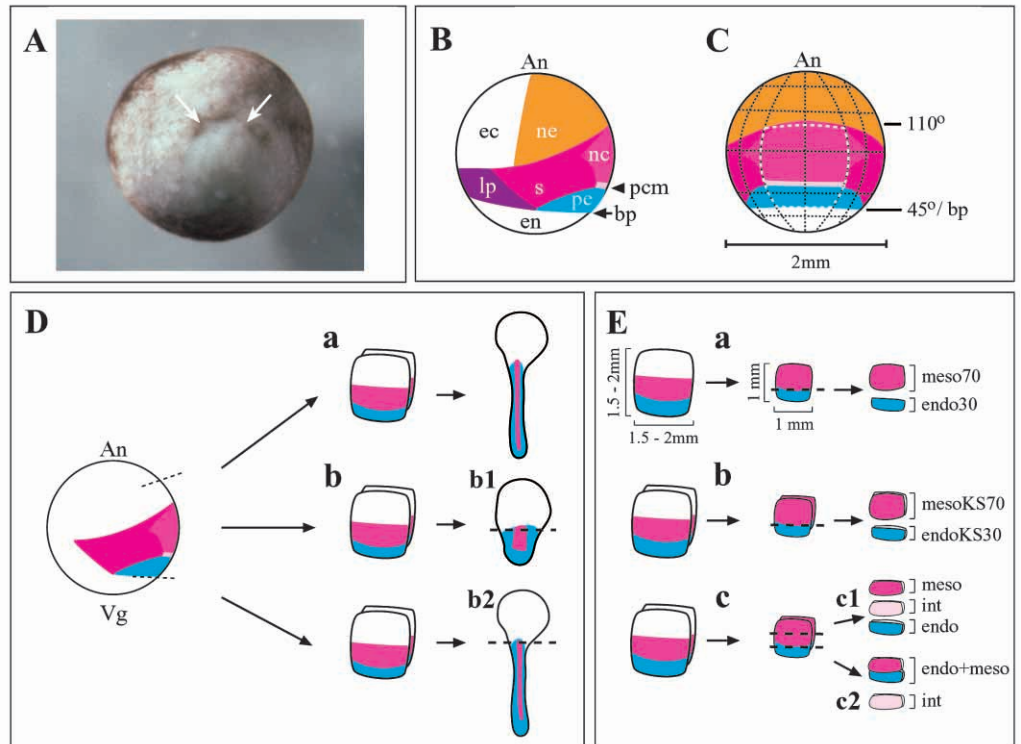
intermediate axial mesoderm plus endoderm and immediately suprablastoporal endoderm (Fig. 1E, part c). In one set of experiments, each region was raised in isolation (Fig. 1E, part c1). In another subset, the most animal mesoderm was combined immediately with the suprablastoporal endoderm to produce fused explants (Fig. 1E, part c2).

Immunofluorescence and image acquisition

All explants were fixed at stage 41 in 4% paraformaldehyde in phosphate-buffered saline, and processed for immunofluorescence according to previously published methods (Barlow and Northcutt, 1997). Explants were cryosectioned at 16 μm and processed with rabbit anti-calretinin (1:2000, Swant), which recognizes taste buds in axolotls (Barlow et al., 1996), and mouse anti-parvalbumin (1:5000, Sigma) to visualize muscle (Barlow and Northcutt, 1997). Secondary antibodies used were goat anti-rabbit Alexa-546 and goat anti-mouse Alexa-488, respectively (Molecular Probes). Sections were counterstained with Hoechst 33248 (Molecular Probes) at 1:50,000 for 30 seconds. Images of immunostained sections were acquired with either an Olympus confocal microscope or a Hamamatsu cooled CCD camera. Images were colorized, contrast enhanced and merged using Adobe Photoshop 6.0 for Macintosh.

RESULTS

Taste bud development in exogastrulae

In axolotls, taste buds are identifiable as aggregates of

calretinin-immunoreactive (CR-IR), fusiform cells with a characteristic onion-shaped morphology and an apical taste pore (Barlow et al., 1996), and the presence of taste buds has been used as a indicator of the specification of pharyngeal endoderm (Barlow and Northcutt, 1997). To test the role of vertical signals from the presumptive neurectoderm in the specification of pharyngeal endoderm, exogastrulae were created and examined for the presence of taste buds. In all exogastrulae, taste buds were well differentiated with CR-IR fusiform cells and distinct taste pores (Fig. 2A-C, Fig. 3). Taste buds were present in large numbers in exogastrulae, and were indistinguishable morphologically from control taste buds (Barlow et al., 1996). This result suggested that vertical signals from overlying neurectoderm during gastrulation were not necessary for the specification of pharyngeal endoderm, but left unanswered the question of persistent planar signals from neurectoderm.

To test for planar signals during gastrulation, evaginating endomesoderm was isolated at progressive stages of exogastrulation. In this way, putative planar signals were titrated temporally to assess if and how long these signals were required for the specification of pharyngeal endoderm. Although some early (stage 10.5), intermediate (stage 11.5) and late (stage 13) isolates could each generate CR-IR taste buds with normal morphology (Fig. 2D-F), the percentage of

isolates with taste buds was dramatically reduced in tissue removed from early exogastrulae (Fig. 3). These data suggested that persistent planar signals might be essential for the specification of pharyngeal endoderm. However, in my hands, and as described by others (Holtfreter, 1933; Nieuwkoop and Florschutz, 1950) (R. E. Keller, personal communication), exogastrulation was a notoriously capricious phenomenon with no well understood pattern of early cell movement between groups of embryos (data not shown). Owing to this variability in initial cell movement, only some endomesodermal isolates probably contained presumptive pharyngeal endoderm capable of generating taste buds. These observations caused me to use another approach for manipulating planar signals during gastrulation, one whereby the endomesoderm could be obtained reliably.

Taste bud development in Keller explants

Cell movements in Keller sandwiches are substantially more predictable than in exogastrulae, and furthermore, the geometry of putative inductive signals can be controlled better in these explants (Keller, 1991). Keller sandwiches developed
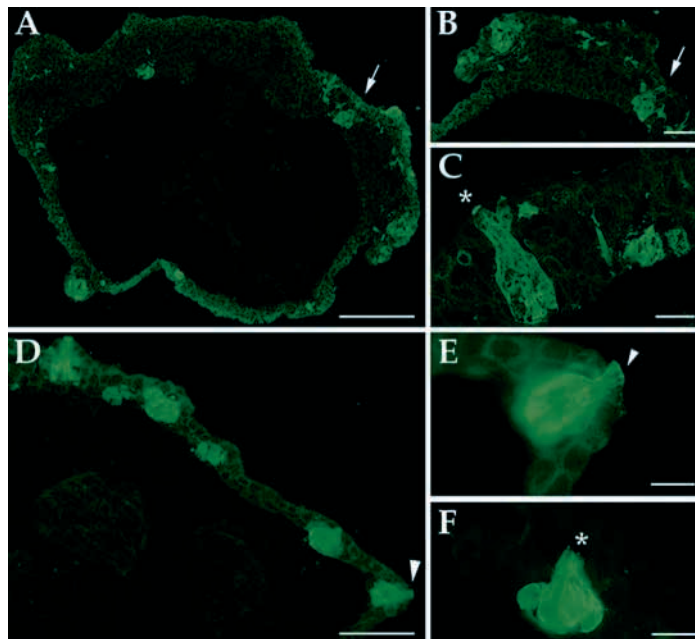

Fig. 2. Taste buds form normally in exogastrulae and endomesodermal isolates of exogastrulae. (A) A transverse section through the endomesodermal region of an intact exogastrula has been immunostained for calretinin to reveal the distribution of differentiated taste buds, which are present in large number. The arrow indicates the apical region of the taste bud shown at higher magnification in B.
(C) This CR-IR taste bud found in an intact exogastrula displays the characteristic accumulation of CR-IR material in the apical microvilli (*), which is commonly seen in vivo (Barlow et al., 1996).
(D) Numerous CR-IR taste buds were found in this endomesodermal isolate from a stage 11.5 exogastrula. The arrowhead indicates the apical region of the taste bud shown in E at higher magnification.
(E) Again, this taste bud has intense CR-IR in the apical microvilli, throughout the cytoplasm of numerous fusiform cells within the bud.
(F) Another CR-IR taste bud from an endomesodermal isolate, illustrating cytoplasmic immunostaining within fusiform cells, as is the case in vivo. Scale bars: 150 µm in A; 100 µm in B,D; are 25 µm in C,E,F.

well-differentiated taste buds, as evidenced by their multicellular morphology and CR-IR (Fig. 1D, part a; Fig. 4A-C). The ability of this tissue to make taste buds was consistent with earlier conclusions from exogastrulae; reciprocal vertical signals from neurectoderm during gastrulation were not necessary for the specification of pharyngeal endoderm.

I next separated the endomesoderm from the neurectoderm of Keller sandwiches at successive stages of gastrulation, and allowed the former to develop in the subsequent absence of putative, planar signals (Fig. 1D, part b). Unlike results from exogastrulae, CR-IR taste buds were found in the majority of Keller isolates (Fig. 3, Fig. 4D,E). Even very early Keller sandwich isolates were able to make taste buds. Three out of six isolates made at 30 minutes (stage 10) generated taste buds at the appropriate stage. However, these early isolates made very few taste buds, one to three buds per explant, rather than the dozen or more found in later isolates (data not shown). In addition, earlier explants appeared to be devoid of notochord and muscle, while later isolates generally possessed these tissues. Notochord was identified histologically only, by the presence of a web of large vacuoles in a characteristic wheel shape, while muscle was revealed with parvalbumin immunostaining (Fig. 4B, Table 1). As axial mesoderm is known to dorsalize adjacent mesoderm, causing the latter to become muscle (Gilbert and Saxen, 1993; Harland and Gerhart, 1997), I next tested the idea that signals from presumptive notochord similarly were specifying pharyngeal endoderm.

Taste bud development in dorsal lip explants

As described in detail in the methods section, the radial dimensions of the regions fated to become pharyngeal endoderm, prechordal plate [eye muscles and head mesenchyme (Adelmann, 1932)], and notochord have been mapped in axolotl gastrulae (Pasteels, 1942). I used these measurements and an average gastrula diameter of 2 mm to
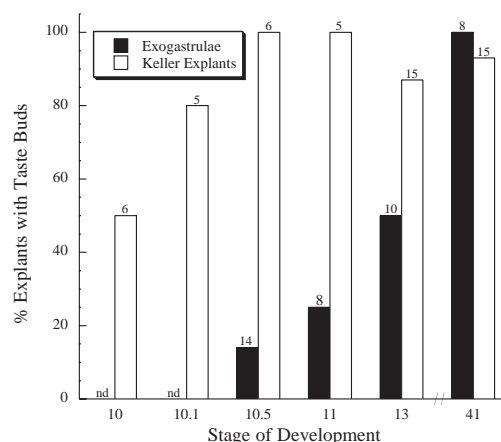
Fig. 3. The percentage of Keller isolates with taste buds differs from that of exogastrula isolates. Virtually all intact Keller sandwiches (white bars) and exogastrulae (black bars) produced taste buds. By contrast, while most Keller isolates, regardless of the stage of isolation, generated taste buds, the percentage of exogastrula isolates with taste buds declined as the presumed endomesoderm was removed at progressively earlier stages. (nd, not done; sample sizes are above histogram bars.)

calculate the surface area for each of these regions (Fig. 1C). I then made a series of dorsal lip explants designed to test if signals from axial mesoderm are important for specification of pharyngeal endoderm (Fig. 1E).
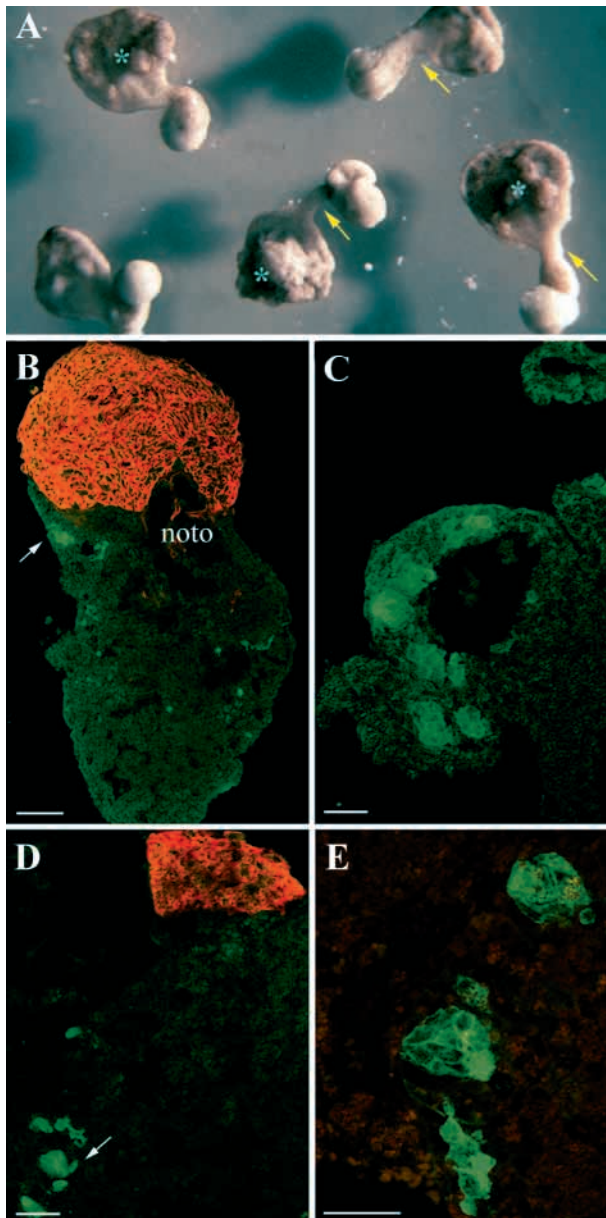
Fig. 4. Both Keller sandwiches and endomesodermal isolates thereof give rise to CR-IR taste buds. (A) Live Keller sandwiches 24 hours after construction have undergone normal convergence and extension of the mesoderm (arrows) and neurectoderm (asterisks). (B) This transverse section through an intact Keller sandwich explant at stage 41 or hatching was immunostained for calretinin (green) to show the presence of taste buds, and parvalbumin (red), which reveals the location of muscle. Notochord (noto) is also easily recognize via its distinct morphology. (C) Numerous multicellular CR-IR taste buds (arrow) are evident in most Keller sandwiches. (D) Immunostained sections of hatchling stage Keller sandwich endomesoderm isolated at stage 13 also revealed CR-IR (green) taste buds with apical processes (arrow), and parvalbumin-IR muscle (red). (E) At higher magnification, each taste bud is clearly multicellular, comprising fusiform, CR-IR cells. Scale bars: 100 µm in B; 50 µm in C,D,E.

Initially, dorsal lip explants that included presumptive pharyngeal endoderm, prechordal plate and notochord were removed at the onset of gastrulation when bottle cell apices had formed a pigmented line, but involution had not yet begun (stage 10; Fig. 1A). These explants were next divided into presumed endodermal (Endo30) and axial mesodermal territories (Meso70; Fig. 1E, part a). Endo30 dorsal lip explants alone failed to give rise to taste buds, and were also devoid of mesodermal derivatives (Fig. 5A,B, Fig. 6). Nonetheless, endodermal dorsal lip explants differentiated as endoderm, i.e. the cells contained large yolk granules typical of amphibian endoderm (Adams, 1924; Holtfreter, 1996; Nieuwkoop and Ubbels, 1972), while the majority of presumed mesodermal dorsal lip explants produced notochord and muscle (Fig. 5C-E, Fig. 6). Unexpectedly, these mesodermal explants also gave rise to taste buds. This result could have occurred if part of the endodermal domain was included in the initial explant, or if the axial mesoderm were able to regulate and give rise to endoderm when explanted, as has been demonstrated for other tissues (Holtfreter, 1996). In either case, these data suggested that local signals from the axial mesoderm were essential for pharyngeal endoderm specification, and that removing the presumptive endoderm from contact with mesoderm had prevented specification, while maintaining persistent contact between mesoderm and endoderm allowed specification. However, endodermal dorsal lip explants were relatively small when compared with axial mesoderm explants. We have found that pharyngeal endoderm explants from neurulae generate taste buds only when explant size exceeds a certain volume (M. A. Parker and L. A. B., unpublished). Thus, the failure of dorsal lip endodermal explants to generate taste buds may have been due to their small volume, rather than to the absence of axial mesoderm.

To address this size issue, dorsal lip sandwich explants were constructed to increase the volume of the endodermal region. Specifically, Keller sandwiches were constructed and trimmed and then divided into endodermal (EndoKS30) and mesodermal (MesoKS70) bilayered components (Fig. 1E, part b). These doubled endodermal explants rarely produced taste buds, and only did so when axial mesoderm was present, while, as above, the presumed mesodermal explants generated taste buds (Fig. 6). Again, these findings supported the idea that signals from axial mesoderm were responsible for specification of pharyngeal endoderm. These data also suggested that the extent of the pharyngeal endodermal field must extend more animal than its fate map domain (Pasteels, 1942), and additional dorsal lip explant permutations confirmed this idea.

When the suprablastoporal presumed endoderm explant was increased to 50% of the dorsal lip domain (Endo50), this region differentiated similarly to the Endo30 and EndoKS30 explants, and remained endodermal. Only a few of these explants differentiated taste buds, but did so only when notochord was evident (Fig. 6). The mesodermal halves of the dorsal lip explants in these experiments (Meso50) always differentiated notochord and muscle, but the percentage that generated taste buds was reduced compared with larger, Meso70 explants (Fig. 6). Interestingly, Endo50 explants occasionally produced solitary CR-IR cells, which were irregularly shaped and had more extensive cytoplasm compared with CR-IR cells within taste buds (Fig. 5F; compare with Fig. 5D,E). The identity of these cells is uncertain. Solitary CR-IR cells of unknown

Table 1. Occurrence of taste buds and differentiated mesodermal derivatives in isolates of Keller sandwiches
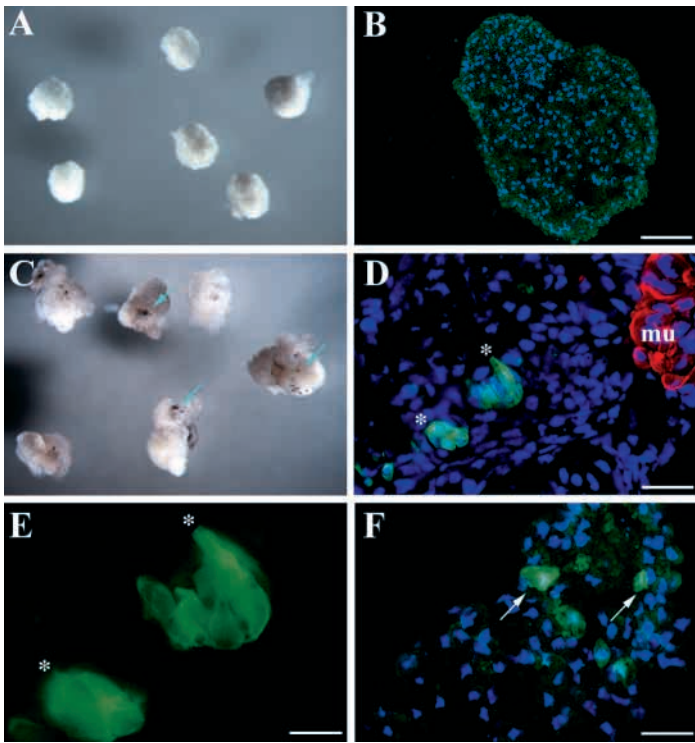
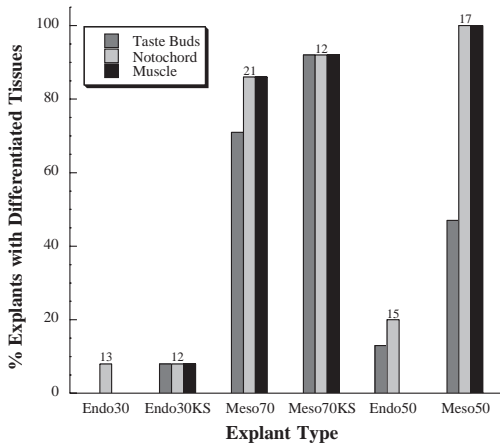| Types of differentiated tissue in isolates at stage 41	Stage of development at time of isolation					
	10	10+2 hour	10.5	11.5	13	41
Taste buds	5/6=83%*	4/5=80%	6/6=100%	5/5=100%	13/15=87%	11/15=73%
Notochord	0/6=0%	2/5=40%	0/6=0%	3/5=60%	13/15=87%	11/15=73%
Muscle	0/6=0%	2/5=40%	1/6=17%	3/5=60%	12/15=80%	11/15=73%

*Two out of six had only one taste bud each.

function are present in the pharynx of larval axolotls (L. A. B., unpublished), but they possess bipolar processes, unlike solitary CR-IR cells in these endodermal dorsal lip explants. These cells may be immature taste cells, which had partially differentiated in response to a low level of signaling from

Fig. 5. Presumed mesodermal portions of dorsal lip explants make taste buds, whereas isolated endodermal components do not. (A) Small endodermal dorsal lip explants (Endo30s) persist as round balls of cells through to hatchling stages. (B) Cryosections of Endo30 explants were devoid of CR-IR; neither CR-IR taste buds nor solitary CR-IR cells were evident. However, yolk granules, indicative of endodermal tissue, autofluoresce in the green channel. Nuclei are stained with Hoechst (blue). (C) Mesodermal explants undergo substantial morphogenesis, and possess melanin granules typical of ectoderm (arrows). (D) Taste buds with numerous fusiform, CR-IR cells (green, * marks apical region of each taste bud) are present in this section of a mesodermal dorsal lip explant counterstained with Hoechst (blue). Parvalbumin-IR muscle (red-mu) is also present in this section. (E) The taste bud on the right in D is shown at higher magnification to illustrate the fusiform nature of CR-IR (green) cells within the bud, as well as the characteristic apical processes of these cells (*). (F) A few solitary, irregularly shaped, CR-IR cells (green) are shown in this cryosection of an Endo50 endodermal dorsal lip explant counterstained with Hoechst (blue). This explant did not develop taste buds, however. Scale bars: 100 μm in B; 50 μm in D,F; 25 μm in E.

mesoderm inadvertently included in the endodermal explant. However, this is unlikely, given that taste buds in intact axolotls are evident in histological section as multicellular aggregates well before these cells begin to express calretinin (L. A. B. unpublished). Regardless of their identity, while these solitary cells occurred occasionally in Endo50 explants (probably when axial mesoderm was present but not detectable), they were never seen in Endo30 dorsal lip explants, which were typically devoid of mesoderm.

The varying results obtained with different dorsal lip explant manipulations are best understood in the context of what is known about movement of dorsal lip cells in Xenopus early gastrulae. In Xenopus, the extent of pharyngeal endoderm on the surface is greater at stage 10–, and then condenses in area by stage 10, as the bottle cells form. At the same time the axial mesodermal field is moving vegetally, toward the dorsal blastopore lip (Keller, 1975; Keller, 1981). A comparable shift in territories may be occurring in axolotl embryos. If these explants were made slightly earlier than the stage at which Pasteels constructed the fate map (Pasteels, 1942), the pharyngeal endodermal

Fig. 6. Mesodermal dorsal lip explants generate taste buds and mesodermal tissues, whereas endodermal dorsal lip explants are generally devoid of both. Mesodermal dorsal lip explants (Meso70, animal 70% of dorsal lip explant; Meso70KS, Keller sandwich Meso70 explant) typically possess taste buds (dark-gray bars), notochord (pale-gray bars) and muscle (black bars). Endodermal isolates (Endo30, suprablastoporal 30% of dorsal lip explant; Endo50, suprablastoporal 50% of dorsal lip explant; Endo30KS, Keller sandwich Endo30 explant) generally do not contain any of these tissues. A smaller percentage of 50% mesodermal explants (Meso50) generate taste buds than do the larger Meso70 explants, consistent with the inclusion of less endoderm in the Meso50 explants.

region would be larger than predicted by the map, and the axial mesoderm field would be shifted animally. The data presented here are consistent with this scheme, and, furthermore, illustrate the difficulty in precisely locating discrete presumptive regions in rapidly developing gastrulae. Regardless of the exact size and position of the presumptive pharyngeal endodermal field, these results suggested that pharyngeal endoderm required signals from axial mesoderm for subsequent differentiation of taste buds, as taste buds did not form in explants apparently devoid of mesoderm in any of the endodermal dorsal lip explants.

To test if contact of pharyngeal endoderm by axial mesoderm was necessary for the differentiation of taste buds, and to eliminate ambiguity about the boundary between presumptive pharyngeal endoderm and axial mesoderm, a final series of dorsal lip explants was constructed (Fig. 1E, part c). In these explants, I removed the intermediate region, which contained some endoderm, all prechordal mesoderm and some presumptive notochord, according to the fate map (Pasteels, 1942), and allowed the most animal axial mesoderm (presumptive notochord) and immediately suprablastoporal endoderm (presumptive pharyngeal region) to fuse (Fig. 1E, part c2). Each of these regions, animal mesoderm, intermediate zone and suprablastoporal endoderm, was also cultured alone (Fig. 1E, part c1).

The intermediate region (Int) produced explants with taste buds and mesodermal derivatives, as expected roughly from the fate map (Fig. 8). Suprablastoporal endoderm alone (Endo) developed as endoderm, but did not produce taste buds (Fig. 7A,D, Fig. 8). By contrast, animal mesoderm (Meso) differentiated as notochord and muscle, as well as nervous tissue, but failed to give rise to taste buds (Fig. 7B,E,F, Fig. 8). When the suprablastoporal endoderm and animal mesoderm were fused (Endo+Meso), these explants now differentiated taste buds, as well as notochord and muscle (Fig. 7C,G,H). Given our previous results (that pharyngeal endoderm isolated after gastrulation is already specified to give rise to taste buds) (Barlow and Northcutt, 1997), these new data indicate that signals from axial mesoderm during gastrulation are necessary for specification and subsequent differentiation of pharyngeal endoderm.

DISCUSSION

Signals from neurectoderm during gastrulation do not specify pharyngeal endoderm

In amphibian embryos, the presumptive pharyngeal endoderm sits at the dorsal lip of the blastopore and is the first set of cells to

involute, followed by the axial mesoderm (prechordal mesoderm and notochord) (Cleine and Slack, 1985; Delarue et al., 1992; Keller, 1975; Pasteels, 1942; Saint-Jeannet and Dawid, 1994; Vogt, 1929). During gastrulation, the endomesoderm neuralizes the ectoderm (Spemann and Mangold, 1924; Harland, 1997). This signal travels both within the plane of the epithelium connecting the endomesoderm and ectoderm, and vertically, from the underlying endomesoderm to the adjacent ectoderm (Doniach, 1992; Nieuwkoop, 1997; Nieuwkoop and Koster, 1995; Papalopulu and Kintner, 1993; Poznanski and Keller, 1997; Ruiz i Altaba, 1992). However, there has been some suggestion that neurectoderm signals back to endomesoderm (Poznanski and Keller, 1997). I reasoned therefore that perhaps signals from neurectoderm to presumptive pharyngeal endoderm during gastrulation were essential for the specification of the latter.
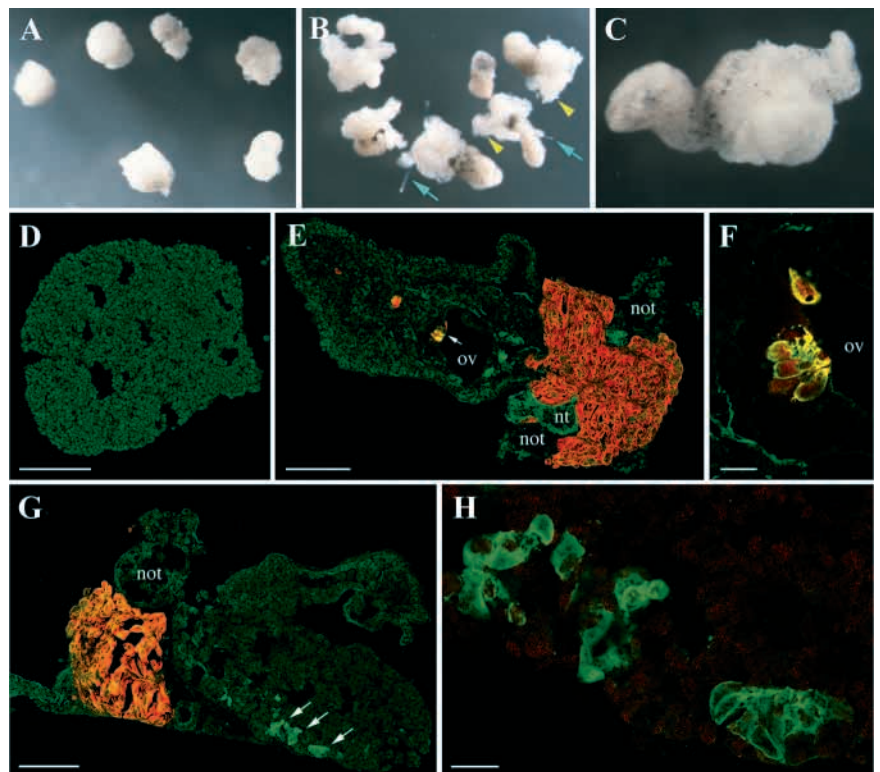
Fig. 7. Dorsal lip endoderm generates taste buds only when fused with dorsal lip mesoderm. (A) Dorsal lip endodermal explants remained as rounded balls throughout the culture period. (B) Mesodermal explants underwent extensive morphogenesis, and developed obvious notochords (arrows), and rough regions of muscle (arrowheads). (C) Fused explants again underwent morphogenesis, but the notochord and muscle elements were typically not as apparent, which is probably due to the presence of the endodermal epithelium. (D) No taste buds are evident in this typical section of an En30 explant immunostained for calretinin. (E) In this cryosection of an animal mesoderm dorsal lip explant, while CR-IR taste buds are absent, notochord (not) and parvalbumin-IR muscle (red) are clearly present. In addition, adjacent tissue has been neuralized, as evidenced by the presence of a neural tube (nt) with CR-IR neurons and axons (green), as well as of the otic vesicle (ov) with parvalbumin- and CR-IR hair cells (arrow; yellow). (F) This micrograph shows a higher magnification view of the parvalbumin- and CR-IR hair cells shown in E. (G) A section through a fused mesoderm-endoderm explant reveals 3 CR-IR taste buds (green; arrows), as well as notochord (not) and parvalbumin-IR muscle (red). (H) This micrograph is a high magnification view of the three taste buds shown in G. Although the plane of section is not optimal, these organs clearly comprise multiple CR-IR cells. Scale bars: 200 µm in C,D,E; are 25 µm in F,H.
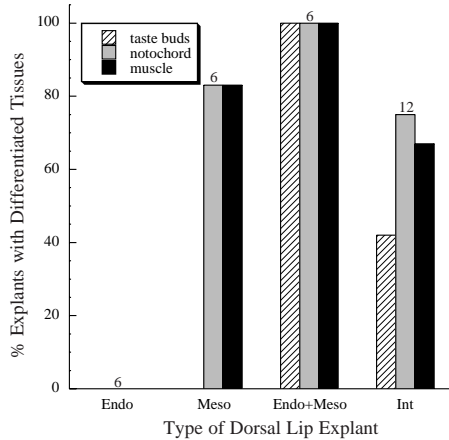
Fig. 8. Taste buds only form when presumptive pharyngeal endoderm is fused with presumptive axial mesoderm. Endoderm alone (Endo) is devoid of taste buds and differentiated mesoderm. Mesoderm alone (Meso) lacks taste buds (striped bars), but develops notochord (gray bars) and muscle (black bars). When these two regions are fused (Endo+Meso), now all explants generate all three differentiated tissues. Intermediate regions (Int) cultured alone typically generated mesodermal derivatives, and reasonably frequently gave rise to taste buds. Sample sizes are above histogram bars.

To test this idea, putative vertical and planar signals were disrupted, and the impact of these manipulations on taste bud genesis was assessed. Vertical signals were interrupted using both exogastrulae and Keller sandwich explants. Taste buds formed readily and normally under either condition, and these results allowed dismissal of the hypothesis that vertical, neurectodermal signals specified pharyngeal endoderm. Isolated endomesoderm from Keller sandwiches also produced taste buds, despite the lack of continued contact with neurectoderm, demonstrating that persistent planar signals were also not necessary for specification of pharyngeal endoderm. However, these results suggested that persistent signals from the axial mesoderm during gastrulation might induce the capacity to differentiate taste buds in pharyngeal endoderm.

Signals from the axial mesoderm during gastrulation specify pharyngeal endoderm

The rationale to test the role of axial mesoderm in the specification of pharyngeal endoderm was to disrupt contact between them, and assess the ability of the latter to generate taste buds. One problem with this approach initially was locating precisely these presumptive regions in early axolotl gastrulae. Although Pasteels had concisely fate mapped a comparable stage in this species (Pasteels, 1942), it was unclear from his descriptions exactly when he performed his experiments. This ambiguity had a profound impact on the studies presented here, because it is well known in Xenopus that presumptive territories of the pharyngeal endoderm and axial mesoderm shift substantially and quickly as gastrulation gets underway (Keller, 1975; Keller, 1976; Keller, 1981). It became clear that the stage used in my experiments, when bottle cells apices are visible as a rough line, was slightly earlier than that chosen by Pasteels, and was at a phase in development when the presumptive pharyngeal endoderm

occupied a greater region than that observed by Pasteels. This result also called into question the location of prechordal mesoderm and presumptive notochord.

While the location of notochord could be determined empirically in these studies, because it is subsequently identifiable via its distinct morphology, the precise position of prechordal mesoderm was difficult to discern. Prechordal mesoderm gives rise to cranial connective tissue and to extrinsic eye muscles in vivo (Adelmann, 1932; Couly et al., 1992; Noden, 1988). The absence of muscle in presumed pharyngeal endoderm explants may have been due to the absence of critical inductive signals for prechordal mesoderm differentiation, rather than the absence of these cells in the explants. Further, dye-labeling studies have not resolved the limit between the pharyngeal endoderm and prechordal mesoderm, which is thought to lie immediately animal to the pharyngeal endoderm (Delarue et al., 1994; Keller, 1975; Keller, 1976; Løvtrup, 1975; Pasteels, 1942; Vogt, 1929). This is in part due to the very narrow domains of each of these presumptive tissues at late blastula stage, the shifting of dorsal regions vegetally and the extensive intermingling of cells within this region early on (Delarue et al., 1992).

Thus, to alleviate problems of a shifting fate map, and the difficulty in detecting prechordal mesoderm, I simply removed the ambiguous middle domain of the dorsal lip explants, and either raised the prospective pharyngeal endoderm and axial mesoderm in isolation, or allowed them to develop together in a fused explant. While this approach allowed me to test directly if contact with axial mesoderm was necessary for pharyngeal endoderm specification, it did not provide a means for assessing the degree to which prechordal mesoderm was present in the axial mesodermal component. Pharyngeal endoderm alone did not give rise to taste buds, notochord or muscle, although it did maintain its endodermal character. By contrast, axial mesoderm explants differentiated notochord, muscle and often neural tissue [as has been noted by others (Holtfreter, 1996)], but failed to give rise to taste buds. Only fused explants produced taste buds, and did so only in the endodermal domain of fused explants. These results suggested that the ability of pharyngeal endoderm to give rise to taste buds is dependent upon signals from axial mesoderm, either prechordal mesoderm, notochord or both. Interestingly, these data also demonstrate that differentiation of notochord is not dependent upon signals from the pharyngeal endoderm.

The nature of the signal(s) from axial mesoderm is not known, but a number of possible candidates exist. Many secreted factors are expressed in the Organizer of Xenopus embryos at the onset of gastrulation, such as noggin, chordin, cerberus, follistatin, dickkopf 1 and several Xnrs (Bouwmeester et al., 1996; Glinka et al., 1998; Hemmati-Brivanlou et al., 1994; Lamb et al., 1993; Osada and Wright, 1999; Sasai et al., 1994; Smith et al., 1995; Vodicka and Gerhart, 1995; Zoltewicz and Gerhart, 1997). These gene products have well-recognized roles in both neural induction and mesoderm dorsalization (Glinka et al., 1998; Schneider and Mercola, 1999; Zoltewicz and Gerhart, 1997). However, one or more of these signaling molecules may also be involved in the specification of pharyngeal endoderm by axial mesoderm during gastrulation.

There is substantial precedent for mesodermal patterning of the gut axis, although, most of these inductive events occur

later in endodermal differentiation. For example, both lateral plate and cardiac mesoderm induce axis specific differentiation of the intestine (Ishizuya-Oka and Mizuno, 1984; Matsushita, 1995; Takata, 1960) and liver (Gualdi et al., 1996), respectively. In the case of liver formation, cardiac mesoderm signals to endoderm via fibroblast growth factor (FGF) 1 and FGF2 (Jung et al., 1999b). Somewhat earlier, but still after the gut tube has formed, notochord contact with gut endoderm represses Shh expression in the presumptive dorsal pancreas, via FGF2 and activin β2 signaling, allowing pancreas differentiation (Hebrok et al., 1998; Kim et al., 1997). Even earlier, immediately after gastrulation, adjacent mesoderm and ectoderm act to pattern the endoderm, and, again, do so via the release of FGF4 (Wells and Melton, 2000). Interestingly, FGF has diverse inductive properties during neural and mesendoderm induction (Harland and Gerhart, 1997), and thus FGFs may also play a role in specification of pharyngeal endoderm during gastrulation.

Specification of pharyngeal endoderm is secondary to induction of presumptive pharyngeal endoderm

In previous studies, the ability of pharyngeal endoderm to give rise to taste buds has been used as an indicator of its specification (Barlow and Northcutt, 1997). My current results suggest that the ability to differentiate taste buds is acquired secondarily to the induction of presumptive pharyngeal endoderm. Although explanted presumptive pharyngeal endoderm failed to produce taste buds, it nonetheless was identifiable as endoderm owing to the presence of characteristic large yolk granules (Adams, 1924; Barlow and Northcutt, 1995; De Beer, 1947). When a comparable region of the dorsal lip was explanted from early axolotl gastrulae by Holtfreter (Holtfreter, 1996), this tissue developed as endoderm and did not give rise to mesodermal derivatives when assessed histologically, implying that the presumptive pharyngeal endoderm was induced before the onset of gastrulation. In addition, numerous gene products are expressed in complicated, partially overlapping subregions of the Organizer before gastrulation begins, including, but not restricted to, Cerberus, Noggin, Xnr3, goosecoid, Xbra and Xotx2 (Blitz and Cho, 1995; Bouwmeester et al., 1996; Cho et al., 1991; Isaacs et al., 1999; Lamb et al., 1993; Schneider and Mercola, 1999; Smith et al., 1995; Vodicka and Gerhart, 1995). Thus, both molecular and experimental data support the idea that pharyngeal endoderm is induced before gastrulation.

It is more than likely that this early induction of presumptive pharyngeal endoderm, as a component of the Organizer, occurs via the same process that induces dorsal or axial mesoderm. In classical recombination experiments, dorsal vegetal blastomeres induce both dorsal mesoderm and pharyngeal endoderm in animal caps (Nieuwkoop, 1969a; Nieuwkoop, 1969b; Nieuwkoop and Ubbels, 1972). Dorsal mesoderm is thought to be induced through the synergism of several molecular pathways, including Wnt/β-catenin signaling, and TGFβ signaling (Vg1, activin and Xnrs) (Agius et al., 2000; Crease et al., 1998; Larabell et al., 1997; Moon and Kimelman, 1998; Zorn et al., 1999). Interestingly, members of the TGFβ family induce the formation of bottle cells, which contribute to pharyngeal endoderm (Keller, 1981), when these genes are expressed ectopically in early gastrulae (Kurth and Hausen, 2000). Thus, these signaling

cascades may also be involved in the induction of presumptive pharyngeal endoderm.

Implications for the development of the taste periphery

Pharyngeal endoderm acquires the ability to generate taste buds at an extremely early stage when compared with the timing of differentiation of these receptor organs. While taste buds develop around the time of hatching in axolotls, pharyngeal endoderm is specified approximately 12 days earlier, during gastrulation. Thus, exceedingly early axial mesoderm signaling initiates a cascade of events that must first produce an array of taste bud progenitors; these cells then must give rise to multicellular taste buds. These progenitors have yet to be identified, and the manner in which taste buds are generated from them is also unknown. However, the autonomous formation of taste buds in amphibian endoderm is consistent with recent reports of patterned gene expression in the developing tongue epithelium of mice.

Early on, well before the appearance of taste buds, epithelial thickenings develop in rows on the embryonic mouse tongue (Mistretta, 1972). These thickenings become papillae, which house taste buds (Kinnamon, 1987). Several genes are expressed focally in these papillae as they form (at E13.5), including *Shh, Ptc* and *Gli1* (Bitgood and McMahon, 1995; Hall et al., 1999; Jung et al., 1999a), and *BMP4* (Hall et al., 2000; Jung et al., 1999a). Additionally, onset of focused gene expression occurs before innervation, implying that this early aspect of patterning of the tongue epithelium is nerve independent (Hall et al., 1999). If papillae contain taste bud progenitors, then these data support an early, and nerve-independent mechanism of patterning of presumptive taste buds in mammals, as has been demonstrated in amphibians (Barlow et al., 1996). Interestingly, *Shh, Ptc* and *Gli1* are expressed diffusely in the tongue epithelium and mesenchyme as early as E12, and subsequently resolve to the focal pattern (Hall et al., 1999), implying that other genetic mechanisms function to restrict Shh signaling to papillae. Identifying the genes that regulate this change in Shh pattern may elucidate a linkage between early specification of pharyngeal endoderm and subsequent epithelial patterning.

An additional implication of the results presented here pertains to the dual origins of the oro-pharyngeal epithelium, and suggests that, once specified, pharyngeal endoderm acts to pattern other tissues in the head. In all vertebrates, the mouth opening develops when the most anterior pharyngeal endoderm fuses with the adjacent oral ectoderm (Adams, 1924; Noden and De Lahunta, 1985). The resultant oropharynx thus is lined anteriorly by ectoderm, and more posteriorly by endoderm (Adams, 1924; Balinsky, 1965; Couly and LeDouarin, 1990; De Beer, 1947; Johnston, 1910). In axolotls, taste buds form in both the ectodermal and endodermal epithelia; however, only the endoderm is capable of generating taste buds autonomously (Barlow, 2000). Oral ectoderm only generates taste buds when paired with endoderm in fused explants, and does so only where the ectodermal epithelium is continuous with endoderm (Barlow, 2000). These data point to an inductive role for pharyngeal endoderm, in that contact with endoderm is necessary for the formation of ectodermal taste buds. In urodele amphibians and in mammals, anterior endoderm is also necessary for proper patterning and development of

ectodermally derived teeth (De Beer, 1947; Imai et al., 1998; Sellman, 1946). These late inductive properties of pharyngeal endoderm extend the early inductive role it plays as a component of the organizer. In *Xenopus* embryos, presumptive pharyngeal endoderm possesses Organizer properties, and will induce dorsal axial structures when grafted to ectopic ventral regions (Shih and Keller, 1992). Thus, after pharyngeal endoderm is specified during gastrulation, it continues to play an important role in organizing the developing vertebrate head.

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