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REVIEW

Development and evolution of the vertebrate primary mouth

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Abstract

The vertebrate oral region represents a key interface between outer and inner environments, and its structural and functional design is among the limiting factors for survival of its owners. Both formation of the respective oral opening (primary mouth) and establishment of the food-processing apparatus (secondary mouth) require interplay between several embryonic tissues and complex embryonic rearrangements. Although many aspects of the secondary mouth formation, including development of the jaws, teeth or taste buds, are known in considerable detail, general knowledge about primary mouth formation is regrettably low. In this paper, primary mouth formation is reviewed from a comparative point of view in order to reveal its underestimated morphogenetic diversity among, and also within, particular vertebrate clades. In general, three main developmental modes were identified. The most common is characterized by primary mouth formation via a deeply invaginated ectodermal stomodeum and subsequent rupture of the bilaminar oral membrane. However, in salamander, lungfish and also in some frog species, the mouth develops alternatively via stomodeal collar formation contributed both by the ecto- and endoderm. In ray-finned fishes, on the other hand, the mouth forms via an ectoderm wedge and later horizontal detachment of the initially compressed oral epithelia with probably a mixed germ-layer derivation. A very intriguing situation can be seen in agnathan fishes: whereas lampreys develop their primary mouth in a manner similar to the most common gnathostome pattern, hagfishes seem to undergo a unique oropharyngeal morphogenesis when compared with other vertebrates. In discussing the early formative embryonic correlates of primary mouth formation likely to be responsible for evolutionary–developmental modifications of this area, we stress an essential role of four factors: first, positioning and amount of yolk tissue; closely related to, second, endoderm formation during gastrulation, which initiates the process and constrains possible evolutionary changes within this area; third, incipient structure of the stomodeal primordium at the anterior neural plate border, where the ectoderm component of the prospective primary mouth is formed; and fourth, the prime role of *Pitx* genes for establishment and later morphogenesis of oral region both in vertebrates and non-vertebrate chordates.

Key words: collar; ectoderm; endoderm; oral membrane; primary mouth; stomodeum; wedge.

Oro-pharyngeal domain and mouth in development and evolution

The design of the mouth opening, its size and functional capacities are determining factors for survival of every animal and, correspondingly, often key variables in the evolutionary history of many clades, including vertebrates. In many cases, minute rearrangements in developmental mechanisms producing these structures became major sources of substantial phylogenetic divergence. The two crown groups of bilateralians, protostomes and deuterostomes, differ exactly in this respect. The production of yolk-rich eggs in deuterostomes (and namely in vertebrates) has postponed oral formation into late embryonic stages and, at the same time, provided a leeway for alternative positioning of the mouth and timing of its formation, which could become a broad field for refinement and structural rearrangements of the developmental mechanisms involved.

The development of the oral opening in deuterostomes proceeds via regulated interactions between cell populations

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of the ectoderm and endoderm lineages, and is implemented into the pathways producing another structure characterizing the anterior pole of deuterostome embryo, the pharyngeal slits (Swalla & Smith, 2008). Pharyngotremia, or perforation of the anterior archenteron with pharyngeal slits, represents, together with deuterostomy, radial cleavage and regulative eggs, the essential characteristic of deuterostome body organization (e.g. Romer & Parsons, 1986). In terms of developmental dynamics, pharyngeal slits are produced by an autonomous endoderm regulation (Veitch et al. 1999; Piotrowski & Nüsslein-Volhard, 2000; Graham & Smith, 2001; Graham et al. 2005), which, especially in vertebrates, is supplemented by an independent placodal patterning in the anterior ectoderm (Schlosser, 2005). These endoderm and ectoderm interactions are further modified by intervention of migrating neural crest cell population into interpouch vacuities to produce novel skeletal designing mechanisms, which, specifically in the preotic region, completely overpower the initial endoderm patterning. Correspondingly, the invasion of neural crest mesenchyme into the region of presumptive mouth, another key innovation of vertebrates, overwrites the primary mechanisms of the oral opening with dozens of novel morphogenetic modules, mostly produced by regulated interactions along the epithelial–mesenchymal boundary (e.g. Stock, 2001; Fraser et al. 2010). As a result, the mouth of vertebrates represents a very complex structure equipped with specialized products of epithelial–mesenchymal interactions – teeth, jaws, glands and sensory cells specifically designed in each particular clade. In accord with previous proposals (Dickinson & Sive, 2006), we term this set of mostly neural crest-derived food-processing adaptations characterizing the adult mouth of particular vertebrate organisms, the 'secondary mouth'.

The secondary mouth can be exemplified by an oral siphon with velar tentacles covered by tunica as in ascidians; velum with tentacles, oral cirri and pre-velar region including derivatives of the preoral pit as in amphioxus; upper and lower lips with collar of tentacles and laterally placed velum as in lampreys; or jaw-joint including jaws with lips and teeth as in gnathostomes. Yet, the focus of this article is none of the above-mentioned structures, but rather the structure that precedes them both in ontogenetic and phylogenetic respects – the 'primary mouth' (in the sense of Dickinson & Sive, 2006). The primary mouth preforms the oral opening by the interplay of the primary embryonic tissues (ectoderm and endoderm), and establishes the topographic setting and organizational platform for the intervention of novel mechanisms producing the secondary mouth.

During chordate embryogenesis, the prospective mouth region is established at the discrete anterior domain at the border zone of neural ectoderm, non-neural ectoderm and anterior endoderm (Fig. 1, left), and its early specification

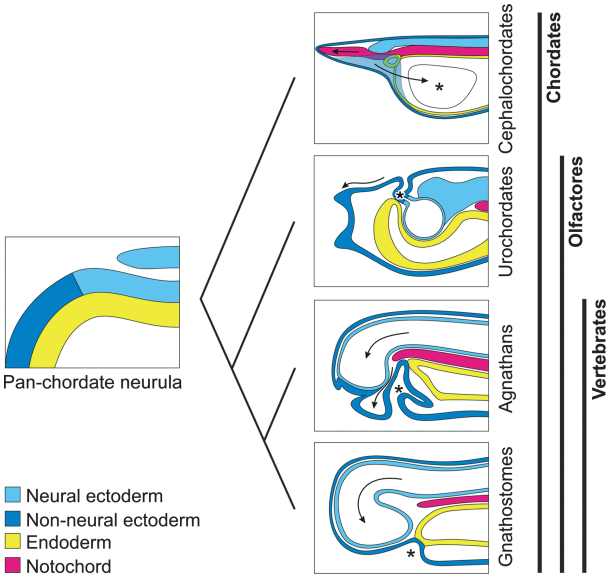

Fig. 1 Developmental positioning of the mouth in chordates. The sagittal plane is shown, anterior to the left. In all chordates and around the neurula stages (left box), the nascent primary mouth region is situated anterodorsally, and comprises foregut endoderm and non-neural ectoderm directly adjacent to neural ectoderm. The primary mouth (*) further undergoes individual lineage-specific relocation due to the growth and differentiation of surrounding tissues. In cephalochordates, the anterior larval region is augmented by the rostral prolongation of the notochord and the mouth is shifted to the left side. In urochordates, the mouth stays at its anterodorsal position and differentiates into the oral siphon primordium. The ectoderm directly anterior to the oral siphon gives rise to prominent attachment organs. In vertebrates, the mouth is shifted ventrally by the massive growth and rostral prolongation of the brain. In agnathans, moreover, the extensively growing upper and lower lips together with velar structures (or nasopharyngeal septum in the case of hagfishes) further modify and elaborate the oral region.

seems to include the same molecular pathways in all groups. Aside from this, the major clades of chordates differ significantly with respect to the final position of mouth opening (Fig. 1, right). In urochordates, mouth development is associated with the neuropore (Manni et al. 2005; Veeman et al. 2010), in cephalochordates it appears on the left side of the pharynx (e.g. Lankester & Willey, 1890; Willey, 1891; Hatschek, 1893; Urata et al. 2007; Yasui & Kaji, 2008), while in vertebrates the prospective mouth is positioned medially and ventrally to the developing brain at the anterior end of the pharynx.

The primary mouth is generally thought to be formed both by an invagination of ectoderm that forms the stomodeum and by an anterior expansion of the foregut endoderm, i.e. comparable morphogenesis to that producing the pharyngeal slits. The process often terminates with a bilaminar membrane separating the ectoderm stomodeum and endoderm pharynx, the so-called stomo-pharyngeal, oropharyngeal or oral membrane, whose disappearance initiates the development of the secondary mouth. However,

formation of the primary mouth does not always proceed in this straightforward way, and alternative developmental scenarios may take place. Despite the potential significance of such information for our comprehension of the chordate evolution, the actual forms of interplay between ectoderm patterning the oral cavity and endoderm developmental dynamics, their heterochronies and heterotopies at particular stages of early oral development, and/or rearrangements of the signalling cascades responsible for the plethora of states characterizing particular vertebrate clades are still largely unknown. This review is intended to uncover the underestimated diversity in primary mouth morphogenesis, and to address potential formative correlates that may take part in the evolution and development of this neglected, though important, embryonic structure.

Modes of primary mouth formation in vertebrates

In vertebrates, the morphogenesis of the primary mouth is initiated during the course of early embryogenesis at the time of late neurulation. It takes place at the anterior-most cranial domain where the ectoderm and endoderm meet directly without intervening mesoderm or mesenchyme (Fig. 2, left panel). This domain, well-defined also by the early expression of *Pitx* genes, is subsequently shifted ventrally by the expansion of the growing neural tube and forms a distinct invagination. Mouth development in vertebrates is generally understood to progress through: first, a stage of deep stomodeal invagination abutting the underlying endoderm foregut lining; followed by, second, the reduction of this epithelial contact zone to a thin, one–two-cell-thick oral membrane; and third, perforation and rupture of this membrane, which opens the primary mouth. The pattern of primary mouth formation via stomodeum and oral membrane is indeed widely distributed over many groups of vertebrates, and it is best exemplified in *Xenopus*, which has recently become arguably the most prominent model vertebrate for early oral organogenesis (Dickinson & Sive, 2006, 2009). However, a significantly different pattern of mouth formation can, for example, be observed among urodele amphibians (salamanders), which represent the sister clade of frogs. The mouth also forms in a dissimilar manner in ray-finned fishes, a clade including the majority of vertebrate species (Fig. 2). We will review developing oral regions of vertebrates from a comparative point of view in order to identify shared and derived developmental processes, and also to reveal the remarkable diversity of vertebrate primary mouth formation.

Mouth development via definitive stomodeum and rupture of oral membrane

Xenopus *as the model organism*

In *Xenopus*, an anuran, which currently represents a model system for studying the vertebrate primary mouth (Dickinson & Sive, 2006, 2009), the prospective oral ectoderm is found in the anterior-most part of the neurula at the border between the transverse neural fold and adjacent epidermis (Figs 1 and 8). Individual cells of this ectoderm region are fated to give rise not only to the stomodeum, but also to the head epidermis, cement and hatching

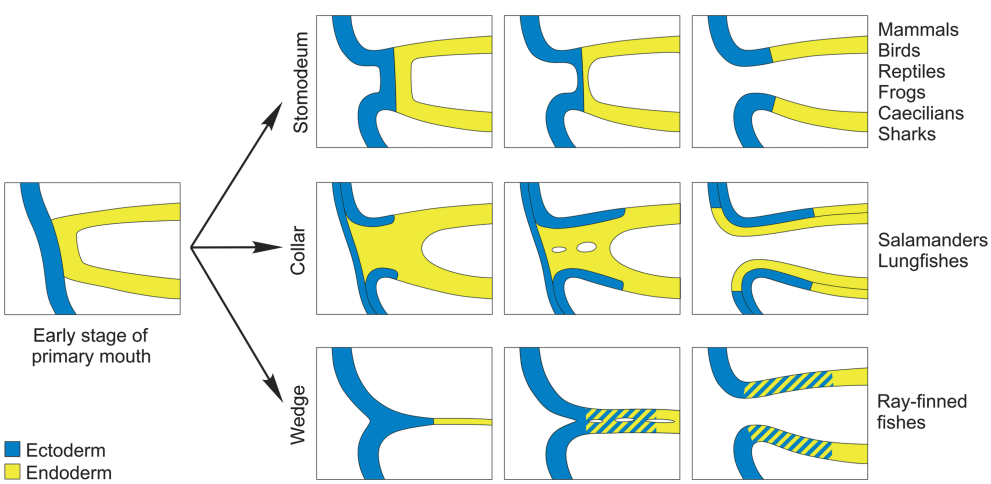

Fig. 2 Main modes of primary mouth formation in jawed vertebrates. The sagittal plane is shown, anterior to the left. The initial stage of primary mouth formation is shared among vertebrates, and involves a direct contact between outer ectoderm and foregut endoderm (left box). Its further morphogenesis in diverse vertebrate groups can generally be schematized to proceed in three main alternative developmental modes. The mouth formation via stages of stomodeal invagination and perforation of the oral membrane is the most common. In salamanders, lungfishes and few frog species, the primary mouth forms via the stomodeal collar and horizontal detachment of oropharyngeal epithelia. In ray-finned fishes, primary mouth formation includes a contact between the stomodeal wedge and the endoderm sheet, and the mouth opens via horizontal detachment of these epithelia.

glands, and adenohypophyseal and olfactory placodes (Eagleson et al. 1986; Drysdale & Elinson, 1991; Schlosser & Ahrens, 2004; Dickinson & Sive, 2007). During the course of further development, this region subdivides and the cells become more and more restricted according to their prospective fates (Pieper et al. 2011). The future stomodeal subregion then consists of a relatively thick ectoderm directly juxtaposed to a multi-layered endoderm foregut with a distinct basal lamina in between (Dickinson & Sive, 2006). Dissolution of this basal lamina, which provides an opportunity for subsequent extensive cellular rearrangements, starts relatively early in this species (Dickinson & Sive, 2009). Only then do the ectoderm cells invaginate in the form of a relatively shallow stomodeum, while the number of both the ectoderm and endoderm cells is gradually reduced. The reduction of the number of cells is caused by increased programmed cell death in the inner and outer ectoderm layers, while no apoptosis has been found in the endoderm (Dickinson & Sive, 2006). The resulting oral membrane consists of one stomodeal ectoderm layer anteriorly and one foregut endoderm layer posteriorly. The membrane thins to a single-layered unit at some points by means of intercalation of cells of both germ-layers. The eventual perforation of the oral membrane is initiated by a single opening, which is gradually enlarged in *Xenopus* (Dickinson & Sive, 2006). However, the oral membrane of another frog species, *Rana japonica*, is perforated by several small openings temporarily giving the membrane a net-like appearance (Watanabe et al. 1984). The remnants of the former membrane can be recognized only for a short period after perforation, and the limits of the ectoderm and endoderm epithelial linings are no longer discernible.

Mouth development via definitive stomodeum and rupture of oral membrane in other jawed vertebrates

In many other vertebrate groups, such as mammals, birds, reptiles, caecilians or chondrichthyans (sharks), the mouth develops classically via formation of the stomodeum and oral membrane similarly to *Xenopus*, with only minor differences that apparently reflect some lineage-specific embryonic settings (Cook & Neal, 1921; Teipel, 1932; Waterman, 1977; Waterman & Balian, 1980; Waterman & Schoenwolf, 1980). In these groups the initial contact of stomodeal ectoderm and foregut endoderm is formed below the closing neural folds, but is subsequently transferred by the increasingly growing head-fold so that the prospective oral membrane is finally established between the foregut endoderm and the ventral head ectoderm. The stomodeum develops in this ventral ectoderm as a shallow invagination, which becomes deeper and wider due to further growth of the brain and also due to the increasing volume of immigrating mesenchyme forming the surrounding jaw structures (e.g. Waterman & Schoenwolf, 1980; Ballard et al. 1993). The stomodeum and closely abutting foregut endoderm collectively form the oral membrane, which finally ruptures, but, interestingly, variable mechanisms seem to be responsible for its rupture in different vertebrate species.

A number of apoptotic cells can be found within the epithelia of the oral membrane in *Xenopus*, *Rana* and mouse (Watanabe et al. 1984; Poelmann et al. 1985; Dickinson & Sive, 2006). The apoptosis may cause its thinning, generating weak spots in the membrane and its subsequent perforation. While some cells of the membrane undergo apoptosis, the remaining non-apoptotic cells intercalate among each other, and incorporate into the epithelia of the upper and lower jaws. On the other hand, no apoptosis has been found during regression of the oral membrane in chick and hamster (Waterman, 1977; Waterman & Schoenwolf, 1980) and, moreover, the chick oral membrane even contains some proliferating cells (Miller & Olcott, 1989). In general, the rate of proliferation in the oral membrane seems to be much lower than that in the surrounding epithelia, suggesting that the heavily proliferating ectoderm and endoderm epithelial linings of the upper and lower jaws are pulling the less proliferating oral membrane apart, finally causing its rupture. This might further indicate that processes of cell intercalation within the membrane and its fusion with the surrounding epithelia are the result and not the cause of its rupture (Waterman, 1985; Miller & Olcott, 1989).

Interestingly, similar differential proliferation rates were also identified in the case of chick branchial membranes (closing plates, Miller et al. 1993), i.e. derivatives of pharyngeal groove ectoderm and pouch endoderm, which are situated between adjacent pharyngeal arches. Correspondingly to oral membrane, branchial membranes also represent transient structures, and their rupture creates gill slits in the primarily aquatic vertebrates possessing functional gills. In chick, it was shown that branchial membranes also undergo cell interdigitations of the ectoderm and endoderm linings and progress to a single cellular layer that eventually ruptures. However, it was concluded that cellular reorganization rather than massive degradation is the main mechanism responsible for their rupture (Waterman, 1985). Branchial membranes in birds and mammals, nevertheless, perforate only temporarily and are subsequently closed when neural crest mesenchyme cells invade the pharyngeal arches.

Mouth development via stomodeal collar formation

Salamanders

The general pattern of mouth formation in salamanders (urodele amphibians) differs significantly from those of the above-mentioned vertebrates (Fig. 2). The oral area initially consists of a double-layered ectoderm, while the inner region of the prospective mouth is filled with a compact mass of 'oral endoderm' (Fig. 3A). The stomodeum with a well-defined lumen does not develop, and only a shallow groove is visible externally (Takahama et al. 1988). Mouth

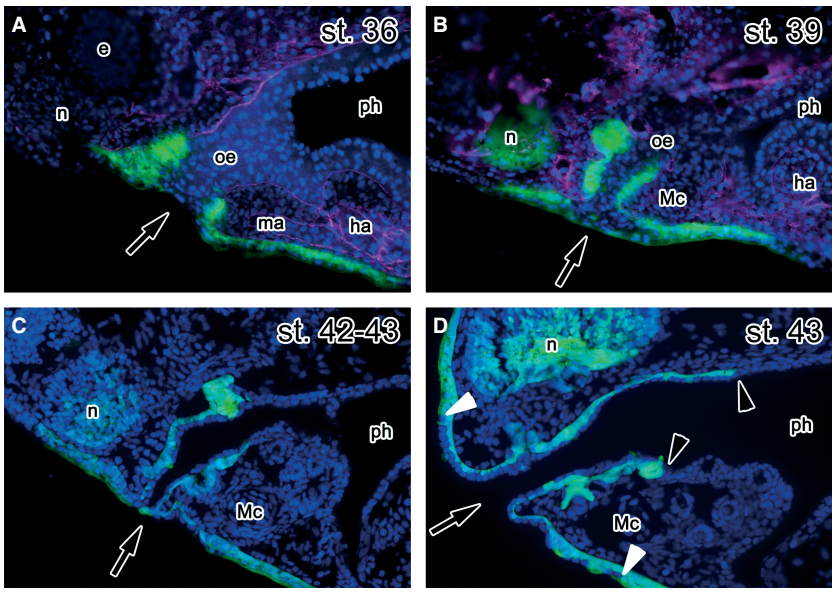

Fig. 3 Details of the stomodeal collar formation during mouth development in axolotl. The prospective oral ectoderm (green channel) was transplanted at early neurula stages from a GFP-transgenic embryo (Sobkow et al. 2006) and fate-mapped during the course of later embryonic development (see Soukup et al. 2008 for a transplantation assay). The magenta channel in (A) and (B) displays basal laminae (fibronectin). Sagittal sections, anterior to the left, black arrows point to the prospective or formed oral opening. (A) Early formation of the stomodeal collar. The outer ectoderm layer covers the oral region, while the inner layer involutes and becomes the basal layer of the future oral cavity. (B) A stage with a well-formed stomodeal collar. (C) Embryo with an almost opened mouth. Prospective oral cavity forms as a cleft inside the oral endoderm mass (oe in A and B), and separates the upper and lower jaws. (D) Open mouth stage. Note the anterior oral endoderm cells that burst outside the mouth and contribute to the lips and adjacent epidermal covering (white arrowheads). Black arrowheads point to the ectoderm internal limits of the former stomodeal collar, now a part of the basal layer of the oral cavity lining. e, eye; ha, hyoid arch; ma, mandibular arch; Mc, Meckel's cartilage; n, nasal epithelium; oe, oral endoderm; ph, pharyngeal cavity. Staging after Bordzilovskaya et al. (1989).

development starts when the inner (basal) layer of the formerly double-layered oral ectoderm undergoes involution and migrating ectodermal cells gradually cover the oral endoderm mass as a 'sleeve' forming the so-called stomodeal collar (Figs 2 and 3A,B; Adams, 1924; Reisinger, 1933; Soukup et al. 2008). No basal lamina is found between the outer ectoderm layer and the oral endoderm in the oral area at this time, but distinct basal laminae are shared by the inner ectoderm layer and the oral endoderm cells separating the oral epithelia from the surrounding head mesenchyme (Fig. 3A,B).

Opening of the mouth occurs as a result of the formation of small cavities that arise among the initially compact oral endoderm mass and that fuse to finally form walls of the future mouth. Degenerating cells were observed only infrequently and only in the oral ectoderm, suggesting that a process of active remodelling rather than cell death is responsible for opening of the mouth (Takahama et al. 1988). None of the previous researchers observed a structure reminiscent of a double-layered oral membrane (Kingsley & Thyng, 1904; Greil, 1905; Johnston, 1910; Landacre, 1921; Adams, 1924, 1931; Marcus, 1930; Reisinger, 1933; Ströer, 1933; Balinsky, 1947; de Beer, 1947; Chibon, 1970), but Takahama et al. (1988) proposed that the oral membrane might be represented by the outer layer of the oral ectoderm together with the entire solid oral endoderm mass. The process of horizontal cleft formation was then compared with the rupture of a typical oral membrane. In the Mexican axolotl, it seems that the oral membrane does exist as a double-layered anterior structure composed of the outer ectoderm and the oral endoderm linings (Fig. 4); it represents only a transient structure and it is unique on account of its very superficial position: it connects the upper lip epithelium to the lower jaw even anteriorly to the lower lip (Fig. 4D). Such an external position of the oral membrane in salamanders may on the other hand explain the presence of the endoderm cells within the lip epithelia and its extent up to the outer head surface (Figs 3 and 4).

Another noteworthy feature of salamander development is that the oral membrane does not always represent the last connection between the upper and lower mouthparts. In some axolotl specimens it was observed that the roof and floor of the mouth cavity are still connected by incompletely detached oral endoderm cells even after perforation of the external oral membrane (Fig. 4E). These cells can eventually take a form of epithelial bridges that, interestingly, were also observed during mouth opening in a basal actinopterygian fish (Kralovic et al. 2010), suggesting that such epithelial connections possibly represent incidental structures that form during the general process of epithelial splitting.

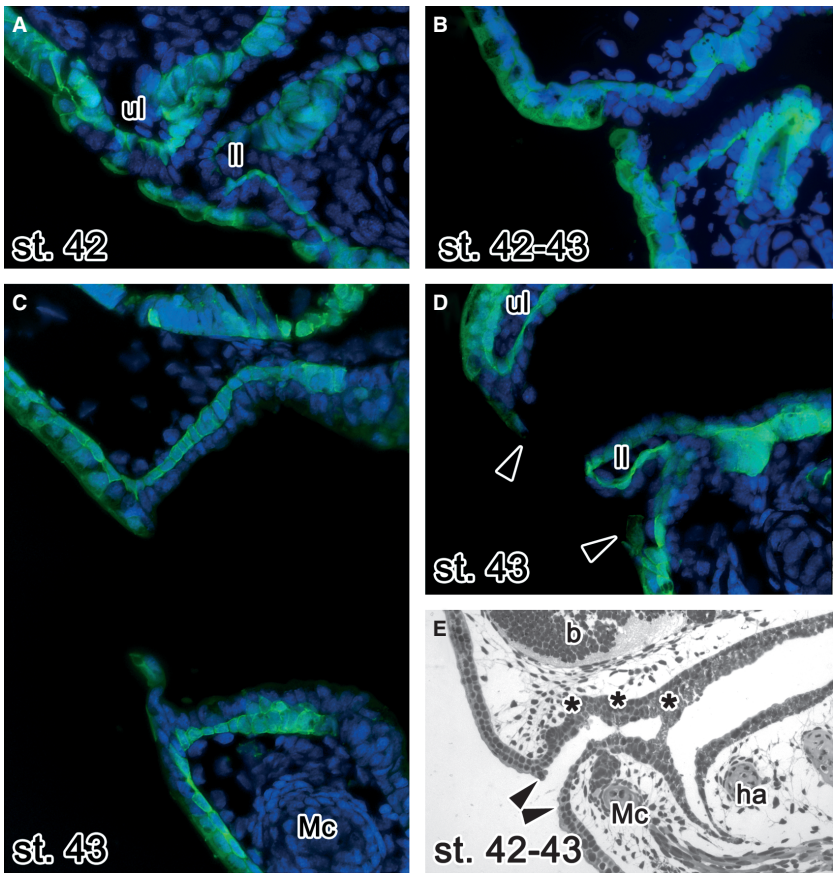

Fig. 4 Opening of the primary mouth in axolotl and perforation of the oral membrane. The oral ectoderm is in the green channel (as in Fig. 3), sagittal sections, anterior to the left. (A, B) The newly identified oral membrane consists of the outer ectoderm layer and the anterior cells of the oral endoderm lining. It is situated superficially at the anterior-most end of the oral cavity as an epithelial connection between the upper lips and the region external to the lower lips. (C) The oral membrane is perforated at stage 43 and its remnants can be observed only temporarily joining the surrounding epithelia. (D) Laterally, the remnants of the oral membrane indicate its former position at the upper, and in front of, lower lips. (E) Histological section of the axolotl with an already ruptured oral membrane but with a not yet fully separated endoderm epithelia inside the mouth, which form epithelial bridges. Arrowheads point to the remnants of the former oral membrane and asterisks mark epithelial bridges. b, brain; ha, hyoid arch; ll, lower lip; Mc, Meckel's cartilage; ul, upper lip.

The distinctive way by which mouth formation is realized in salamanders, consequently also causes an alternative distribution of respective epithelial linings. Generally it is assumed that the oral cavity is lined by the ectoderm epithelium anteriorly and endoderm epithelium posteriorly with a sharp border represented by the oral membrane (e.g. Romer & Parsons, 1986; Kardong, 1995). In salamanders, the posterior part of the oral cavity is indeed lined by endoderm, whereas the anterior mouth lining is composed of cells of dual germ-layer origin: the ectoderm basal layer (former stomodeal collar) and the endoderm apical layer (former solid oral endoderm mass; Figs 3 and 4). The ectoderm–endoderm border zone in salamanders is consequently rather complex, comprising the previous extent of the stomodeal collar together with the above-described oral membrane.

Mouth development via stomodeal collar in other vertebrates

Mouth formation via developmental stages analogous to the stomodeal collar has also been reported for lungfish embryos, which, as in salamanders, have their oral region plugged by a mass of yolk-laden oral endoderm cells. In lungfishes, this foregut endoderm mass contacts the double-layered ectoderm, which successively takes a form of a shallow stomodeal plate (Kerr, 1902, 1910; Greil, 1913). Based on histological evidence, it was reported that the inner ectoderm layer disappears from the contact zone with the oral endoderm and, by the tailbud stage, it forms a continuous sheet with cells of the basal oral endoderm (Kerr, 1902, 1910; Greil, 1913). The remaining apical ectoderm layer covering the oral endoderm was described as diminishing shortly before the opening of the mouth, leaving the underlying endoderm cells exposed to the external surface (Kemp, 2002). The oropharyngeal cavity is then opened by a horizontal cleft that forms inside the oral endoderm mass and spreads from behind. Interestingly, the yolk-laden endoderm cells can be found at the very tips of the mouth, suggesting a substantial endoderm contribution to the oropharyngeal cavity in lungfishes (Kemp, 2002), a situation equivalent to that in salamanders (Soukup et al. 2008).

Comparable developmental morphogenesis of the oral epithelia forming a stomodeal collar-like structure instead of a clear stomodeum was also reported for the Tailed Frog *Ascaphus truei* (Reiss, 1997), which belongs to the basalmost anuran lineage. In this species with an unusual ventrally placed sucker mouth, the stomodeum is shallow and ventrally placed. The outer layer of the oral ectoderm contacts a ventral part of the flattened anterior mouth endoderm mass, while the inner ectoderm layer expands posteriorly on the endoderm dorsal surface. Moreover,

dense ectoderm bands were also found running along the endoderm wall from the corners of the mouth (Reiss, 1997). Interestingly, for the Agile Frog (*Rana dalmatina*), oral formation was described as developing via the stomodeal collar-like structure with some ectoderm bands running inside the mouth over the foregut endoderm (Reisinger, 1933). Moreover, the same author observed that at least some ectoderm cells invade the mouth in the case of the Midwife Toad (*Alytes obstetricans*) in a salamander-like manner, aside from the fact that its oral formation otherwise develops via a definitive stomodeum and rupture of classic oral membrane. Similarly to the situation in lungfishes, all these intriguing pieces of data are derived from histological descriptions and, thus, caution should be taken when drawing decisive conclusions.

Mouth development via ectodem wedge and detachment of initially compressed oropharyngeal epithelia

In the ray-finned fishes (Actinopterygii), mouth formation is strongly influenced by the fact that the whole oropharyngeal region is mechanically constrained by the developing brain dorsally and by the yolk sac and pericardium ventrally, leaving rather limited space for the oropharyngeal structures. In zebrafish, a recent paradigmatic fish model system, the earliest described stage of mouth development involves a wedge formed by several ectoderm cells beneath the cranial end of the head merged with the anterior part of unicellular archenteric endoderm layer (Fig. 2; Waterman & Kao, 1982; Warga & Nüsslein-Volhard, 1999; Wallace & Pack, 2003). From this initial contact the oral ectoderm begins to form a small stomodeum, which enlarges and deepens by separation of the ectoderm wedge cells that, however, are not clearly separated from the endoderm lining. The border between alternate oral epithelial linings is hindered by the absence of a distinct basal lamina, which probably dissolves already at earlier stages (Senior, 1909; Waterman & Kao, 1982). The single-layered oropharyngeal epithelium further undergoes tubulation by ventromedial folding and fusion of its lateral edges, finally forming a squeezed tube with no lumen (Senior, 1909; Edwards, 1929; Sucré et al. 2009). The lumen of the oropharynx appears when roof and floor oral epithelia start detaching from each other leaving tenuous epithelial connections between them. In other words, small cavities arise among oropharyngeal epithelia, merge and enlarge to finally develop into lumen of the oropharynx. Opening to the outer environment is therefore achieved by the breaking down of various epithelial bridges instead of perforation of a single and definitive oral membrane. In zebrafish, however, some of the last epithelial connections can be found at the posterior-most stomodeum (Waterman & Kao, 1982), but whether these represent the ectoderm–endoderm bordering zone and thus are comparable to an oral membrane remains to be determined.

Importantly, early development of endoderm foregut lining is radically different in teleosts on one hand, and the non-teleost actinopterygians on the other (with *Amia* representing an intermediate state; see Nelsen, 1953; Cooper & Virta, 2007). In the first case, a single-cell-thick endoderm lining arises during gastrulation, forming a squeezed tube with no lumen, as described above for zebrafish. The foregut lining of bichirs, sturgeons, paddlefish and gars arises, on the other hand, from a widely hollowed archenteric endoderm, similar to the foregut formation in amphibians (Kerr, 1907; Detlaff et al. 1993). This feature undoubtedly represents an ancestral condition for the ray-finned fishes, and the accelerated development of brain together with a massive yolk-ball is one of the characteristics of teleost lineage. Aside from this, a general mode of the oropharyngeal cavity formation comprising detachment and separation of the oropharyngeal epithelia and formation and subsequent rupture of the epithelial bridges between them was also observed in the Senegal bichir (*Polypterus senegalus*; Kralovic et al. 2010), which appears among the basal-most actinopterygian clades. This mode of mouth development might therefore be regarded as a blueprint for all ray-finned fishes.

Noteworthy epithelial rearrangements have been reported to occur during and after perforation of the mouth and also gill slits in carp (*Cyprinus carpio*; Edwards, 1929), a close relative to zebrafish. In the region between the hyoid and first branchial arch, the lateral head ectoderm was observed to contact the pharyngeal endoderm by a wedge of cells in a similar way to that described for the zebrafish oral region. However, before opening of the first gill slit, the ectoderm wedge cells push themselves between the apical sides of the compressed endoderm epithelial linings. The ectoderm cells were seen to populate the whole pharyngeal cavity when the hyobranchial gill slit became open. According to Edwards (1929), this cell behaviour should take place in the oral region as well. The resulting oropharyngeal epithelium should, therefore, be of double-germ-layer origin with ectoderm squamous cells apically and endoderm columnar cells basally.

Interestingly, a completely different situation regarding oropharyngeal epithelial morphodynamics was reported for another teleost fish, *Pterophyllum scalare*, a derived teleost and distant relative to the carp. According to Colle-Vandevelde (1966), the apical layer of the double-layered ectoderm oral epithelium stays in contact with the underlying pharyngeal endoderm lining, together forming the oral as well as branchial membranes. The basal ectoderm was depicted as passing over the membranes and becoming fused with the basal layer of pharyngeal endoderm. These morphodynamics should consequently lead to oral linings of double-germ-layer origin but with the apical layer formed by endoderm and the basal layer by ectoderm, i.e. differing from the carp (Edwards, 1929), but comparable to salamanders (see above).

Mouth development in agnathans

Agnathans (jawless fishes) have been, and still are, recognized as among the most important animals for understanding vertebrate evolutionary history (e.g. Janvier, 1996; Mallatt, 1996; McCauley & Kuratani, 2008). Absence of the jaw is regarded as a primitive trait for vertebrates, and thus these animals are expected to exhibit other primitive character states as well. Lampreys and hagfishes, modern agnathan representatives, were a key subject of the earliest embryology research (reviewed by Gorbman, 1997; Kuratani et al. 2001; Ota & Kuratani, 2006; Richardson et al. 2010), but whilst lampreys recently reached the level of model animals in evolutionary and developmental biology (e.g. Nikitina et al. 2009), hagfish embryonic material has not been accessible until only very recently and still is very deficient (Ota & Kuratani, 2006; Ota et al. 2007). Consequently, hagfish embryology remains very incompletely known. Moreover, the phylogenetic relationship between lampreys and hagfishes is still not completely resolved. They either form a monophyletic clade Cyclostomata that represents a sister group to Gnathostomata (the 'cyclostome hypothesis' currently supported mostly by molecular data: Mallatt & Sullivan, 1998; Kuraku et al. 1999; Delarbre et al. 2002), or, according to the 'craniate hypothesis' (supported mostly by morphological arguments: e.g. Løvtrup, 1977; Janvier, 1981, 1996; Donoghue & Sansom, 2002; Gess et al. 2006; Near, 2009), hagfishes represent a separate clade, a sister group to vertebrates (i.e. gnathostomes plus lampreys). The lamprey primary mouth clearly forms via a deep stomodeum and rupture of the oropharyngeal membrane, and bears a resemblance to the main mode of the gnathostome development, whereas hagfish embryos probably develop their primary mouth in a strikingly different manner. Because of both taxonomic and developmental uncertainties, we deal with lampreys and hagfishes separately.

Lampreys

The development of the oral region in the ammocoete larvae differs from that in gnathostomes (Fig. 1). At first, a thickened ectoderm anterior to the forming stomodeum develops into a nasohypophyseal plate, which gives rise to the prospective adenohypophyseal and olfactory placodes (Honma et al. 1990; Kuratani et al. 2001). The nasohypophyseal plate stays tightly connected to the forebrain and is passively brought to the top of the head by an extensively growing upper lip, which separates the nasohypophyseal plate from the stomodeum and forms a prominent part of the lamprey head (for a review, see Kuratani et al. 2001). The lamprey nasohypophyseal plate thus develops outside the prospective mouth whereas, in gnathostomes, the nasohypophyseal complex is separated into the nasal and adenohypophyseal placodes, where the latter is incorporated into the stomodeum. The expansion of the oral ectoderm forming the stomodeum and its independence from the adenohypophyseal anlage probably represents the most profound difference between the lamprey and gnathostome early oral development (e.g. Romer & Parsons, 1986; Kuratani et al. 2001).

The stomodeum in the lamprey is formed by the relatively deeply invaginated single-layered oral epithelium, which at the posterior blind end abuts against the single-layered pharyngeal endoderm together forming an oral membrane (Fig. 5). Because the stomodeum primarily forms on the ventral side of the head, is rather deep, and the foregut lining reaches the anterior notochord dorsally, the oral membrane has a large extent (Fig. 1; compare agnathans and gnathostomes). The oral membrane is finally broken through and the resulting vertical slit is flanked by extended velar outgrowths. The velum, which develops in a position of former oral membrane, finally forms a specialized structure consisting of paired muscular flaps, and internal and external velar bars in each flap (Mallatt, 1996; Kuratani et al. 2001).

Hagfishes

Our understanding of the development of mouth and oral cavity in hagfishes is very fragmentary and controversial. The controversy stems from the fact that, until recently, all information on hagfish development arose from just a few reports more than 100 years old (Dean, 1899; von Kupffer, 1899, 1900, 1906; Stockard, 1906). Moreover, the original histological sections were redrawn schematically (von Kupffer, 1899), re-examination of the same embryos lead to different interpretations (Gorbman, 1983; Gorbman & Tamarin, 1985, 1986), and confusion was extended by further schematization in the secondary literature (see reviews by Gorbman, 1997 and Ota & Kuratani, 2006, 2008, the only recent authors who succeeded in re-examination of hagfish development with a new embryonic material). Regardless of uncertainty in developmental characteristics of the hagfish oropharyngeal region, the results can be summarized as follows.

The early hagfish embryo is represented by flat layers of epidermal, neural and endodermal tissues lying on a large yolk-ball. At the developmental stage when the head process starts to arise, the foregut endoderm forms a flattened tube with a lumen arising at its anterior-most portion. This archenteric space further progresses posteriorly and, thus, the anterior mouth–nasopharyngeal area comes into continuation with the pharyngeal cavity. Then, however, the nasopharyngeal canal is separated from the oropharyngeal cavity when the proliferating neural crest mesenchyme forms the nasopharyngeal septum as a part of the secondary mouth, a process comparable to the lamprey situation (Fig. 5). In the hagfish, however, the now separated spaces do not yet open to the exterior, and thus the prospective oral cavity, olfactory epithelium and adenohypophysis arguably arise from endoderm (Gorbman, 1983). It is only at a rather late stage of hagfish mouth formation that the

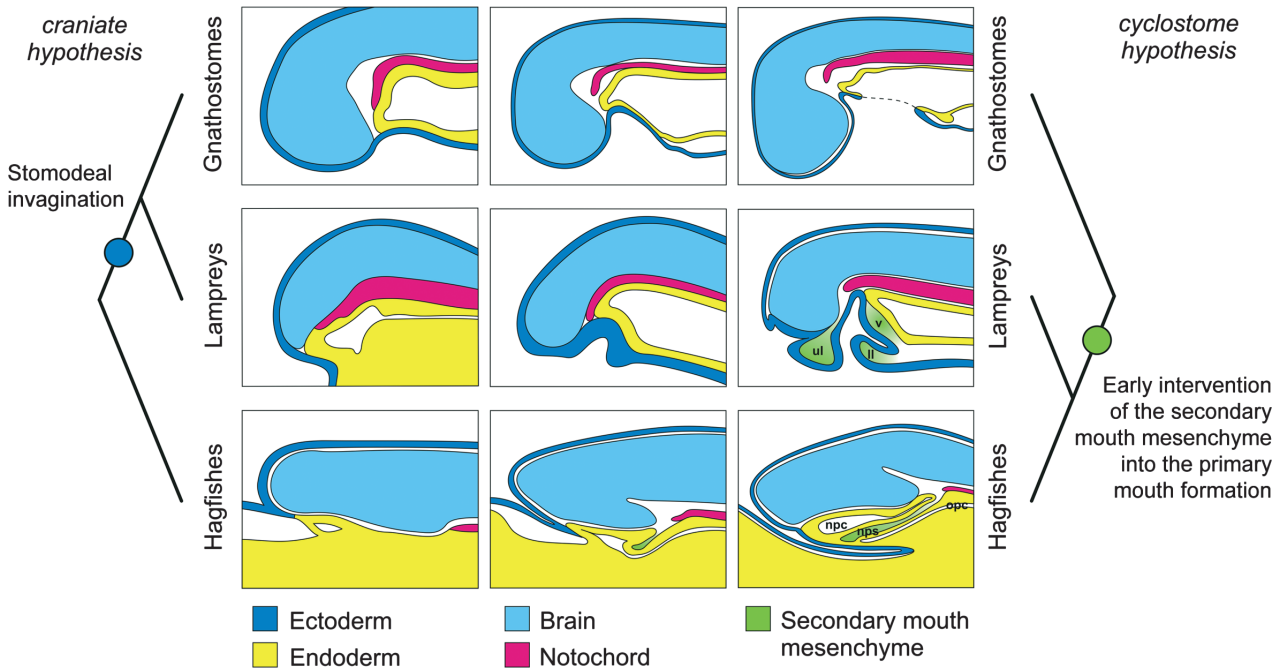

Fig. 5 The main mode of primary mouth formation in gnathostomes (shark) compared with mouth formation in lampreys and hagfishes. The sagittal plane is shown, anterior to the left. The shark illustrates mouth formation via stomodeal invagination and perforation of the oral membrane. In lampreys, similar morphogenesis occurs including deeply invaginated stomodeum, but the forming oral membrane is made complex by the velum, which represents a part of the secondary mouth. This holds true for hagfishes as well. Here, however, the primary mouth formation arguably appears entirely in the endoderm domain, with ectoderm reaching this area rather late via the subcephalic cleft. The forming oral membrane later perforates to open the separate oropharyngeal and nasopharyngeal cavities. ll, lower lip; npc, nasopharyngeal cavity; nps, nasopharyngeal septum; opc; oropharyngeal cavity; ul, upper lip; v, velum.

forward growth of the head process brings the subcephalic ectoderm into contact with the anterior endoderm, forming an oral membrane, which later perforates to open the oropharyngeal and nasopharyngeal cavities to the outer environment. Whether anything like stomodeal invagination known from gnathostomes and lampreys also exists in hagfishes and what would its relationship be to the subcephalic cleft ectoderm remains to be elucidated.

von Kupffer (1899, 1900, 1906), however, hypothesized that, in hagfish, the oral membrane, comprising the subcephalic ectoderm and the foregut endoderm linings, disappears before the formation of the nasopharyngeal septum and new secondary membranes develop during septal morphogenesis. Yet, regardless of improbable appearance of the secondary ecto-endodermal membranes, not known in any chordates, no further studies in hagfish development provided any empirical support for the above hypothesis (Dean, 1899; Stockard, 1906). Gorbman (1983) argues that von Kupffer (1899), who recognized the endoderm derivation of this area, was apparently troubled by it and tried to explain it by formulating a hypothesis involving an early ectoderm invagination followed by secondary closures of both nasopharyngeal and oropharyngeal tubes. Re-examining the then available embryonic material, Gorbman (1983, 1997), however, concluded that 'there is no evidence that the stomodeal and nasopharyngeal spaces were ever open prior to the stage shown in von Kupffer's figure.' Moreover, Gorbman (1983) also provides convincing histological evidence for the immediate topographic context of the infundibular evagination of the brain and a thickened cellular layer of the endoderm. The sole up-to-date reports on hagfish development (Ota et al. 2007, 2011; Ota & Kuratani, 2008) do not provide any details on the matter. Hence, until new data appear, we tentatively propose to follow the above-mentioned conclusions by Gorbman (1983, 1997) as a default view of the primary mouth formation in hagfish.

This 'default' view suggests considerable differences in developmental dynamics between the two agnathan groups and, at the same time, distinct differences between hagfish and other vertebrates, lampreys included (Fig. 5); it predicts that hagfishes undergo disparate and quite unique oropharyngeal morphogenesis when compared with other vertebrates. The primary mouth formation appearing entirely in the domain of endoderm developmental dynamics without formative intervention of the ectoderm placodal patterning would provide one of the strongest arguments for a separate position of hagfishes and for the 'craniate hypothesis' of vertebrate phylogeny.

It is worth mentioning that both groups exhibit certain similarities in development of the secondary mouth structures that apparently arise before the formation of the oral

opening, i.e. at the stage when the primary mouth still undergoes development. Namely, this involves the nasopharyngeal septum in hagfish and velum in lampreys augmenting the ectoderm–endoderm contact zone by their growth. Although in hagfish and lamprey the respective structures form in the domain of the mandibular arch, their morphogenesis differs considerably. The ammocoete velum represents two lateral dorsoventrally placed flanks between the oral and pharyngeal cavities, and it is reminiscent of the former position of the oral membrane (Dohrn, 1886; Damas, 1944). The velum of hagfishes, on the other hand, very probably arises within the endoderm lining and forms as two ventral outgrowths from the dorsal pharyngeal wall (von Kupffer, 1900; Stockard, 1906). Yet, the final word on this matter must be postponed; new developmental data on hagfish are needed.

Primary mouth formation from the phylogenetic perspective

Mapping the above-surveyed alternatives of the primary mouth formation onto a phylogenetic tree of vertebrates (Fig. 6) reveals several intriguing issues.

1. First, a support for the basal divergence of hagfishes and vertebrates (lampreys + gnathostomes) if the 'default view' of the hagfish mouth development (*sensu*; Gorbman, 1983) is accepted. The conditions characterizing the hagfish are: (a) a key role of early differentiation of the anterior endoderm with formation of the endoderm nasopharyngeal cavity and its separation by the nasopharyngeal septum from the ventral endoderm oropharyngeal cavity; (b) delayed persistence of oral membrane without deep stomodeal invagination; (c) and opening of the mouth cavity via rupture of the oral membrane – a character shared with most of the other vertebrates.

2. Second, lampreys exhibit essential differences with respect to gnathostomes, particularly in: (a) a strict separation of stomodeal ectoderm from the nasohypophyseal plate containing olfactoric and adenohypophyseal placodes, which form a common developmental unit retaining its primary topographic position during evolution. The topographic separation of stomodeal region from the nasohypophyseal plate in lamprey resembles the situation in the hagfish, though these clades supposedly differ both in origin and developmental dynamics of these structures. The lamprey corresponds to gnathostomes, or the hypothetical gnathostome ancestor, in (b) a deep stomodeal invagination resulting in a bilaminar oral membrane.

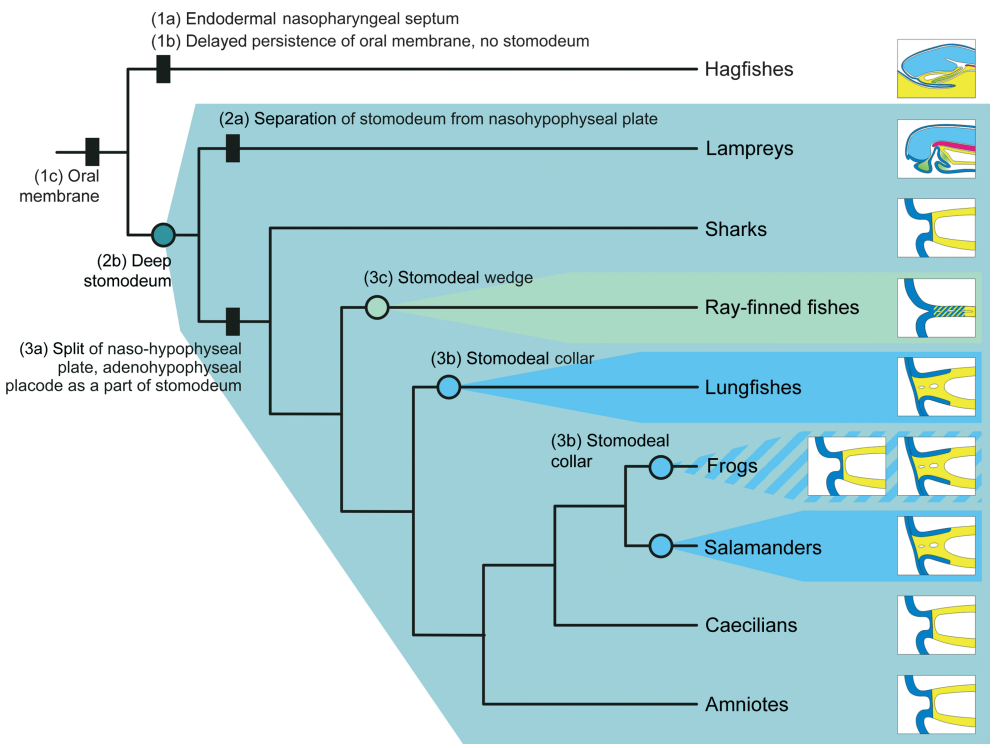

Fig. 6 Phylogenetic distribution of the primary mouth formation characteristics. Phylogenetic relationships after Near (2009), where the 'craniate hypothesis' is preferred with hagfishes as a sister group to lampreys + gnathostomes. See the text for coding of the characters. Opening of the primary mouth via rupture of the double-layered oral membrane (character 1c) is an ancient plesiomorphic character of craniates. Stomodeal invagination (character 2b) is probably apomorphic for lampreys and gnathostomes, while it was further modified once into the stomodeal wedge (in the ray-finned fish lineage and most notably in teleosts, character 3c) and separately several times into the stomodeal collar (in lungfishes, salamanders and some frogs, character 3b).

3. Third, because the arrangements corresponding to character (2b) appear in the vast majority of vertebrate clades, we propose it a synapomorphy of vertebrates and a plesiomorphy (ancestral state) of gnathostomes. The major apomorphy of gnathostomes is then: (a) a separation of the nasohypophyseal plate and incorporation of the adenohypophyseal anlage into the expanding stomodeal field. Besides the common mode of primary mouth formation in gnathostomes (i.e. characters 1c + 2b + 3a), at least two other modes evolved: (b) a process of stomodeal involution followed by formation of the stomodeal collar in salamanders, lungfishes and some frogs; and (c) an intimate connection between ectoderm and endoderm (stomodeal wedge), with a subsequent dissociation of roof and floor oropharyngeal epithelia in ray-finned fishes. The extent of variation and taxon-specific arrangement of the mode of mouth formation among ray-finned fishes are unfortunately largely unknown, although this lineage contains half of all vertebrate species. However, despite the scarcity of information, the character (c) has probably been acquired already at the base of the ray-finned fish clade.

Interestingly, character (3b) probably evolved several times independently in lineages leading to lungfishes, salamanders and/or frogs (Fig. 6) and, thus, represents a convergently acquired homoplastic trait. All these lineages undergo comparable morphogenesis of the pharyngeal region (including extensive development of branchial arches or larval external gills), and their early ontogeny (namely the content of yolk, gastrulation or endoderm formation) is markedly alike. We therefore expect that the respective mode of primary mouth formation is influenced by a set of various contextual factors, one of them evidently represented by the amount of yolk and by spatial and molecular settings of the early developing embryo, as discussed below.

General patterns of gastrulation and particularly the yolk content of embryos prefigure modes of primary mouth development

In the previous section, three main modes of mouth formation were identified for vertebrates (Fig. 2). In order to reveal factors determining and constraining the mouth in development and evolution, the formation of prospective oral regions was followed from the time of gastrulation, and developmental correlates of mouth morphogenesis were explored (Fig. 8; Table 1).

During the process of gastrulation, chordate embryos must bring their endoderm into the future anterior region so that a direct contact zone between the ectoderm and endoderm can be established for the prospective mouth formation (Fig. 1). However, particular patterns of endoderm formation vary and strongly depend upon the amount and positioning of yolk in eggs and embryos. Therefore, in groups, where the yolk areas are fully internalized into the embryo during gastrulation and where the whole egg cytoplasm is cleaved (mesolecithal eggs, holoblastic development), the endoderm is formed with a distinct archenteron, a situation seen in amphibians, basal ray-finned fishes, lampreys or lungfishes (Table 1). The archenteric cavity then progresses into the lumen of the whole alimentary canal, and its anterior-most lining directly contacts the surface ectoderm. This contact demarcates the position of future mouth that opens anteriorly. Alternatively, in those groups, where the yolk (vegetal) part of eggs is uncleaved and serves for nutrition only, the embryo develops from the cleaved animal part itself (telolecithal eggs, meroblastic development), no foregut cavity forms initially, but a compact archenteric layer or archenteron mass develops in the anterior part of the head instead. The foregut cavity has to be formed secondarily (Nelsen, 1953), for example, as described above for teleosts, via ventromedial bending of the lateral edges of the foregut endoderm sheet and their subsequent fusion into a tube. This situation is known for hagfishes, sharks, teleosts and amniotes.

Both protochordate groups (cephalochordates and urochordates) undergo embryonic development from small eggs with a minimum amount of yolk and equal holoblastic cleavage, which is in strong contrast to the vertebrate embryos where such a situation is achieved only secondarily in placental mammals. Vertebrates, on the contrary, display increased maternal investments into their offspring by massive deposition of yolk into the eggs (Takeuchi et al. 2009), which necessarily leads to different cleavage and gastrulative cellular behaviour. These two processes are then exemplified either by unequal holoblastic cleavage with an amphibian-type gastrulation, or meroblastic cleavage with an amniote-type gastrulation. It is beyond the scope of this paper to discuss the archetypal condition for vertebrates in detail as both conditions may, based on the distribution on the cladogram, represent an ancestral character state. However, the holoblastic development is generally regarded plesiomorphic for vertebrates (Collazo et al. 1994; Takeuchi et al. 2009).

Development of the primary mouth via rupture of the oral membrane, which is preceded by either deep or shallow stomodeal invagination, is found in embryos with both amphibian-type (lampreys, frogs and caecilians) and amniote-type (hagfishes, sharks and amniotes) gastrulation (Table 1). These two types seem to differ in the amount of yolk platelets in the oral endoderm cells – while hagfish, shark and amniote oral endoderm is yolk-free, that of lampreys, caecilians and frogs is composed of yolk-rich cells, which clearly represent an obstacle for further morphogenesis of the primary mouth. Yet, in the majority of the cases, the oral membrane is composed of a very thin endoderm lining, sometimes even organized into a single-cell-thick layer, as exemplified in lamprey. A noteworthy feature of all these embryos (except for anurans) is an early and exten-

Table 1 Correlations between the modes of primary mouth formation and some key features of embryonic development in individual vertebrate lineages.

		mode of mouth formation	endoderm during gastrulation	position of embryo	head process
amniotes	mammals	stomodeum	archenteric layer	yolk stalk	yes
	birds	stomodeum	archenteric layer	yolk stalk	yes
	reptiles	stomodeum	archenteric layer	yolk stalk	yes
amphibians	frogs	stomodeum collar	archenteron	yolk internalized	no
	salamanders	collar	archenteron	yolk internalized	no
	caecilians	stomodeum	archenteron	yolk internalized	yes
lungfishes		collar	archenteron	yolk internalized	no
ray-finned fishes	teleosts	wedge	archenteric layer	lying on yolk	no
	non-teleosts	wedge	archenteric layer archenteron	lying on yolk yolk internalized	no
sharks		stomodeum	archenteric layer	yolk stalk	yes
agnathans	lampreys	stomodeum, velum	archenteron	yolk internalized	yes
	hagfishes	no stomodeum, velum	archenteric layer	yolk stalk	yes

The comparative features of the mode of primary mouth formation include: first, endoderm during gastrulation, which can primarily represent a hollow pocket with an archenteron or a single sheet archenteric layer (hypoderm in amniotes); second, position of embryo and its connection to the yolk, which can be manifested in three ways – the yolk can be internalized and become a part of the embryonic body, the embryo can be connected to the yolk-ball by a yolk stalk, or the embryo can lie directly on the yolk-ball without a yolk stalk; and third, head process, which may or may not arise from the main yolky trunk region during early embryonic development.

sive growth of the head process, which removes the prospective primary mouth far from the trunk. We speculate that this morphogenetic event enables development of the primary mouth at a distance from the yolk-rich trunk region and a concurrent participation of only a small number of endoderm cells (Fig. 7). Indeed, this mode of primary mouth formation, although modified in terms of position and development, is found in urochordates as well as in cephalochordates (Fig. 1), and represents a plesiomorphic condition for vertebrates (Legros, 1898; Göppert, 1906; Manni et al. 2005; Veeman et al. 2010). Within vertebrates, such a mode of primary mouth formation represents a ground state condition from which the other modes are derived.

One such derived mode is the formation of primary mouth via the stomodeal collar and horizontal detachment of oropharyngeal epithelia, which occurs in lineages with embryos undergoing holoblastic cleavage and amphibian-type gastrulation; more specifically, in those lineages where the primary mouth development does not correlate with the formation of a distinct head-fold during early embryogenesis (Fig. 7; Table 1). The oral region is meanwhile filled by a mass of yolk-laden cells, which resides within the mandibular arch domain and abuts the oral ectoderm anteriorly. On account of the accumulation of oral endoderm, no deep invagination forming a stomodeum can develop and, consequently, the ectoderm cells tend to undergo involution in a form of deep epithelial layers (Fig. 3). Shallow stomodeum and multilayered oral endoderm can be found also in *Xenopus* (Dickinson & Sive, 2006), and it should be emphasized that the default classic mode of mouth formation via invaginating stomodeum and rupture of the oral membrane might not be present in all anuran species and that, instead, involution of the inner ectoderm layer and the presence of the stomodeal collar-like structure might be a more common feature than recently appreciated. Morphogenesis of the stomodeal collar apparently represents an alternative way to bring the ectoderm inside the mouth in those lineages, where yolk cells were deposited within the oral region and thus block the formation of deep stomodeal ectoderm invagination.

Meroblastic development often leads to embryos consisting of almost transparent sheets and layers (in hagfishes,

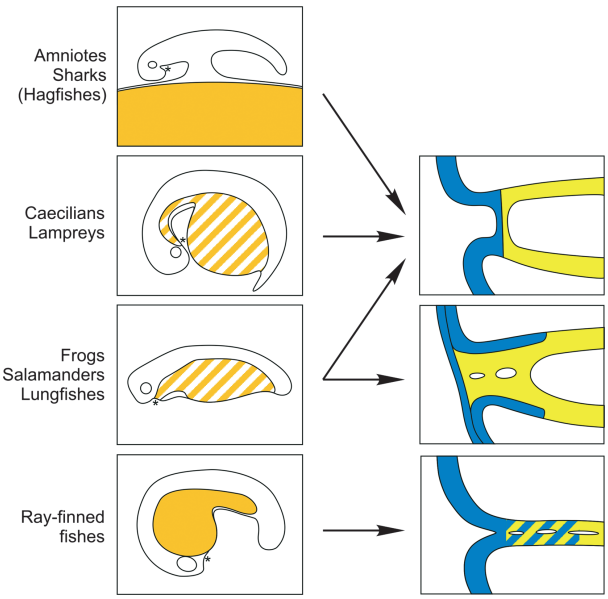

Fig. 7 Relationship between embryonic development, amount of yolk and primary mouth formation. Position of the primary mouth is marked by an asterisk. The amniote, shark and hagfish embryos develop separately from the yolk-ball (dark yellow), and their oral region is situated in a distinct head process far from the yolk. In caecilians and lampreys, the yolk is internalized during gastrulation and all endoderm cells contain yolk platelets (dark yellow/white hatching). As in the previous case, however, the primary mouth is situated in a distinct head process. In frogs, salamanders and lungfishes, the yolk is internalized, but the head develops adjacent to the trunk region without a distinct head process. All the above-mentioned lineages, except for salamanders and lungfishes, develop their primary mouth via stages of stomodeal invagination and rupture of the oral membrane (Fig. 2). In salamanders, lungfishes and some frogs, however, the primary mouth forms via stages of stomodeal collar development and later horizontal detachment of the oropharyngeal epithelia. Ray-finned fish embryos develop their oral region in close proximity to the yolk, and no head process is apparent at early stages. Their primary mouth develops via stages of stomodeal wedge formation and horizontal detachment of the oropharyngeal epithelia due to the extremely high amount of yolk and general compression of the oropharyngeal region.

sharks and amniotes), or to development of extensive extra-embryonic tissues covering the yolk. In these embryos, the developing head is already elevated above the yolk at early stages of development, or the embryo is separated from the yolk-ball by a distinct yolk stalk (Fig. 7; Table 1). This, however, does not hold true for the teleost fishes, which undergo meroblastic cleavage of the egg and rather similar embryogenesis. However, their embryonic axis lies directly on the yolk-ball and the oropharyngeal region is pressed in between the brain, the yolk and the heart. Moreover, teleost fishes are the only vertebrate group where meroblastic development is not correlated with the obligate enlargement of the egg volume (Collazo et al. 1994). This leads to the formation of compact mass of tissue especially in the head. Consequently, the oropharyngeal region is dorsoventrally flattened from the very beginning, and its cavitation occurs only later by horizontal detachment of the oropharyngeal epithelia and without a discernible oral membrane. In fact, the presence of the ecto-endoderm oral membrane has not been decisively described in any teleost species, yet. The overall organogenesis of the oropharyngeal region in teleosts thus supposedly represents a morphogenetic response to the mechanically constraining environment, where this region resides. Yet, the apomorphic situation of teleosts, which represents an evolutionary trend towards meroblastic cleavage and the succeeding formation of endodermal sheet during gastrulation (epiboly), evolved from the holoblastic cleavage and consequent internalization of the endoderm with a fully formed archenteric cavity. This can be exemplified in basal ray-finned fishes, and might be regarded as halfway between the holoblastic and meroblastic development (Collazo et al. 1994; Cooper & Virta, 2007). In this sense, the basal-most lineages, the bichirs and sturgeons, undergo early embryogenesis comparable to amphibians with formation of the archenteric cavity (Kerr, 1907; Detlaff et al. 1993; Diedhiou & Bartsch, 2009). Interestingly, although in the basal-most ray-finned fish lineages the anterior wall endoderm initially contacts the oral ectoderm in a way suggestive of primary mouth formation via stomodeal invagination and oral membrane rupture, the ecto-endoderm contact zone is subsequently gradually dorsoventrally compressed into a thin sheet, and the primary mouth opens via detachment of the roof and floor epithelia similar to the situation in teleosts (Kerr, 1907; Kralovic et al. 2010).

Molecular regulation of the primary mouth formation

Specification of the anterior neural plate border

Embryonic origin of the prospective chordate primary mouth can be traced back to early neurula stages. The cells fated to become oral epithelia and associated derivatives are located at the anterior-most part of the embryo, where ectoderm and endoderm are directly juxtaposed with no mesoderm or mesenchyme cells intervening into this contact zone at any time of development. Fate-mapping studies especially in amphibians have identified the prospective primary mouth encompassing the border zone between the transverse neural fold, the associated anteroventral ectoderm and the underlying archenteric endoderm (Soukup et al. 2008; Veeman et al. 2010; Pieper et al. 2011).

Recent studies have identified some of the tissues and factors responsible for patterning of the anterior-most part of the vertebrate embryo. At the end of gastrulation, the whole embryonic ectoderm can be divided into three domains: the neural plate representing the prospective central nervous system; the non-neural ectoderm giving rise to the future epidermis; and the intermediate neural plate

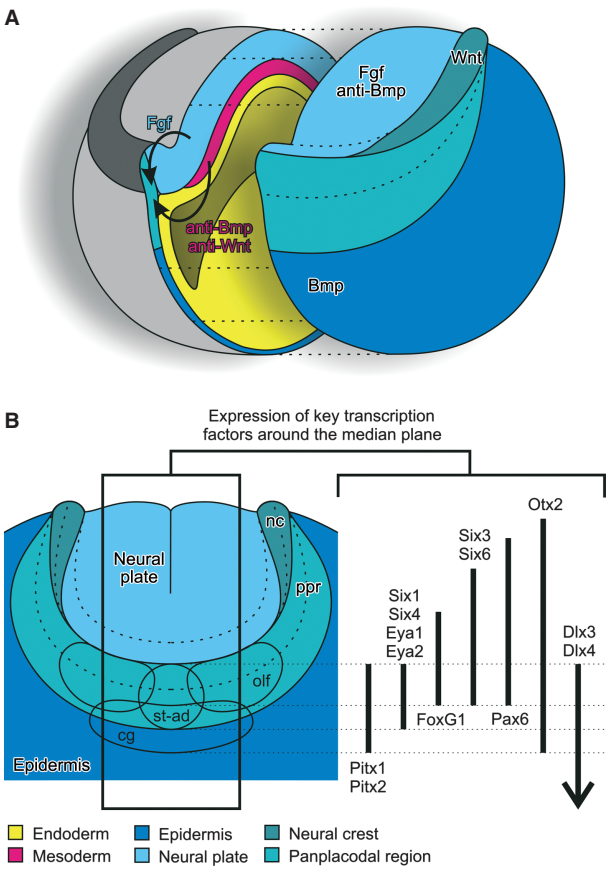

Fig. 8 Induction and molecular specification of the prospective primary mouth region in vertebrates. (A) Anterolateral view of the early amphibian neurula cut through the median plane with the demarcation of endoderm (yellow), mesoderm (red) and ectoderm (blue) regions. The left and right parts show relation of the different ectoderm regions (see the colour code) to the external morphology. The activity of the major signalling pathways is depicted in various regions. The prospective oral ectoderm (part of the pan-placodal region) is specified by a simultaneous upregulation of Fgf signalling from the neural plate and downregulation of Bmp and Wnt signalling by the inhibitors secreted from the cephalic mesoderm. (B) Anterior view at the amphibian neurula (same colour coding as in A). The subregions of the pan-placodal region are not strictly determined yet with occurrence of overlapping areas of several fates. The diagram at the right shows the dorsoventral extent of expression of several transcription factors around the median plane. cg, cement gland; nc, neural crest region; olf, olfactory placode; ppr, pan-placodal region; st-ad, stomodeo-adenohypophyseal placode. Expression data in (B) redrawn from Schlosser (2005, 2006).

border differentiating into neural crest laterally and pan-placodal region anteriorly (Fig. 8). The prospective primary mouth is situated within this horseshoe-shaped pan-placodal region ventral to the adenohypophyseal placode (Pieper et al. 2011). The pan-placodal region is defined by the expression of *Six1/2*, *Six4/5* and *Eya* gene families (Ahrens & Schlosser, 2005; Schlosser, 2005, 2006; Streit, 2007), which are a part of the Six-Eya-Pax-Dach pathway with *Pax* and *Dach* orthologues expressed in individual subregions of this pan-placodal region. The *Six* and *Eya* genes are responsible for multiple functions during the development of placodes, including size regulation, changes in cell shape or cytodifferentiation (Schlosser, 2005, 2006).

The establishment of the anterior neural plate border has been thoroughly studied in *Xenopus* owing to the early emerging cement gland and its unambiguous morphological and molecular discrimination (reviewed in Sive & Bradley, 1996; Wardle & Sive, 2003). The cement gland forms at the contact zone between the anterior neural plate border and non-neural ectoderm, and is influenced by signals from both areas with *Otx2* expressed in the anterodorsal domain and *Bmp4* in the ventral domain of the embryo. Gammill & Sive (2000) proposed that *Otx2* can be expressed only at low and intermediate levels of *Bmp4* gradient, and speculated that on the basis of these combinatorial expression profiles, the anterior neural plate should be specified by *Otx2* at low levels of *Bmp*, the ventral epidermis solely by Bmp signalling and the cement gland together with the pan-placodal primordium from a region where *Otx2* is expressed together with intermediate *Bmp* thresholds.

However, an active modulation of Bmp levels has been found within the pan-placodal region to account for the position where cement gland, placodes and oral epithelium develop, and the role of other co-expressing signals has been emphasized (Fig. 8; Ahrens & Schlosser, 2005; Litsiou et al. 2005). Kwon et al. (2010) have recently shown that Bmp signalling has a dual role: first, it has to be activated for induction of the pre-placodal competence throughout the non-neural ectoderm at late blastula/early gastrula stages (see also Patthey & Gunhaga, 2009); and then, its levels have to be attenuated by expression of its inhibitors at late gastrula stages. At this time, overexpression of *Bmp* blocks expression of the pan-placodal marker *Six1*, while elevated levels of Bmp inhibitors expand *Six1* expression at the expense of the neural crest field (Brugmann et al. 2004; Glavic et al. 2004; Ahrens & Schlosser, 2005). Esterberg & Fritz (2009) have identified *Dlx3b* and *Dlx4b* expressed at the zebrafish neural plate border as key modulators of *Bmp* levels through activation of Bmp inhibitor *Cv2*. This circuit is directly upstream of the pan-placodal specification Six-Eya-Pax-Dach pathway. Recent analysis of protein-binding sites of the Six1–14 enhancer in mouse has suggested Dlx5 as a direct activator, and Msx1 and Pax7 as repressors of *Six1* expression (Sato et al. 2010), further emphasizing the role of *Dlx* genes in establishment of the pan-placodal region. Moreover, although not yet clearly identified, tissue culture experiments in chick have suggested secretion of other Bmp inhibitors not only within the pan-placodal region but also from the prechordal mesoderm (Litsiou et al. 2005). Aside from Bmp inhibition, the *Six1* expression and, thus, specification of the pan-placodal region is further dependent on *Fgf8* from the anterior neural plate (Ahrens & Schlosser, 2005; Litsiou et al. 2005; Kwon et al. 2010). Fgf8

together with Bmp inhibitors, but not Fgf8 alone, are able to induce ectopic *Six1* expression in anteroventral ectoderm, whereas knock-down of Fgf signalling results in loss of pan-placodal markers in *Xenopus* (Ahrens & Schlosser, 2005). Moreover, canonical Wnt pathway has been shown to direct the decision of neural plate border into either pan-placodal or neural crest fates (Brugmann et al. 2004; Litsiou et al. 2005). The Wnt/β-catenin antagonist *Dkk1* secreted from the prechordal mesoderm is required for preventing the formation of neural crest in the transverse neural fold, which is normally fated to become the pan-placodal region (Carmona-Fontaine et al. 2007). Taken together, interplay between activated Fgf and inhibited Bmp and Wnt signalling is a basis for specification of the pan-placodal region and activation of the Six-Eya-Pax-Dach pathway (Fig. 8A). Once specified, the pan-placodal region is further subdivided into distinct cell populations giving rise to a variety of placodes, head epidermis, cement gland and primary mouth ectoderm (Pieper et al. 2011). During the course of embryonic development, the regions giving rise to these organs are first blurred (meaning that a single cell can contribute to several organs), but the respective fates gradually become spatially restricted (Whitlock & Westerfield, 2000; Pieper et al. 2011).

Given that the similar induction and specification mechanisms of the pan-placodal region have been identified in different vertebrate species (*Xenopus*, zebrafish, chick and mouse), the pan-placodal region almost certainly seems to represent a conserved spatiotemporal domain of vertebrate embryonic period (Schlosser, 2006). In urochordates, the vertebrate sister group, *Six* and *Eya* genes are expressed in a similar manner, although they may provide different placode-specific functions (Bassham & Postlethwait, 2005; Mazet et al. 2005; Schlosser, 2007), while in amphioxus, these genes are expressed in endodermal derivatives and the vertebrate type pan-placodal region is not found (Kozmik et al. 2007; Schlosser, 2007). The pan-placodal region thus probably evolved in the common ancestor of vertebrates and urochordates, and represents a defining feature of the taxonomic group Olfactoria.

Specification of the primary mouth and the role of *Pitx* genes in oral development

Although, due to scarcity of detailed comparative information, hypothesizing on the structure of the molecular regulation controlling the primary mouth formation would be premature, some of its components can almost certainly be identified. First, this concerns the essential role of Pitx signalling. As described above, the prospective primary mouth forms from the median portion of the pan-placodal ectoderm and the rostral foregut endoderm. Such an extreme anterior position of the oral region is specifically marked by the expression of the pituitary homeobox (*Pitx*, formerly *Ptx*) genes (Fig. 8B; Dickinson & Sive, 2007). *Pitx* genes belong to a family of paired-like homeodomain class of transcription factors, which includes *Pitx1*, *Pitx2* and *Pitx3* paralogues. These genes can produce a number of different proteins thanks to alternative splicing and alternative translation initiation sites (Gage et al. 1999b; Shiratori et al. 2001; Cox et al. 2002; Angotzi et al. 2008; Lamba et al. 2008). *Pitx* genes are involved in morphogenesis of diverse organs, including eye, brain, heart or limbs, and are responsible for early embryonic patterning and left–right asymmetry (Ryan et al. 1998; Gage et al. 1999a; Lanctôt et al. 1999b; Lin et al. 1999; Lu et al. 1999; Burdine & Schier, 2000; Shapiro et al. 2004). Most notably, however, *Pitx* paralogues are expressed at the anterior part of the pan-placodal ectoderm at neurula stages (Schweickert et al. 2001a; Jaszczyszyn et al. 2007; Angotzi et al. 2008), and their expression is subsequently restricted to the ectoderm anlagen of the developing stomodeum and adenohypophysis and endoderm anterior pharynx. Further on, the *Pitx*-expressing epithelia contribute to vertebrate ectoderm and endoderm derivatives of the oral cavity, like tooth germs, tongue or palate (Lanctôt et al. 1997, 1999a; St. Amand et al. 2000; Fraser et al. 2004).

While the functions of *Pitx* genes during the morphogenesis of organs associated with the mouth are becoming clearer (see below), their roles in the development of primary mouth are still to be elucidated. This might partially be due to the relatively simple morphogenesis of the invaginating stomodeum and consequently a lack of morphologically clearly discernible developmental landmarks when compared with the morphogenesis of, for example, teeth or the adenohypophysis. Therefore, the roles of *Pitx* genes in primary mouth morphogenesis can only be inferred by analogy to their known functions in associated organs.

Functional studies in vertebrates have demonstrated the requirements of *Pitx* genes for the development of the murine adenohypophysis and *Xenopus* cement gland. All three *Pitx* genes are expressed in the *Xenopus* primary mouth and adenohypophysis, while only *Pitx1* and *Pitx2* paralogues can be found in the early developing cement gland (Hollemann & Pieler, 1999; Pommereit et al. 2001; Schweickert et al. 2001b). Overexpression of either *Pitx1* or *Pitx2c* induces formation of enlarged or ectopic cement glands, while more severe phenotypes display reduced trunk and tail regions (Chang et al. 2001; Schweickert et al. 2001b). Double knock-down of both of these genes, on the other hand, inhibits the formation of ectopically induced cement glands (Schweickert et al. 2001b). *Pitx* genes are thus both necessary and sufficient for the induction of *Xenopus* cement gland formation. Concerning the adenohypophysis, different *Pitx* paralogues are required for its development in different vertebrates, showing conserved functions and evolutionary shuffling of these genes in pituitary organogenesis (Gage et al. 1999a; Hollemann & Pieler, 1999; Lanctôt et al. 1999a; Szeto et al. 1999; Pommereit et al. 2001; Schweickert et al. 2001b; Suh et al. 2002;

Jaszczyszyn et al. 2007; Angotzi et al. 2008). The adenohypophysis in mouse is dependent on the expression of *Pitx1* and *Pitx2* paralogues with cooperative and functionally overlapping functions (Suh et al. 2002; Charles et al. 2005). While mice deficient in *Pitx1* show milder pituitary phenotypes with normal formation of Rathke's pouch, *Pitx2*$^{-/-}$ mutant embryos have relatively normal Rathke's pouch as well, but its later expansion is arrested and increased cell death occurs (Gage et al. 1999a; Szeto et al. 1999; Charles et al. 2005). A very small Rathke's pouch forms after disruption of both genes, suggesting a synergistic action of these paralogues and specific *Pitx* dosage requirements for proper pituitary morphogenesis (Suh et al. 2002).

Pitx genes have multiple roles in pituitary development by regulating the expression of different downstream factors at different time points (Suh et al. 2002). At early stages, when the prospective murine adenohypophysis is in the form of Rathke's pouch and therefore morphologically resembles a much earlier invagination of the stomodeum, *Pitx1* and *Pitx2* regulate the expression of factors responsible for proliferation and Rathke's pouch expansion. *Pitx2* expression is induced at early to late G_1 phase of the cell cycle by Wnt/β-catenin signalling, and directly co-activates the expression of growth-control genes like *Cyclin D1*, *Cyclin D2* and *c-Myc* (Kioussi et al. 2002; Baek et al. 2003). *Pitx2* as well as cell cycle-control mRNAs represent unstable molecules with a rapid turnover and short half-life, a feature that enables quick and precise response to the changing developmental and genetic cues. After activation of *Pitx2* expression by *β-catenin*, the Pitx2 protein acts as a stabilizing factor of both *Pitx2* mRNA and the cell cycle-control mRNAs like *Cyclin D1*, *Cyclin D2* and *c-Jun* (Briata et al. 2003). The early adenohypophyseal morphogenesis is therefore regulated by Wnt/β-catenin signalling at both transcriptional as well as RNA stabilization levels.

A question thus arises as to whether the Wnt/β-catenin/Pitx pathway identified as triggering proliferation in the adenohypophysis functions in a similar way also during the invaginating stomodeum. *Pitx* orthologues have been found at the median pan-placodal region and the developing primary mouth in a number of vertebrates (Lanctôt et al. 1997; Schweickert et al. 2001b; Boorman & Shimeld, 2002a; Jaszczyszyn et al. 2007; Angotzi et al. 2008). However, Wnt signalling is specifically downregulated in the pan-placodal region by secretion of its inhibitor *Dkk1* from the cranial mesoderm (Fig. 8A; Carmona-Fontaine et al. 2007), and it has to be inhibited also at later stages during the process of dissolution of the basal lamina between ectoderm and endoderm of the oral membrane prior to opening of the primary mouth (Dickinson & Sive, 2009). The *Pitx* expression in the primary mouth is therefore probably regulated by a mechanism different from the Wnt/β-catenin signalling. During the early patterning of the murine mandibular arch, the *Pitx1* and *Pitx2* are positively regulated by *Fgf8* and negatively regulated by *Bmp4* (St. Amand et al. 2000). These genes in turn regulate expressions of *Fgf8* in positive and *Bmp4* in negative feedback loops, a mechanism responsible for proper positioning of teeth (St. Amand et al. 2000; Liu et al. 2003). In the mouse upper and lower jaw epithelia, the *Pitx2*-expressing cells display increased proliferative and migratory properties so that their daughter cells expand from the mandibular arch area both outwards into the facial epithelium and inwards contributing to the roof and floor epithelial linings of the oral cavity (Liu et al. 2003). The establishment and maintenance of the zone of expression of *Pitx* genes in the anterior neural plate border may therefore be activated by the Fgf signalling similar to its activation in the mandibular arch and teeth, and may in turn repress Bmp in this region. Similarly to *Pitx* genes, the activation of *Six1* during establishment of the pan-placodal region is also dependent on both synergistic activation of Fgf and repression of Bmp signalling (Ahrens & Schlosser, 2005; Litsiou et al. 2005; Kwon et al. 2010).

Whatever induces the *Pitx* genes in the embryonic anterior region, their expression has been identified in a number of vertebrate species from lamprey to mouse, suggesting a conserved function of *Pitx* genes in the early specification of the stomodeo-adenohypophyseal ectoderm and anterior pharyngeal endoderm. Moreover, *Pitx* orthologues have been found associated with the oral apparatus of urochordates and cephalochordates (Yasui et al. 2000; Boorman & Shimeld, 2002b; Bassham & Postlethwait, 2005; Christiaen et al. 2005), illuminating their archetypal instructive roles for stomodeal invagination, adenohypophyseal placode development and oropharyngeal morphogenesis in chordates.

Concluding remarks: phylogenetic implications of primary mouth formation

Until now, our interest in comparative aspects of primary mouth formation was restricted to vertebrates. Yet to reveal the phylogenetic background of the involved developmental pathways requires a comparison with the pathways in basal chordates. Although detailed information on patterns and processes of primary mouth formation is available for just a few species and the actual taxonomical coverage is quite scarce for the discussion of the phylogenetic implications, several interpretations do arise.

First, the early steps of mouth formation putatively represent a shared trait of vertebrates and urochordates. Both vertebrates and urochordates show a topographically intimate connection between the stomodeum and adenohypophyseal placode appearing in identical positions: at the anterior margin of pan-placodal region in vertebrates; and at the anterior margin of neuropore in urochordates (Manni et al. 2004, 2005; Schlosser, 2007). In both lineages, therefore, the primary mouth is associated with the invagination of the epithelial cell population derived from the

stomodeal ectoderm and formation of distinct stomodeum. In contrast, in amphioxus, the mouth forms as an endoderm outpocketing in the topographic and structural context of the primary pharyngeal slits at the left side of the anterior pharynx. The only putative placode appearing in amphioxus, the preoral ciliary pit, invaginates only after completion of primary mouth formation, where it forms the Hatschek's pit, i.e. the homologue of vertebrate adenohypophysis (Gorbman, 1999; Holland & Holland, 2001). In this regard, the correspondence of primary mouth formation seems to support monophyly of Olfactoria (vertebrates + urochordates) and distinct position of cephalochordates (Fig. 1). The essential separation of mouth formation from the morphogenetic processes of pharyngotremia can tentatively be considered as the key apomorphy of olfactors. Of course, it should be remembered that the vertebrate pharyngeal region exhibits a number of apomorphies as well.

The plesiomorphic condition for deuterostomes indicated by initial endoderm outpocketings, which adopt a narrow slit-like morphology and are elaborated along the dorsoventral axis (Graham et al. 2005), is to form a pharyngeal apparatus designed for filter-feeding and ventilation by cilliary propulsion. In both respects, its efficiency increases with the number of slits and height of cilia-bearing interpouch bars, while no special requirements are imposed upon shape and size of oral opening. The branchial basket in cephalochordates and urochordates reflects this scheme (Swalla & Smith, 2008). In contrast, the pharyngeal apparatus in vertebrates is exclusively designed for the ventilation by branchiomeric musculature attached to rigid branchial bars. Here, the capacity of ventilation is positively related to the volume of the musculature and the extent of particular pouches. The pharyngeal region of vertebrates consequently tends to exhibit a reduced number of particular slits, extensive enlargements of their volumes and the height of branchial region. This also characterizes the earliest sister fossil taxon of vertebrates, the Cambrian genus *Haikouella* (Mallatt & Chen, 2003). Here, enlargement of the branchial region is accompanied by the enlargement of oral opening and rostral expansion of a suprapharyngeal brain. It can be consequently expected that mouth formation involved both the invagination of ectoderm population at the anterior margin of expanding brain as well as the instructive role of endoderm. The situation in *Haikouella* thus fits the developmental evidence demonstrating that: first, the source ectoderm population for oral formation appears at the anterior margin of neural plate within the zone of the primordial pan-placodal field (Schlosser, 2005, 2006; Christiaen et al. 2007), but also that, second, the morphogenesis of the pharyngeal endoderm may play the role of the key patterning factor (Graham et al. 2005) preforming the space for stomodeal invagination. Third, we can also hypothesize that the signalling modules including co-expression of *Pitx* transcription factors had already been established.

The ultimate role of endoderm morphogenesis in mouth formation is supposedly retained in hagfishes and, as discussed above, the hagfish development in that respect may provide a very robust argument for the 'craniate hypothesis' of vertebrate phylogeny. Yet, validity of that statement is essentially dependent upon data on early development in these animals, which are unfortunately still missing. Correspondingly, little is also known about primary mouth formation in the vast majority of other vertebrate clades. The precise developmental data both in taxonomic and methodological respects are just what we urgently need in order to uncover dissimilarities in developmental processes producing morphological variation and being responsible for evolutionary change.

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