Charles University in Prague
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ORAL MORPHOGENESIS IN THE MEXICAN AXOLOTL:

DEVELOPMENTAL ORIGIN OF TOOTH GERMS IN EVOLUTIONARY CONTEXTS

ORÁLNÍ MORFOGENEZE AXOLOTLA: PŮVOD ZUBŮ V EVOLUČNÍM KONTEXTU

Master thesis

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Herewith I declare that I carried out this Master thesis only by myself, with the use of cited literature.

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ABSTRACT

Mouth in the majority of vertebrates develops throughout an ectodermal stomodeum which posteriorly contacts the foregut endoderm, together forming an oropharyngeal membrane. This ecto-/ endodermal membrane gradually thins and become eventually perforated, which causes opening of the stomodeal cavity into the pharynx. Teeth are then understood as organs arising within the stomodeal part of the mouth, where the ectodermal epithelium produces tooth enamel and neural crest mesenchymal cells form dentine and tooth pulp. This project was meant to study the dynamics of the ectoderm and endoderm during the formation of mouth and teeth in the Mexican axolotl. By utilizing transplantations of the oral ectoderm from GFP-transgenic embryos and injections of fluorescent tracer DiI into the endoderm, it was possible to follow the fate of both germ-layers during the course of embryonic development into details. By using this approach it was demonstrated that the mouth in the axolotl develops in a different way, i.e. via the stomodeal collar. Teeth were found to arise within the stomodeal collar ectoderm as well as in the more posteriorly situated endodermal areas. Moreover, some tooth germs were generated also directly at the ecto-/ endodermal boundary. Thus, the formation of teeth does not seem to primarily depend on the distribution of different germ-layer epithelia, but, more likely, on strictly defined places within the oropharynx. The evolutionary origin of teeth should not, therefore, be derived from denticles, which hypothetically settled the oral area in a mechanistic kind of shift, either from the external ectoderm or from the pharyngeal endoderm. Teeth should rather be assumed as neural-crest-derived elements, which arose primarily in the oropharyngeal area only after an odontogenic potential for their production was achieved here and which were probably never dependent on the germ-layer derivation of the epithelium.

ABSTRAKT

U většiny obratlovců se ústa vyvíjejí pomocí ektodermálního stomodea, které posteriorně kontaktuje faryngeální entoderm a tvoří s ním orofaryngeální membránu. Tato ekto-/ entodermální membrána se postupně ztenčuje, až dojde k jejímu protržení, což způsobí otevření stomodeální dutiny do faryngu. Zuby jsou chápány jakožto orgány vznikající ve stomodeální části úst, kde ektodermální epitel produkuje sklovinu zubu a mezenchym neurální lišty tvoří dentin a zubní pulpu. V této práci byla studována dynamika ektodermu a entodermu během vývoje úst a zubů u axolotla mexického. Transplantací orálního ektodermu z GFP-transgenních embryí a injikací fluorescenční značky DiI do entodermu příjemce bylo možné sledovat osud obou zárodečných vrstev během embryonálního vývoje. Tímto přístupem bylo zjištěno, že se ústa u axolotla vyvíjejí odlišným způsobem, a to pomocí ektodermálního stomodeálního límce. Zuby pak vznikají jak v ektodermální oblasti stomodeálního límce, tak i v posteriornějších entodermálních místech. Navíc, některé zubní zárodky jsou generovány přímo na ekto-/ entodermální hranici. Tvorba zubů tedy nezávisí na rozložení epitelů různého zárodečného původu, ale spíše na konkrétních definovaných místech v rámci orofaryngu. Evoluční původ zubů by tedy neměl být odvozován z dentikul, které hypoteticky osídlily ústa mechanistickým přesunem, ať už z povrchového ektodermu nebo z faryngeálního entodermu. Zuby by tedy spíše mohly být chápány jakožto elementy, které se objevily primárně v orofaryngeální oblasti poté, co zde vznikl odontogenní potenciál pro jejich tvorbu a které pravděpodobně nikdy nezávisely na zárodečném původu konkrétního epitelu.

CONTENTS

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1. INTRODUCTION

Teeth represent one of the most important evolutionary novelties within the vertebrate clade. The appearance of these cone-shaped hard structures within gnathostomes can be understood as a milestone that, together with jaws, enabled diversification of vertebrates as active predators and, thus, completely changed relations within the Paleozoic seas. Although it is generally accepted that teeth evolved from denticles[1], the question about the place of origin of these denticles is still a hot topic of for recent scientific discussions. Although some researchers suggest that teeth evolved from external *ectodermal* denticles that reached the oral area in an "outside-in" manner (*sensu* Reif 1982), others propose that rather pharyngeal *endodermal* denticles were those to give rise to teeth according to an "inside-out" scenario (Smith & Coates 1998).

Tooth development is most extensively studied in the mouse, where teeth are assumed to develop from stomodeal ectodermal epithelium that secretes enamel and neural crest mesenchyme that produces dentine and tooth pulp (Thesleff & Sharpe 1997; Jernvall & Thesleff 2000). This model organism, however, possesses highly derived dentition not only among mammals, but also among other vertebrates. Nevertheless, the aspects of mouse tooth development are apodictically applied also to other species. Therefore, teeth are understood as structures developing from the ectoderm and neural crest whatever their position is in whatever species. However, such contribution of the germ-layers to teeth sometimes seems to be very improbable. Teeth developing deep within the pharynx of some extant fishes would suggest their derivation from rather endodermal (than ectodermal) epithelium. Moreover, the precise germ-layer origin of oral teeth cannot be easily identified due to difficulties to satisfactorily distinguish ectoderm from endoderm at critical stages of tooth germ initiation. Hence, after perforation of the oropharyngeal membrane, which constitutes the natural boundary between ectoderm and endoderm, the distribution of these germ-layers is unknown even in such model animals like mouse, chick or zebrafish.

Based on this theoretical background, the purpose of this project was to contribute to our knowledge on the development and evolution of vertebrate teeth by testing the direct contribution of ectoderm and endoderm to tooth germs. The most suitable model organism for such analysis seemed to be the Mexican axolotl *Ambystoma mexicanum* (Shaw, 1789), from several reasons.

[1] denticles = dentine including elements

1) The axolotl as other urodele amphibians represent interesting model organisms, because the development of mouth does not advance through the stages of ectodermal stomodeum as in other vertebrates (Johnston 1910; Adams 1924). Moreover, past studies have questioned the dogmatic view on teeth as derivatives of ectoderm and neural crest cells (Adams 1924; de Beer 1947).

2) Urodeles are maybe the most suitable vertebrates to study formation of mouth and teeth and development in general. Their development is generally slow and its rate can be easily controlled by temperature, they have large mesolecital eggs that are easy to manipulate and enable experimental-embryological approaches.

3) A new strain of fluorescent transgenic axolotls (Sobkow et al. 2006) is recently available. Transplantations of certain tissue from such fluorescent embryo into the non-fluorescent host enables following the fate of this tissue during the course of embryonic and larval development into great details.

4) I have worked with axolotl embryos and studied the development of mouth and teeth in urodeles already during my Bachelor degree (Soukup 2006).

During this project, I have utilized GFP (Green Fluorescent Protein) transgenic embryos of the Mexican axolotl (Sobkow et al. 2006) together with experimental embryological approaches and performed a fate-mapping of ectoderm and endoderm during the course of mouth and tooth development. Taking an advantage of transplantations of labelled tissues and injections of cell tracer, this project was meant to study:

1) the dynamics of ectoderm and endoderm during the formation of mouth in urodeles, and

2) the contribution of ectoderm and endoderm to developing teeth.

2. CURRENT KNOWLEDGE ON THE SUBJECT

2.1. Development of a tooth

Teeth, together with scales, hair, feathers, claws and multiple glands among others, are considered to be derivatives of integument (Kardong 1995). However, despite the diversity in form and function, all these structures develop at the interface of epithelium and underlying mesenchyme and seem to be regulated by common developmental mechanisms, i.e. sequential reciprocal interactions between these tissues (Pispa & Thesleff 2003).

The first visible sign of tooth development is a local thickening of the epithelial layer, which forms a placode. Mesenchymal cells directly under this thickening later condensate into a papilla. Still later, the thickened epithelial layer starts to invaginate and proliferate into the underlying mesenchyme to form a tooth bud and subsequently a tooth cup. Continued folding of the epithelial-mesenchymal junction finally outlines the future shape of the tooth. In mammals, folding of the epithelial layer is ruled by a transitional structure called enamel knot. Enamel knot represents a cluster of non-dividing cells within the epithelium (Jernvall et al. 1994) that secrete multiple growth factors both to surrounding epithelia and mesenchyme and directs either activation or inhibition of proliferation in these tissues (Jernvall et al. 1998; Jernvall & Thesleff 2000; for a computational-developmental model see Salazar-Ciudad & Jernvall 2002). On the account of reciprocal interactions, mesenchymal cells form a dental pulp and become odontoblasts that generate dentine. Epithelial cells, on the other hand, differentiate into an enamel organ, where ameloblasts produce enamel. Secretion of protein matrix and anorganic material takes place at the epithelial-mesenchymal junction.

The epithelial-mesenchymal contact is, as mentioned above, a place of reiterative reciprocal signalling from the initiation of tooth germ to hard tissue deposition (Thesleff & Sharpe 1997; Jernvall & Thesleff 2000; Tucker & Sharpe 2004). Signalling involves multiple molecules belonging to fibroblast growth factor (FGF), transforming growth factor β (TGFβ), hedgehog (Hh), Wnt and tumor necrosis factor (TNF) families. It is beyond the scope of this study to review all the genetic mechanisms that govern the development of the tooth. For the purpose of this study it is more interesting to have a look at tooth induction and its tissue origin. Mouse tissue recombination studies identified that the first instructive signal for odontogenesis comes from the oral epithelium to the adjacent mesenchyme (Mina & Kollar 1987; Lumsden 1988). This signal goes hand in hand with the first regionalization of the mandibular arch by antagonistic signalling of Bmp4 and Fgf8 (Tucker et al. 1999) and leads to a specification of places of tooth development (Neubüser et al. 1997; Tucker et al. 1998).

The odontogenic potential, however, subsequently shifts from the epithelium into the mesenchyme (Peters & Balling 1999). Mesenchymal expression of multiple homeobox-containing transcription factors then defines the type of developing tooth (Sharpe 1995; Thomas & Sharpe 1998).

It is generally accepted that tooth enamel epithelium (later ameloblasts) is a derivative of ectoderm, whereas dental mesenchyme (later odontoblasts) is produced by neural crest cells[2]. Such scheme is however apodictically applied to all vertebrates, although germ-layer derivation of teeth has been shown in a relatively low number of species. By means of precise fate-mapping[3] methods, the dental mesenchyme has been shown to be a neural crest derivative only in rodents (Imai et al. 1996; Chai et al. 2000) and urodeles (Chibon 1967a; 1967b), but its neural crest origin is assumed also for other vertebrates (Smith & Hall 1990; 1993). Similarly, ectodermal origin of enamel epithelium was demonstrated by precise fate-mapping only in urodeles (Chibon 1970), but ectodermal origin is proposed also for mouse (e.g. Jernvall & Thesleff 2000), bony fishes (oral dentition; Sire & Huysseune 1996; Sire et al. 1998; Sire 2001) and other vertebrates as well (Reif 1982; Kardong 1995; Pispa & Thesleff 2003). However, teeth of many actinopterygians are found deep within pharynx, beyond the possible influence of ectoderm, where the epithelium is proposed to be rather of endodermal origin (Huysseune et al. 2002). No fate-mapping study has, however, been performed to confirm this suggestion.

The epithelial germ-layer derivation of such pharyngeal teeth[4] is not well known, however, even the germ-layer origin of teeth in the oral area is questionable. In the oral area, ectoderm can be clearly distinguished from endoderm during initial stages of development, because these two germ-layers are in contact at an oropharyngeal membrane. After rupture of this membrane, which causes opening of the mouth, the natural limit between both germ-layers is lost. Later distribution of ectoderm and endoderm in the mouth cannot be determined by means of classical histology, because cell compartments, characteristic for ectoderm and

[2] Neural crest is a transient population of cells that originates at the limit of neural and non-neural ectoderm during the closure of the neural tube (Le Douarin & Kalcheim 1999). These cells undergo an extensive latero-ventral migration through the whole body (Cerny et al. 2004) to reach multiple positions within the embryo. At these positions, neural crest is the source of multiple cells and tissues, such as pigment cells, cartilage, bone, nerves etc. (Le Douarin & Kalcheim 1999).

[3] Fate-mapping is a term for mapping the fate of desired tissue. At the initial stage, this tissue is labelled by a stable marker, which can be easily followed during the course of development and which is finally found within all the derivatives of this tissue (Stern & Fraser 2001).

[4] Pharyngeal teeth are teeth found within the pharyngeal cavity of various fishes. They can be situated either loosely in the pharyngeal mucosa or associated with branchial arches.

endoderm at initial stages of development, are gradually using up. Cells of both germ-layers, thus, become indistinguishable already before any signs of development of teeth.

The germ-layer origin of vertebrate teeth is, however, key to the understanding of tooth evolution. Possible endodermal contribution to tooth formation revealed by fate-mapping studies could have direct influence on our understanding of tooth evolutionary scenarios. A series of these studies performed throughout various vertebrate taxa could indicate the role of germ-layers during the evolution of vertebrates, whether or not there is the endodermal contribution to teeth, or whether there were shifts in either contribution of ecto-/ endoderm in relation to developing teeth.

2.2. Evolutionary Origin of Teeth

Recent opinions on the evolutionary origin of teeth in extant vertebrates are predominated by two opposing theories. It used to be generally accepted that teeth evolved from external dermal denticles and that is why they should develop from ectodermal epithelium ("outside-in" theory, *sensu* Reif 1982). More recently, however, it was proposed that teeth could evolve from denticles situated in the pharyngeal region, which would support their endodermal derivation ("inside-out" theory, Smith & Coates 1998; 2000; 2001). No experimental evidence has, however, been put forth to support endodermal origin of teeth (until nowadays) and, therefore, the "classical" ectodermal evolutionary origin is rather accepted, although the influence of the latter is increasing. These theories are discussed in details bellow.

2.2.1. External "Ectodermal" Origin of Teeth (Outside-in Scenario)

It was proposed already a long time ago, however, mainly based on tissue composition, that teeth are structures homologous to external dermal denticles, but it had taken quite a long time, until a suitable theoretical model explaining the mechanism of evolutionary denticle-to-tooth-transition was brought in. The nowadays widely accepted "Odontode Regulation Theory" (Reif 1982) offered such a model and replaced the previous "Lepidomorial Theory" (see Janvier 1996 and Donoghue 2002 for aspects of Lepidomorial Theory).

The Odontode Regulation Theory is based on studies of dentition and squamation in sharks and comprises an odontode as its most fundamental unit. Odontode is the primary element of the dermal skeleton of all vertebrates (Reif 1982). It is a composite hard structure,

which consists of dentine surrounding central pulp, acellular or cellular bone at the base anchoring it into dermis and a hypermineralized cap composed of enamel or enameloid (which, however, need not be necessarily present). It develops at the interface of outer ectodermal epithelium and underlying neural crest mesenchyme. Although the odontode was first defined as a "hard tissue unit corresponding very closely to teeth and difficult to distinguish from them by any rational criteria" (Ørvig 1967), it was later extended to include teeth as well (Reif 1982). Teeth were understood to have not evolved independently *de novo*, but rather by means of a modification of the morphogenetic program for denticles. The term odontode thus comprises two structures: dermal denticles (scale-like elements of external skeleton found mostly on the body surface) and teeth. The main difference between these manifestations of the odontode is that dermal denticles are always generated superficially (e.g. Reif 1980), whereas teeth develop from a deep epithelial invagination called dental lamina (Reif 1982). For more information about odontodes and their evolutionary changes and derivatives see Ørvig (1977) and Huysseune & Sire (1998).

According to the Odontode Regulation Theory, the evolution of teeth from dermal denticles starts from a situation of undifferentiated odontodes arranged uniformly on the body surface. These odontodes form inhibitory fields around themselves preventing formation of other odontodes in their vicinity (Osborn 1978). New odontodes can be added only apart from the inhibitory field, i.e. during the growth of the animal or after shedding of the preceding odontode. This mechanism is, however, very undesirable especially along the jaw margins, where odontodes could have functioned as grasping or crushing elements. Loss of odontode naturally enables formation of a new one, but, at this place, the developing germ would be very vulnerable. Moreover, the development of the new odontode at the jaw margin just after the loss of its predecessor is not efficient, because it would take quite a long time for the successive germ to become functional. The epithelial invagination along the whole length of the jaws (called dental lamina), however, solve the problem of superficially developing odontodes. Odontode germs are, thus, generated deep within the dental lamina, far away from inhibitory fields of other superficially forming odontodes, and in the area, where they cannot be easily damaged. These are generated in advance of need, quite quickly compensate their predecessors and gradually increase in size according to the body growth. Dental lamina thus offers a series of replacement germs (now teeth), a "tooth family", while functioning tooth is still at the place.

Originally, the evolution of teeth as derivatives of the dental lamina was proposed to take place only after the evolution of jaws (Reif 1982; Mallatt 1996). Jaws are seen to have

evolved from anterior pharyngeal arch(es), as they enabled closure of the mouth during forceful expiration through pharyngeal slits (Mallatt 1996). It was only later, when jaws enlarged and were augmented by continuously replacing teeth to become an apparatus for grasping the prey. Hence, teeth as derivatives of dental lamina are proposed to be a synapomorphy of Chondrichthyes and Teleostomi, since Placodermi, the basal jawed vertebrates possessed only non-tooth bearing gnathalia (Reif 1982, but see Smith & Johanson 2003).

The term "odontode" has, however, gradually become more or less theoretical, in spite of being concretely defined, and is nowadays mainly used as a model of predecessor or archetype of both dermal denticles and teeth. This can be seen for example in the studies of dermal skeleton in fishes (Sire & Huysseune 1996; Sire et al. 1998; Sire 2001), where teeth are suggested rather to precede than to evolve from dermal denticles. By comparison of developmental stages of individual dermal elements, Sire & Huysseune (2003) propose that teeth evolved from isolated external odontodes (represented by placoid scales of sharks) 450 My ago, while dermal denticles, having more similar ontogeny and structure with teeth, arose much later, ca 100 My ago. Thus, the following evolutionary sequence was suggested: (1) isolated odontodes, (2) teeth, (3) dermal denticles. Teeth would in this case represent only intermediate products later giving rise to dermal denticles. Such suggestion is based also on the presence of teeth found on the surface of the head of some teleosts (Sire 2001). Dermal denticles are then proposed to have evolved from teeth that escaped from their original positions in the mouth and were spread along the body surface. Since the extra-oral teeth are found in several non-related species (Sire 2001), it was suggested that this change in position and subsequent hypothetical tooth-to-denticle shift could happen even many times during the evolution of vertebrates.

Whether this evolutionary tooth-to-denticle shift happened or not, remains to be elucidated. Nevertheless, according to this generally accepted "outside-in" scenario, the external skin odontodes were captured in the oral cavity and were incorporated into the jaw apparatus. Hence, odontodes, as derivatives of exoskeleton, are assumed to have always been developing in the ectodermal area. Teeth are, thus, understood as derivatives of ectodermal epithelium and underlying mesenchyme from their very evolutionary beginning.

2.2.2. Internal "Endodermal" Origin of Teeth (Inside-out Scenario)

The Odontode Regulation Theory is an influential concept in that it offers logical scenario on the evolutionary change of denticles into composite patterned teeth and clearly

demarks teeth from other dermal skeletal units by features of dental lamina (Reif 1982). On the other hand, important points against this theory were addressed, namely that no transition from denticles into teeth has ever been found even in sharks (see even Reif 1982) and that it includes an assumption that teeth evolved only after the appearance of jaws (Smith & Coates 1998; 2000).

Another hypothesis on the evolutionary origin of teeth was, therefore, put forth (Smith & Coates 1998). According to the "inside-out" scenario (Smith & Coates 1998; 2000; 2001), teeth have evolved from denticles found within the pharyngeal cavity, i.e. areas lined by endodermal (rather than ectodermal) epithelium.

The "inside-out" theory is based on the presence of denticles associated with pharyngeal arches of *Loganellia scotica* (Van der Brugghen & Janvier 1993; Janvier 1996) (an agnathan vertebrate from the group Thelodonti). Pharyngeal denticles of *Loganellia* have been redescribed by Smith & Coates (1998) as a series of hard exoskeletal units arranged in a whorl-like manner representing a family of subsequently developing denticles. The denticle family likely grew in one direction by a precise apposition of successive elements at the end of this series. This pattern organization might propose exact spatial and temporal regulation of initiation, development, polarity, shape and size of successive denticles (Smith & Coates 1998). Such characteristics are, however, claimed to be features of dental lamina, meaning that these denticles should be regarded, according to the definition by Reif (1982), as true teeth. Whether the dental lamina really existed in *Loganellia*, cannot, however, be demonstrated.

Similar families (tooth-whorls) have been found also in basal gnathostomes such as stem Chondrichthyes, Acanthodii, basal Osteichthyes and also recent Chondrichthyes (Janvier 1996, pp. 125, 137-138, 145, 196, 203; Smith & Coates 1998 and citations therein). These tooth-whorls are associated either with branchial arches or with the jaw margins (along their whole length, or in symphyseal or parasymphyseal positions), as clusters of either unicuspid or multicuspid teeth with fused bases. They can vary from being a bow-shaped series of four teeth, or complete spirals consisting of many teeth gradually increasing in size (Reif 1982).

Although teeth are generally regarded as derivatives of ectoderm and neural crest, the inside-out theory proposes evolution of teeth from endodermal pharyngeal areas (Smith & Coates 1998; 2000; 2001). Teeth are seen to originate from patterned pharyngeal denticles similar to those found in *Loganellia*, where the patterning mechanisms were intrinsic to the pharyngeal endodermal epithelium. The patterning mechanisms were then co-opted from the endodermal pharyngeal epithelium into ectodermal oral epithelium. The advantage of this co-

option would be in generating the denticle/ tooth-whorls within the ectodermal jaw epithelium and functioning as grasping apparatus. Teeth of extant vertebrates would, thus, according to the inside-out theory, be derivatives of ancient odontogenic developmental mechanisms co-opted from endodermal regions.

Tooth-whorls are assumed to be an ancient character acquired already before the appearance of jawed vertebrates, which led Smith & Coates (2001) to the proposal that they could represent a synapomorphy of loganellid thelodonts and primitive gnathostomes. Although no tooth-whorls have been found within several groups of this proposed clade (e.g. jawless Osteostraci or basal jawed Placodermi), denticles organized into rows were reported at the rear of the gill chamber of some placoderms (Johanson & Smith 2003; 2005). Based on the different spatial arrangement of these denticles when compared to the pattern of outer skin denticles, the pharyngeal denticles are regarded as derivatives of different patterning mechanisms. These denticles are, therefore, proposed to have developed from endodermal epithelium and are claimed to share the same developmental mechanisms with tooth-whorls of *Loganellia* (Johanson & Smith 2003; 2005).

Although the inside-out theory assumes the shift from pharyngeal denticles into oral tooth-whorls, there appears an inconsistency in proposals of later evolution of these elements (in spite of being addressed by the very same author). Formerly, it was proposed that ancient mechanisms for tooth development underwent shift from endodermal into ectodermal epithelium and that it was primarily the endodermal epithelium, where teeth have evolved (Smith & Coates 1998; 2000). However, just the very same authors proposed later that it is the boundary of ecto- and endoderm, which is central to tooth development (Smith 2003; Johanson & Smith 2003). Hence, the ecto-/ endodermal border was proposed to function as the place of initiation, development and subsequent replacement of teeth within first vertebrate dentitions, whether situated on the pharyngeal arches or in the oral area. Development of the whole dentition was, therefore, proposed to start from a single symphyseal tooth germ, which acts as an inductor of other teeth. A waving ecto-/ endodermal border along the whole length of the jaw functions as the place of initiation of these tooth germs. These founders subsequently induce development of other members, which together form tooth families. This theoretical model is based on the assumption of ecto-/ endodermal border as the place of origin of teeth; however, such proposal is faded by the inability to map the distribution of ectoderm and endoderm in the mouth of fossil vertebrates. Moreover, even in extant vertebrates, the origin of oral epithelia is also not known due to a considerable lack of fate-mapping studies.

Summarizing this "inside-out" theory out, dermal and pharyngeal denticles are proposed to share common regulation of morpho- and histogenesis, and are therefore assumed to be homologous at this level. However, the latter differ significantly from the former in a whorl-like pattern which suggests different mechanisms of development. These whorls were initially formed in the pharyngeal portion of oropharynx prior to the evolution of jaws and were later co-opted for oral areas. Teeth have probably always been associated with the ecto-/ endodermal boundary, no matter whether this border is situated at pharyngeal arches or associated with jaws. Teeth in extant vertebrates would therefore be generated by mechanisms that evolved primarily in the pharyngeal areas.

Despite being based on paleontological data, the origin of teeth from pharyngeal denticles of agnathan vertebrates was questioned by many paleontologists. Purnell (2001) found oral denticles comparable to those of *Loganellia* in some members of the Heterostraci group (another agnathan clade). These denticles were organized into a pattern as well; however, they gradually integrated with external headshield denticles that possessed different pattern. This led to the suggestion that external skin denticles in Heterostraci must have given rise to oral denticles rather according to "outside-in" than "inside-out" scenario (Purnell 2001). Next, Donoghue & Smith (2001) showed that *Loganellia* is a derived thelodont and pointed out that no comparable organized pharyngeal denticles have been found within other members of this clade! Pharyngeal denticles of *Loganellia* and tooth-whorls of basal jawed vertebrates would thus be convergently achieved and could not in this way be considered as a synapomorphy of these lineages. According to these points, the proposal that teeth evolved according to "inside-out" theory clearly needs more developmental as well as paleontological evidence.

2.3. Oral vs. Pharyngeal Teeth in Fishes

After appearance of reports that vertebrate teeth could have evolved from pharyngeal, i.e. endodermal areas, much effort has been put into studies of pharyngeal teeth of extant bony fishes (Laurenti et al. 2004; Jackman et al. 2004; Fraser et al. 2004; 2006a; 2006b; Borday-Birraux et al. 2006; Debiais-Thibaud et al. 2007). Pharyngeal teeth are found either distributed loosely in the pharyngeal mucosa or associated with branchial arches. Small toothlets can be found even in a large pharyngeal sac behind the last branchial arch in stromateoid fishes (Haedrich 1967). The most specialized teeth within fishes seem to be those of cypriniform teleosts (e.g. zebrafish) being situated exclusively on the fifth ceratobranchials

(Huysseune et al. 1998; Van der heyden et al. 2000). Although fate-mapping of endoderm in zebrafish revealed its presence in the pharyngeal region (Warga & Nüsslein-Volhard 1999), it is of great interest that no labelled tissue was found in developing tooth germs[5]. Presence of endoderm in the pharynx does not, however, necessarily mean that teeth do develop from this germ-layer. Edwards (1929) documented ingrowth of ectodermal tissue at the contact areas of ecto- and endoderm between pharyngeal arches and its migration on the surface of endoderm in the carp. Pharyngeal teeth in extant bony fishes are, nevertheless, generally considered as endodermal derivatives (Huysseune et al. 2002; Stock 2001).

Modern studies comparing oral and pharyngeal teeth in fishes could reveal whether there are differences in expression patterns of genes and could thus find out whether these differences are correlated with ectodermal or endodermal derivation of teeth. Such studies performed until now have, however, found mostly similar expression of studied genes. For example, expressions of *Dlx2* and *Dlx4* during mouse tooth development correlate with expression patterns of their zebrafish orthologs *dlx2a*, *dlx2b* and *dlx4a*, *dlx4b* (Borday-Birraux et al. 2006). Spatial and temporal expression patterns of trout *Shh*, *Pitx-2* and *Bmp-4* in tooth germs do not differ from their mouse orthologs (Fraser et al. 2004). Expression sites of *Fgfs* and their downstream genes are also similar within mouse and zebrafish (Jackman et al. 2004). Shared expression patterns of all these genes suggest that they have similar roles within tooth primordia in vertebrates and that these genes are required for the proper development of teeth in all vertebrates (Jackman et al. 2004; Fraser et al. 2004; Borday-Birraux et al. 2006).

Some small differences were, however, found between the gene expression patterns during the development of oral and pharyngeal teeth. Fraser et al. (2004) found a downregulation of *pitx-2* after initiation of a tooth germ in the pharyngeal region, whereas its expression was not altered in marginal, palatal or lingual teeth of a trout. Next, whereas mouse *Dlx1* and *Dlx6* are expressed in dental mesenchyme during dental papilla stage (Zhao et al. 2000), their zebrafish orthologs (*dlx1a* and *dlx6a*) have not even been found in developing tooth germs (Borday-Birraux et al. 2006). The absence of expression of *dlx1a* and *dlx6a* could be, however, related to the relative absence of dental papilla during tooth development in the zebrafish. Zebrafish tooth anlagen, most significantly, lack expression of *fgf8* and its downstream gene *pax9* (Jackman et al. 2004). *Fgf8* is however a crucial gene for the development of mouse teeth in that it is necessary for advancement beyond the tooth bud

[5] This is, however, not due to any technical bias, but because in this paper, the endodermal contribution to pharyngeal teeth was not primarily sought.

stage (Peters et al. 1998). Jackman et al. (2004) concluded that, very probably, other *Fgf* paralogs are expressed in zebrafish tooth primordia to compensate for *fgf8*. The synchronous absence of *pax9* and *fgf8* from zebrafish dental mesenchyme could indicate a fundamental difference between the development of oral and pharyngeal teeth. Alternative explanation is that differences in *pax9* and *fgf8* expression are due to accumulated changes in the genetic control of tooth development because of a long separate history of Actinopterygii and Sarcopterygii. Whether such differences can be found between oral (supposedly ectodermal) and pharyngeal (supposedly endodermal) teeth in fish, remains unknown (zebrafish, the model organism in genetic and developmental studies, possesses pharyngeal teeth only). Laurenti et al. (2004) found expression of *eve1* gene in the tooth epithelium of the first developing tooth in zebrafish. Paralogs of this gene (*evx1* and *evx2*), however, are not expressed during the development of teeth in mouse. This led to suggestions that either different regulatory pathways could contribute to tooth development in mouse and zebrafish, or that oral and pharyngeal teeth evolved as convergent elements being under different molecular control. Similar study on the expression of *eve1* gene in medaka (Debiais-Thibaud et al. 2007), a teleost possessing both pharyngeal and oral teeth, however revealed its presence during the development of oral as well as pharyngeal teeth. *eve1* thus represents a gene that was probably incorporated into developmental mechanism producing teeth within the actinopterygian lineage, and is not an example of a difference between oral and pharyngeal dentitions.

It can, therefore, be concluded, based on the conserved expressions of multiple genes within oral as well as pharyngeal teeth in fishes (Fraser et al. 2004; 2006a; 2006b; Debiais-Thibaud et al. 2007) when compared to similar expressions in the mouse (Jackman et al. 2004; Fraser et al. 2004; 2006a; Borday-Birraux et al. 2006) that teeth are probably homologous structures (at least within Osteichthyes) and that oral and pharyngeal teeth do not differ significantly (but see Tucker & Sharpe 2004). Minor differences in expression patterns between oral (ectodermal) and pharyngeal (endodermal) teeth are probably either due to separate histories of these teeth, or due to positional cues, e.g. influence of Hox-code in the pharyngeal, but not oral, areas (Hunt et al. 1991; Krumlauf 1993).

2.4. Development of Mouth in Vertebrates

During early vertebrate development, from gastrulation onwards, the embryo consists of three germ-layers: ectoderm outwards, endoderm inwards and mesoderm between them.

The only place, where the mesoderm is never present, is the area of prospective mouth (Adelman 1932). Anterior endoderm ends blindly at this place and contacts prospective oral ectoderm. It is only at later stages of development when this oral ectoderm produces a blind pocket-like structure called stomodeum. The stomodeum represents a primitive oral cavity. The stomodeal cavity is, at first, separated from the pharyngeal endoderm by an oropharyngeal membrane, but the membrane is later perforated causing a continuation of oral and pharyngeal cavities.

Oropharyngeal membrane thus represents a transient structure during development of the mouth. It consists of two layers: stomodeal (oral) ectoderm situated anteriorly and foregut (pharyngeal) endoderm situated posteriorly. The membrane gradually thins from a double-layered to a single-layered unit (by intercalation of anterior ectodermal and posterior endodermal cells) until it is finally perforated (Dickinson & Sive 2006). The natural boundary between ectoderm and endoderm is, thus, lost and its later position within the oral area is not precisely known due to the inability to distinguish between cells of these germ-layers.

Interestingly, the mechanism of perforation of the oropharyngeal membrane can be driven by different processes in various vertebrates. For example, in frogs and mouse, the perforation of the membrane is caused by apoptotic cells (Watanabe et al. 1984; Poelmann et al. 1985; Dickinson & Sive 2006). Alternatively, in chick and hamster, the surrounding epithelia seem to proliferate intensively, which causes tension and subsequent rupture of the membrane (Waterman 1977; Waterman & Balian 1980; Waterman & Schoenwolf 1980; Miller & Olcott 1989). Thus, either apoptotic membrane cells, or increased proliferation in surrounding epithelia can lead to formation of minute holes in the oropharyngeal membrane and results in the opening of the stomodeum into the foregut (Waterman & Schoenwolf 1980; Poelmann et al. 1985).

Just in front of the oropharyngeal membrane, there appears an invagination of cells at the median dorsal surface of ectodermal stomodeum. This is a direct predecessor of an anterior lobe of hypophysis (adenohypophysis). It appears either as a pocket-like structure, Rathke's pouch (sharks, Baumgartner 1915; caecilians, Laubman 1925; Teipel 1932; reptiles, Baumgartner 1916; marsupials Parker 1917), or as a solid ingrowth of cells (*Amia*, Reighard & Mast 1908; Smith 1914; frogs, Atwell 1918). Prospective adenohypophysis migrates on the surface of prosencephalon, loses its connection with ectoderm and finally contacts evaginated part of diencephalon, the infundibulum; together forming an anterior pituitary. According to classical morphological observations, adenohypophysis was proposed to be a derivative of external ectodermal (epidermal) epithelium. However, it has been demonstrated by fate-

mapping that its predecessor tissue can be found within the inner layer of transverse neural fold (chick, Couly & Le Douarin 1985; *Xenopus*, Eagleson et al. 1986; mouse, Kawamura & Kikuyama 1992; Kouki et al. 2001).

2.5. Development of Mouth in Urodeles

Although notes on the mouth development in urodeles go as far as to Goette (1869), Orr (1889), Honssay (1893) and Röse (1895) and their aspects have been studied quite extensively (Johnston 1910; Landacre 1921; Adams 1924; 1931; Marcus 1930; 1932; Gerhardt 1932; Reisinger 1933; Ströer 1933; Balinsky 1947; de Beer 1947; Chibon 1970; Takahama et al. 1988), the primary inability to satisfactorily distinguish between ectoderm and endoderm made it often quite difficult to precisely determine the exact distribution of these tissues during development of the mouth. Since majority of these studies was non-experimental and made by means of classical histology, their results did necessarily lead to very different interpretations on the morphodynamics and final arrangement of ectoderm and endoderm. Nevertheless, the general process of mouth development was described quite satisfactorily and in thorough details and was supported by a number of studies.

Mouth in urodeles develops in a completely different manner than in other vertebrates. The whole prospective oral area is filled with a mass or cluster of cells of "oral endoderm". Moreover, the ectoderm does not create a pocket-like stomodeum, but instead it forms the so called "ectodermal" or "stomodeal collar" (Landacre 1921; de Beer 1947; see also schematics on Fig. 17). The collar is formed by single layers of ectodermal cells, each of which is situated directly dorsal or ventral to the endodermal foregut and ensheath it in a ring-like manner.

The development of mouth in urodeles begins by an invagination of the inner ectodermal layer between the foregut endoderm and surrounding tissues, while the outer ectodermal layer stays in place and covers the whole oral area. The invagination occurs along the borders of contact of ectoderm and endoderm and continues on the surface of the oral endoderm. It is not known, whether the cells directly between the oral endoderm and the outer ectodermal layer at the place of prospective oral opening undergo apoptosis or whether they are included into the collar, but after the invagination occurs, there is no inner ectodermal layer between these two tissues. Migrating inner ectodermal cells gradually cover larger and larger part of oral endoderm as a "sleeve" (Adams 1924; for a 3D model of stomodeal collar see Reisinger 1933, Fig. 39). The mouth cavity has not yet been formed. It appears only later

as a horizontal cleft in the middle of the oral endodermal cluster of cells. Although, in one report, it was observed that the cleft is proceeding anteriorly from the lumen of the foregut to finally reach the surface of the embryo (Johnston 1910), Takahama et al. (1988) found several gaps among endodermal cells that become connected to each other, to the outer surface or to the lumen of foregut to finally cause opening of the mouth. Endodermal cells detach, during this process, from each other and join the walls of future mouth. Thus, "the oral cavity is lined by endoderm, but possessing a collar of ectoderm dorsal to its roof and ventral to its floor" (Landacre 1921; p. 26). Degenerating cells were observed infrequently in the oral ectoderm, whereas there were none of these in the endoderm (Takahama et al. 1988). No basal lamina at the contact between ectoderm and endoderm was found in any stage of development (Takahama et al. 1988).

The development of adenohypophysis is rather ingrowth-like than pocket-like and resembles that of fishes and anurans (Reighard 1908; Smith 1914; Atwell 1918). Prospective adenohypophysis is situated in the inner layer of the anterior median part of the ectodermal stomodeal collar, but its possible origin can be seen in the basal layer of transverse neural fold (see 2.4. Development of Mouth in Vertebrates). Cells of prospective adenohypophysis represent in fact the leading edge of the collar and are, thus, first cells which invaginate (Kingsley & Thyng 1904). Adenohypophysial primordium gradually extends posterodorsally along the surface of the oral endoderm until its posterior part gets into contact with an infundibulum. Finally, the contact with the rest of the stomodeal collar is broken and the adenohypophysis continues its development in connection with the infundibulum (Atwell 1921; Copeland 1943).

From the problematica mentioned in the first paragraph of this chapter, there appear differences in observations of the distribution of the germ-layers during the course of development. Although Adams (1924) states that the outer ectodermal layer stays intact and always covers the oral area, Johnston (1910) observes loss of this layer, which would bring endodermal cells exposed to the outer surface of the embryo and parallels this to the beginning of the rupture of oropharyngeal membrane of other vertebrates. The distribution and extent of the ectodermal collar is also insufficiently described in some studies. Adams (1924) reports ectoderm reaching approximately behind the level of inner choanae on the palate and to the middle of the lower jaw. On the other hand, other authors propose much more massive migration of cells of stomodeal collar, reaching up to oesophagus (Marcus 1930; 1932; Gerhardt 1931; 1932). This would lead to ectodermal origin of multiple oral and pharyngeal derivatives. In this way Gerhardt (1931) and Marcus (1930; 1931b) proposed

ectodermal derivation not only of teeth and taste buds, but also of thyroid gland, which was in turn shown not to be the case and stated as a wrong observation (Reisinger 1931; 1933). Moreover, these two authors propose ectodermal lining of the pharynx also in anurans (Marcus 1931a; 1931b; Gerhardt 1932). As the mouth in anurans is mostly seen as developing in a classical way through an oropharyngeal membrane (Watanabe et al. 1984; Dickinson & Sive 2006) and no collar has been reported in other studies (but see Reiss 1997), it therefore seems that this statement is wrong as well.

2.5.1. Development of Teeth in Urodeles

The dentition of axolotl is polyphyodont, i.e. with multiple generations of replacement teeth. Teeth are distributed on the upper and lower jaws but also on the palate. They are arranged into five tooth fields: premaxillary/ maxillary on the upper jaw, vomeral and palatal on the palate, and dentary and splenial on the lower jaw; in the manner that premaxillary/ maxillary and dentary teeth oppose against each other and so do splenial against vomeral and palatal teeth.

Teeth in urodeles start to develop at the end of ectodermal collar migration (Adams 1924). Developing teeth are first visible by an ingrowth of thickened epithelium into the surrounding tissues. The ingrowing epithelium interacts with underlying mesenchyme to form a cup-like structure, a tooth germ (Röse 1895). Whereas mesenchymal cells develop into odontoblasts, the tooth epithelium is later distinguished into an outer enamel epithelium and an inner enamel epithelium, which differentiates into ameloblasts (Smith & Miles 1971). During mineralization, the tooth germ in the axolotl is, at first, composed of enameloid, a tissue that is generated by both odontoblasts and ameloblasts[6]. Later, dentin is produced by odontoblasts and, eventually, the tooth is augmented on top by a thin layer of enamel, a derivative of ameloblasts (Wistuba et al. 2002). Such tooth is thus composed of dentin, enameloid and enamel.

The origin of cells forming tooth germs in urodeles is very interesting. Although odontoblasts generating tooth pulp and dentin were shown to be derived from neural crest (de Beer 1947; Chibon 1967a; 1967b), the origin of epithelium giving rise to ameloblasts (enamel organ) is not certain. Marcus (1930; 1932) and Gerhardt (1932) observed a deep invagination of ectodermal collar up to the level of pharynx making a conclusion of ectodermal origin of enamel epithelia. Although others did not observe purely ectodermal lining of the

[6] In fish, odontoblasts first generate a matrix of collagen, on which the ameloblasts secrete amelogenin-like proteins (Shelis & Miles 1974). Mineralization of enameloid starts after this matrix is ready.

oropharyngeal cavity and supported the "common" range of stomodeal collar, the results on the origin of tooth epithelia nevertheless differ. Takahama et al. (1988) proposed tooth formation only within the ectodermal collar area. Johnston (1910) came to the same conclusion but he stated that tooth germs developing from the collar give rise to teeth of the maxillary, dentary and vomeral tooth fields. Where do palatal and splenial tooth germs originate from, is not indicated, in spite of drawing epithelial ingrowths indicating tooth germs also in endodermal areas (Johnston 1910; Fig. 18). Ströer (1933) did not agree with such interpretations and, based on his experiments on the induction of mouth, he negated the origin of teeth from endoderm. On the other hand, Adams (1924), besides from ectodermal origin of tooth germs, proposed derivation of teeth also in the endodermal portion of the oral cavity. According to her, maxillary, vomeral and dentary teeth are of ectodermal origin, whereas palatal and splenial teeth arise in the endodermal areas. Moreover she found tooth germs developing also at the leading edge of the stomodeal collar. These teeth would thus have epithelial lining of ecto- and endodermal origin simultaneously! Endodermal origin of enamel organs of posteriorly situated tooth germs is supported by experiments, where the contact between ectoderm and endoderm was prevented and both tissues were developing without contact (Adams 1931). Although these embryos did not develop mouth, teeth did arise in the ectodermal as well as endodermal tissues. The results of Adams (1924) were fully accepted and brought into details by other authors (Reisinger 1933; de Beer 1947).

Chibon (1970) performed a fate-mapping study of oral ectoderm labelled by tritiated thymidine to resolve the problem of possible endodermal contribution to tooth formation. According to this procedure, all of the ectodermal cells contributing to mouth formation were visualized and could be easily recognized on histological sections. Results of this study confirmed stomodeal collar formation and ectodermal derivation of teeth. Moreover, there appeared non-labelled tooth germs behind the edge of collar leading to an assumption that these teeth develop within the endodermal epithelium. However, identification of endodermal teeth solely by being non-labelled is only a negative evidence. Direct evidence on the endodermal origin of tooth enamel organs could be obtained, for example, either by visualizing of expression of gene that would be known to play role solely in foregut development, or by other direct labelling of foregut endoderm by a stable marker and its subsequent fate-mapping.

Although direct derivation of teeth from endoderm has not yet been demonstrated, results obtained by studies using *in vitro* cultivation of multiple axolotl tissue types proposed its possible potential for tooth induction and development. In a series of experiments on *in*

vitro cultivation of different tissue types, Wilde (1955) observed developing teeth in a culture containing cranial neural crest together with oral ectoderm and foregut endoderm. No teeth were developing, when cranial neural crest together with oral ectoderm, or cranial neural crest together with foregut endoderm were cultured. Takata (1960) also did not observe *in vitro* formation of teeth when cranial neural crest was co-cultured with endoderm. Graveson et al. (1997) confirmed that teeth can develop *in vitro* only when foregut endoderm and oral ectoderm are co-cultured with cranial neural crest. These studies demonstrate that endoderm has a potential for tooth induction or subsequent development, if not a direct contribution to tooth germs.

Results obtained by means of classical histology support the possible origin of tooth germs from ectodermal as well as endodermal areas. On the other hand, these data cannot be taken into account as decisive, since only precise fate-mapping studies utilizing labelling of tissues by means of stable markers can provide direct evidence on the embryonal origin of studied structures (Stern & Fraser 2001). Only direct evidence is a real evidence. Moreover, there was a number of reports on the germ-layer origin of teeth in urodeles that used identical approaches of classical histology, but such reports did not lead to similar conclusions. This again calls for the need of precise fate-mapping of germ-layers that could have an influence on tooth development. Lack of these studies is just the reason, why endodermal origin of tooth enamel epithelia has not been taken into account until very recently (Soukup et al. in press, see Supplements Research Article).

3. MATERIALS AND METHODS

3.1. Handling of the Embryos

Wild-type, white and GFP-transgenic embryos of the Mexican axolotl were obtained from Elly Tanaka's axolotl colony of Max Planck Institute Dresden. Embryos were let to develop in a tap water either at room temperature, at 7°C or at 4°C according to request. Staging was done according to Bordzilovskaya et al. (1989) and Nye et al. (2003). Neurulae (stage 13-17) were washed in a tap water and, afterwards, maintained in 1-strength Steinberg [100 ml 4-strength Steinberg + 300 ml dH_2O + 4 ml antibiotic/antimycotic (Gibco) + 40 µl 0.1 µM Fortum antibiotics (GlaxoSmithKline)]. Embryos prepared for operations were placed into Petri dish lined by 2% Agar (Sigma) and were manually dejellied by forceps. Next day, immediately before the operation, vitelline envelope was removed by forceps and embryos were positioned into beds in the agar lining for surgery.

3.2. GFP Ectoderm Transplantations

Transplantations of prospective oral ectoderm from GFP transgenic embryos into white specimens were performed at early neurula stages (stage 13-17). Several areas were transplanted in order to ensure grafting of the whole prospective oral ectoderm (Fig. 3).

First, the border of certain area (double-layered ectoderm) in GFP specimen was cut by tungsten microneedles. Then the adequate area was cut and removed from the wild-type embryo. The outlined area (double-layered ectoderm) of the GFP embryo was subsequently transplanted orthotopically into the wild-type specimen. Immediately, a small piece (ca 1×1 mm) of a glass cover slip was used to hold the transplant at accurate position for several minutes to enable healing of the wound. After approximately one hour, the operated embryo was removed from the operating bed and allowed to develop into required stage when fixed and analysed. Reciprocal graftings were performed as controls.

3.3. DiI Injections

A lipophilic dye, DiI (1,1'-dioctadecyl 1-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes), was used for focal injections into oral endoderm to specifically mark endodermal cells during the mouth development. This was done after removing of oral ectoderm from the wild-type embryo; that means before grafting of GFP-positive oral ectoderm. Again, several types of injections were performed to assure precise oral endoderm labelling.

A stock solution of DiI (10 μg DiI in 50 μl 100% ethanol) was sonicated by ultrasound to destroy potentially forming crystals. DiI stock solution was diluted 1:7 with 10% sucrose and sonicated again. This final solution was injected into embryos using microinjector IM 300 (WPI).

3.4. Sectioning

Embryos and larvae at stages 36-46 were overanesthetized by MS-222 (Serva) and immediately fixed in 4% PFA overnight. Specimens were then stored in azidebuffer (0.02% sodium azide in 0.1 M PBS) at 4°C to prevent bleaching of fluorescent signals by PFA.

For vibratome sectioning, embryos were first put in 0.1 M PBS. After 10 min, they were embedded into 2.5% Agar (Sigma) and sectioned on vibratome (Leica VT 1200S or Series 1000, TedPella System). The sections (50 or 100 μm thick) were transferred into 0.1 M PBS in Petri dish and prepared for immunostaining.

Embryos prepared for cryostat sectioning were washed in 0.1 M PBS three times and placed into 15% sucrose at 4°C overnight. Specimens were embedded in Tissue Freezing Medium (Jung) and put into cryostat (-20°C) for several hours to freeze properly. Frozen blocks were sectioned in cryostat (Leica CM 3050S) and acquired sections were adhered onto SuperFrost slides. Slides were dried at room temperature and then transferred into moisture chamber for immunostaining.

3.5. Immunostaining

Vibratome sections (50 or 100 μm thick) were first washed in 1% BSA for 10 minutes. Next, polyclonal rabbit anti-human Fibronectine (DakoCytomation) staining (diluted 1:100 with 0.1 M PBS) was used overnight to label tissue borders. Following day, Cy3-conjugated goat anti-rabbit secondary antibody (Dianova) was applied overnight after one washing step (0.1 M PBS). Finally, sections were counterstained by DAPI (diluted 1:50 with 0.1 M PBS) to visualize cell nuclei.

Cryostat sections (20 μm thick) were washed in 1% BSA for 10 minutes and 0.1 M PBST [0.4% Tween 20 (Sigma-Aldridge) in 0.1 M PBS] for 10 minutes to allow better binding of antibodies. Next, a monoclonal mouse anti-Calbindin-D-28K antibody (Sigma) (diluted 1:100 with 0.1 M PBST) was applied to label ameloblasts of developing teeth (Barlow & Northcutt 1997). After two days, the sections were washed five times in 0.1 M PBS and a secondary antibody was applied overnight. Alexa Fluor 647 goat anti-mouse

(Molecular Probes) was used onto sections from DiI injected larvae; otherwise Alexa Fluor 594 goat anti-mouse (Molecular Probes) was applied (diluted 1:100). Anti-GPF rabbit IgG/ Alexa Fluor 488 conjugate (Molecular Probes) (diluted 1:100) was used after five washing steps (0.1 M PBST) to re-visualize transplanted ectoderm in those sections where the GFP signal was bleached. Finally, the sections were washed five times in 0.1 M PBS, mounted in DAPI mounting medium (Vectashield) and the slides were coverslipped. Next day, the slides were covered by nail lacquer to prevent drying of the mounting medium. Slides were stored at 4°C.

3.6. Resin histology

Wild-type larvae fixed in 4% PFA were dehydrated through a successive series of ethanol and put into JB-4 (Polysciences) A + C solution overnight for infiltration. Next day, specimens were embedded in JB-4 A + C solution into which a polymerization substance was added. Anaerobic conditions ensured polymerization and resulted in a hard plastic resin block. Resin blocks were cut and embedded again to ensure exact embryo position in a block. These blocks were fixed to blockholders and cut by a microtome (Leica RM 2155) (for details see Supplements JB-4 Embedding).

Sections (8 μm thick) were adhered to Poly-L-Lysine (Sigma-Aldridge) coated slides and stained either by Azure B/Eosin (Serva) or Haematoxylin/Eosin (Sigma) (for details see Supplements Histological Staining). Sections were then mounted in DPX Mountant (Fluka) and coverslipped.

3.7. Software, Microscopes

Sections were photographed using fluorescent microscope (Olympus BX 51, SPOT RT Camera; Diagnostic Instruments Inc.). Details of teeth and stomodeal collar were photographed on inverted fluorescent microscope (Olympus IX 81, Orca Hamamatsu Photonics camera) using a Z-stack (thickness step: 1 μm). Acquired figures were then processed either by SPOT Advanced or by Cell^{R} software. Merging, colour change, enhancement and other subsequent adjustments of acquired images were done by Adobe Photoshop 6.0 and CorelDraw 12.

4. RESULTS

4.1. Histological Observations

First, I studied axolotl mouth development by means of classical histology. Serial sections counterstained by either Azure B/ Eosin or Haematoxylin/ Eosin were compared to figures displayed in older studies (see 2.5 Development of Mouth in Urodeles). I tried to distinguish ectoderm from endoderm on the basis of cell components, basically in an identical way compared to classical reports. Ectodermal cells contain a lot of maternal pigment (at least during early stages) and are thus well distinguishable from endodermal cells that possess many yolk granules. Based on occurrence of maternal pigment, formation of ectodermal collar was followed from the beginning of invagination through the course of mouth development. However, even before any sign of tooth development, the maternal pigment disappears from ectodermal cells making identification of the tissue origin quite difficult. Identification of the origin of cells is nevertheless still partially possible, because yolk granules within endoderm are resorbed much later.

Tooth germs were found as conical structures composed of mesenchymal cells (odontoblasts), which were covered by overlying epithelium (ameloblasts) (Fig. 1). The overlying epithelium of anterior tooth germs was made up of non-yolk-laden cells (Fig. 1B, C, D, green arrows), whereas posterior anlagen were covered by cells containing large amount of yolk granules (Fig. 1B, C, D, red arrows). Presence of yolk in the enamel epithelium could reflect contribution of endodermal cells to tooth germs. However, the distinction between cells containing or non-containing yolk granules was not really specific and the exact limit between oral ectoderm and foregut endoderm, thus, could not be determined in detail. Therefore, another approach to study oral and dental development in axolotl was utilized.

4.2. Transplantations of GFP Ectoderm

Since the germ-layer origin of the tissues contributing to oral and dental epithelia and their borders could not be determined by classical histology, I have utilized transgenic animals and fate-mapping approaches. Oral and dental development of axolotl was thus studied by following the fate of specifically labelled tissues. In order to detect the development of the ectodermal collar, I took advantage of recently developed GFP-transgenic axolotls (that express Green Fluorescent Protein in the cytoplasm of all cells; Sobkow et al. 2006) and performed homotopic transplantations of prospective oral ectoderm from GFP-embryos into white hosts (Fig. 2). The operations were performed at early neurula stages (st.

13-17), i.e. when neural folds are prominent, thus, early enough for the appearance of neural crest cells (Cerny et al. 2004), and when prechordal or lateral mesoderm does not fully reach the anterior part of the embryo (Adelman 1932). Ectoderm at this area consists of a double-layered epithelium directly juxtaposed to endoderm making it easy to extirpate and perform transplantations. Moreover, the cells contain maternal pigment and are thus well distinguishable from underlying endoderm.

Four types of transplantations were performed in order to determine the exact range of ectoderm that would invaginate and form the epithelia of mouth (Fig. 3). First transplantations involved the area just ventrally to the transverse neural fold (*n*=15 Fig. 3A). This operation resulted in labelling of the majority of oral ectodermal cells, but anteriorly, there were observed also non-GFP ectodermal cells migrating within the collar (Fig. 3B, C). This suggested incorporation of cells from transverse neural fold, a possible source of precursors of adenohypophysis (Eagleson et el. 1986) that is at the leading edge of the collar (Kingsley & Thyng 1904). According to this proposal, either transverse neural fold only (*n*=2) or transverse neural fold together with the area directly ventrally to it (*n*=8) were grafted (Fig. 3D, G). Sections of these embryos revealed contribution of transverse neural fold not only to outer epithelium, nostrils, brain and collar (Fig. 3F), but also to neural crest mesenchyme, cartilage and odontoblasts (Fig. 3F, I). Since lateral portions of transverse neural fold possibly gave rise to neural crest cells (Chibon 1967a; 1967b), only a middle portion of this tissue together with the ventral area ("T" shape) were included into the transplant (*n*=113; Fig. 3J). This type of transplantation ensured labelling of all the ectodermal cells that contribute to the collar and also surrounding epithelia, so that none of the non-GFP ectodermal cells could migrate into the mouth (Fig. 3K). Analyses of these embryos demonstrated labelled cells within nostrils, brain, collar and the surrounding external epithelia (Fig. 3L).

In order to ensure that the transplantation procedure is precise enough and that no non-GFP-ectodermal cells could contribute to formation of the mouth, the extent of the GFP graft was checked from the ventral side of larvae and could be also clearly visible on the sections (e.g. Fig. 3B, C). Embryos that possessed GFP-graft wrongly transplanted, were not included into analyses. As controls, non-operated GFP-larvae (*n*=3) were analyzed for the presence of GFP to ascertain that there is no loss of the signal within the cells during the course of development. GFP-signal was found within all cells of the larva so that no loss of signal takes place (Fig. 4A-C). Moreover, reciprocal transplantations according to Fig.2A (*n*=6; white graft into GFP embryo) revealed the same results (Fig. 4D).

4.3. Mouth Development in Axolotl

After transplantation of the prospective oral ectoderm from GFP embryo into the white host (Fig. 2), it was possible to follow the fate of these cells during the course of mouth development. First sign of mouth development is represented by an invagination of the inner layer of GFP oral ectoderm along the borders of contact with the oral endoderm (Fig. 5A). This initial situation was first observed at stage 34. Cells of the inner invaginated layer of ectoderm subsequently migrate on the surface of endoderm gradually ensheathing it in a ring-like manner and form the stomodeal collar (Fig. 5B). The outer layer of GFP oral ectoderm does not invaginate, but covers the whole oral area. In the stomodeal collar as well as directly posterior to it, there appears a number of epithelial bulges into the surrounding tissues with cone like structures, the anlagen of developing teeth (Fig. 5B). A horizontal cleft emerges among the cells of endodermal mass from stage 42 (Fig. 5C). During this process, endodermal cells detach from each other and "stick" to the walls of future mouth, which finally results in an oral opening (st. 43).

This specific mode of mouth development leads to interesting relationships of cells within the lining of the oral cavity. The oral epithelium is always double-layered, but the cells forming it are of different germ-layer origin. Whereas posteriorly, both layers of the epithelium are solely composed of endodermal cells, in the anterior portion of the oropharyngeal cavity, on the basis of the process of mouth opening, the detaching endodermal cells become the apical layer of oral epithelium, whereas the cells of the ectodermal collar give rise to the basal layer (Fig. 5D). Few ectodermal cells can be however found in the apical layer as well. The double germ-layer epithelia extend posteriorly up to approximately the level of Meckel's cartilage on the lower jaw and slightly behind the level of choanae on the palate, and anteriorly reach the margins of the mouth. Moreover, endodermal cells can be found, as a continuation of the apical epithelial layer, even outside of the mouth!

4.4. Mouth Opening

The process of mouth opening takes place via appearance of small gaps that join each other to form a cleft (st. 42). During this process, some endodermal cells are found to form already the apical layer of oral epithelium (st. 42-43), whereas others are still undergoing detachment (Fig. 6A-D). So, immediately before complete mouth opening, the upper and lower jaws are still contacting anteriorly (at the body surface), posteriorly (at the posterior limit of the lower jaw) or at both areas even in a single embryo (Fig. 6A-D). The anterior

connection is likely homologous to the oropharyngeal membrane found in other vertebrates. The membrane is visible as a double-layered structure composed of ectodermal cells anteriorly and endodermal posteriorly (Fig. 7A,B). Ruptures in the membrane appear simultaneously at various places (Fig. 6A, D) to form the oral opening. Cells of the ruptured membrane are likely becoming the cells of the oral epithelium (Fig. 7C,D). Whether there are any intercalations of cells between the outer and the inner layer and whether the rupture is caused rather by lowering the number of cells within the membrane due to apoptosis, or by increased proliferation of surrounding epithelia, was not studied. The membrane is situated even anteriorly to the lips (that are best visible in lateral parts, Fig. 7E,F) that form the oral margins.

4.5. Adenohypophysis

In the anterior portion of the stomodeal collar, there is a population of cells, which gives rise to adenohypophysis. At stage 40, these cells represent the median leading edge of the collar and its deepest invaginated part. Cells of the prospective adenohypophysis migrate to come into contact with the infundibulum, without losing their initial contact with other cells of the collar (Fig. 8A). After reaching this point, still before the formation of the cleft, adenohypophysis separates from the collar (st. 41) (Fig. 8B) and develops independently on it (Fig. 8C, D).

4.6. Non-Ectodermal Teeth

Developing tooth germs were first observed ca at stage 37, i.e. still during the formation of stomodeal collar, as epithelial invaginations into the surrounding mesenchyme. Tooth primordia develop within GFP-epithelia (ectodermal collar), however, similar cone-like structures can be found also more posteriorly in non-GFP areas (Fig. 5B, D). To confirm that these cone-like structures represent developing teeth, I used a monoclonal mouse anti-Calbindin-D-28K antibody (Sigma) that was shown to label (besides taste buds) enamel epithelia at specific stages of tooth formation (Barlow & Northcutt 1997). Anti-calbindin demonstrated development of tooth germs from both GFP (ectodermal) as well as non-GFP epithelia. Non-ectodermal teeth were found directly posteriorly to GFP ones on the palate (Fig. 9A-C) and in the postero-medial part of the lower jaw pointing medially (Fig. 9D).

4.7. DiI Injections and GFP Transplantations

To substantially demonstrate that oral teeth in axolotl are situated in the ectodermal as well as endodermal portions of the mouth, both of these tissues had to be specifically labelled. Therefore, I performed double staining, where ectoderm was labelled by GFP (as in the previous approach) and endoderm was tagged by a red fluorescent lipophilic cell tracer DiI (carbocyanine, Molecular Probes). This long-term stable marker, when injected into a tissue, binds to cell surfaces and does not dissociate so that it is widely used for tissue fate-mapping studies (Stern & Fraser 2001).

Embryos of the Mexican axolotl (*n*=91) were operated at early neurula stages (st. 13-17). First, the prospective ectoderm contributing to the mouth ("T" shape) was extirpated from the white embryo (Fig. 10A). Next, DiI was injected into the exposed endoderm (Fig. 10B) and, finally, the wound was covered by ectoderm transplanted from GFP embryo (Fig. 10C, D). Mainly three types of injections were performed to exactly tag the endodermal area, which is expected to give rise to foregut and oral endoderm. Either the whole exposed endodermal layer was injected many times (*n*=17; Fig. 10E), or only three focal injections were made into the area below the transverse neural fold (*n*=40; Fig. 10F), or the DiI was injected four times into the area directly ventrally to the transverse neural fold (*n*=23; Fig. 10G). Injections according to Fig. 10E were not specific enough, since DiI was then found in multiple tissues such as in brain, mesodermal derivatives and even in cartilages among others. Four other types of injections were performed (*n*=11 in total; data not shown), but these were not specific enough. Moreover, in many cases, these embryos did not develop oropharyngeal region properly possibly due to large amount of ethanol that is a dissolvent of DiI. The best oral endodermal labelling was obtained by injections below the transverse neural fold (Fig. 10F), while injections ventrally to transverse neural fold (Fig. 10G) sometimes also labelled oral endoderm.

4.8. Endodermal Derivation of Teeth

According to the double germ-layer labelling procedure (Fig. 10), it was possible to follow the fate of both oral ectoderm (GFP) and endoderm (DiI). This method therefore enabled specific labelling of endodermal layer and its possible contribution to tooth enamel epithelia.

In the sections, DiI was found not only in the endodermal mass and later in the apical layer of oral epithelial lining (Fig. 11, 12A, B), but also in tooth forming areas, i.e. in the

posterior part of lower jaw as well as on the palate (Fig. 11, 12, 13H) . DiI granules were found both in the outer and inner dental epithelium of teeth developing in these areas (Fig. 13). Contribution of DiI-labelled cells to tooth enamel organs thus supports previous indirect evidence and proves direct formation of teeth from endodermal epithelium.

4.9. Dual Origin of Tooth Enamel Epithelia

Dual fate-mapping approach enabled demonstration of derivation of teeth from ectodermal as well as from endodermal areas. However, at the leading edge of stomodeal collar, just at the contact zone of ectoderm and endoderm, there can be found tooth germs that have a dual (ecto-endodermal) origin of their inner enamel epithelia (Fig. 14). Ecto-endodermal teeth are represented either by incomplete covering by GFP ectoderm alone, or, in several cases, there were found GFP as well as DiI-labelled cells in the inner enamel epithelium at the same time, which gives further evidence of dual origin of enamel organ (Fig. 14D).

4.10. Germ-Layer Derivation and Distribution of Teeth

As already mentioned, teeth in axolotl can be grouped into dentary and splenial tooth fields on the lower jaw and into premaxillary/ maxillary, vomeral and palatal tooth fields on the upper jaw and the palate (Fig. 15A). Tooth germs on the palate cannot, however, be satisfactorily distinguished as vomeral or palatal on the sections because of their close proximity; these tooth germs are thus regarded as vomeropalatal in later analyses. Premaxillary/ maxillary tooth field is solely of ectodermal origin and is not present at the beginning; it develops later, when all the other tooth fields are already set up. Vomeropalatal teeth are either ectodermal (anteriorly situated, supposedly vomeral), endodermal (posteriorly situated, supposedly palatal) or dual (in between) (Fig. 15B). On the lower jaw, dentary teeth are in the majority of ectodermal origin with some exceptions (being dual) in the midline; on the other hand, splenial tooth field is mainly endodermal with some teeth of dual origin (Fig. 15C).

A quantitative analysis of the germ-layer origin of teeth of 26 embryos from double fate-mapping approach was performed (Tab. 1 and Fig. 16). This screening revealed that from 1137 teeth, 374 were of ectodermal, 598 of endodermal and 155 of ecto-endodermal origin. Interestingly, endodermal teeth are more frequent than ectodermal teeth, but the proportion of ectodermal teeth increases during the course of development. The high number of mixed ecto-

endodermal origin is also of great interest. Thus, for example, the average larva at stages 42-43, when the opening of the mouth takes place, possesses 56 teeth, from which 22 are of ectodermal, 28 of endodermal and 6 of ecto-endodermal origin (see Tab. 1 for detailed data).

Table 1. **Germ-layer derivation of axolotl teeth.**

st. 39	pmx/mx			vom+pal			den			spl			sum		
(n=4)	min	max	avg	min	max	avg	min	max	avg	min	max	avg	min	max	avg
GFP-ECT	0	0	0	2	5	3,5	0	6	3	0	0	0	3	11	6,5
non-ECT	0	0	0	3	5	3,75	0	0	0	7	12	8,75	11	16	12,5
DiI-END	0	0	0	0	5	1,5	0	0	0	0	5	2	0	6	3,5
non-ECT/ECT	0	0	0	2	3	2,5	1	3	1,75	0	1	0,5	3	7	4,75
DiI-END/ECT	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
sum	0	0	0	10	13	11,25	2	9	4,75	7	15	11,25	20	35	27,25

st. 40-41	pmx/mx			vom+pal			den			spl			sum		
(n=14)	min	max	avg	min	max	avg	min	max	avg	min	max	avg	min	max	avg
GFP-ECT	0	4	1,71	2	6	3,57	4	10	6,35	0	0	0	9	18	11,64
non-ECT	0	0	0	0	7	3,78	0	0	0	1	15	7,85	1	18	11,64
DiI-END	0	0	0	0	6	1,57	0	0	0	1	22	7,07	2	22	8,64
non-ECT/ECT	0	0	0	1	4	2,21	0	2	1,07	0	3	1,07	1	8	4,35
DiI-END/ECT	0	0	0	0	2	0,35	0	2	0,21	0	3	0,64	0	5	1,21
sum	0	4	1,71	10	14	11,5	4	11	7,64	9	26	16,64	27	49	37,5

st. 42-43	pmx/mx			vom+pal			den			spl			sum		
(n=6)	min	max	avg	min	max	avg	min	max	avg	min	max	avg	min	max	avg
GFP-ECT	5	10	6,33	4	8	5,16	7	13	10,33	0	0	0	18	29	21,83
non-ECT	0	0	0	3	8	6,16	0	0	0	7	15	11,83	10	28	18
DiI-END	0	0	0	0	1	0,33	0	0	0	1	16	9,5	1	17	9,83
non-ECT/ECT	0	0	0	2	6	4,16	0	2	0,5	1	11	2	2	8	5
DiI-END/ECT	0	0	0	0	1	0,33	0	2	0,5	0	1	0,16	0	2	1,16
sum	5	10	6,33	12	19	16,16	9	14	11,33	14	30	22	45	65	55,83

st. 44-45	pmx/mx			vom+pal			den			spl			sum		
(n=2)	min	max	avg	min	max	avg	min	max	avg	min	max	avg	min	max	avg
GFP-ECT	11	12	11,5	4	8	12	11	12	11,5	0	0	0	27	31	29
non-ECT	0	0	0	10	13	11,5	0	0	0	10	17	13,5	20	30	25
DiI-END	0	0	0	0	1	0,5	0	0	0	16	16	16	16	17	16,5
non-ECT/ECT	0	0	0	5	8	6,5	2	3	2,5	0	1	0,5	8	11	9,5
DiI-END/ECT	0	0	0	0	0	0	1	1	1	0	0	0	1	1	1
sum	11	12	11,5	24	25	24,5	15	15	15	27	33	30	77	85	81

Table shows numbers of tooth germs according to their germ-layer derivation and position within different tooth fields. GFP-ECT, teeth having GFP-ectodermal enamel epithelium; non-ECT, teeth having non-labelled enamel epithelium (supposedly endodermal); DiI-END, teeth having DiI-endodermal enamel epithlelium; non-ECT/ECT, teeth having enamel epithelium composed of GFP-ectodermal cells as well as non-labelled cells; DiI-END/ECT, teeth having GFP-ectodermal as well as DiI-endodermal enamel epithelium.

5. DISCUSSION

Past studies in urodeles have shown, by means of precise fate-mapping, that odontoblasts of teeth have their origin in cranial neural crest cells (Chibon 1967a; 1967b) and that enamel organs are of ectodermal origin (Chibon 1970). My study however unequivocally demonstrated that teeth situated in the mouth of the axolotl are generated by cells of ectoderm, endoderm as well as by cells of both germ-layers (Fig. 11, 12, 13). My data, thus, represents the first reliable evidence on direct contribution of endoderm to tooth enamel epithelia and thus to tooth initiation and also to formation of the enamel. Although there were already some authors that came to similar conclusions (Adams 1924; Reisinger 1933; de Beer 1947), their work was purely based on descriptive histology. However, such approach does not guarantee any stable and reliable marker of ectodermal or endodermal cells for satisfactorily long time. That is maybe why some authors (Marcus 1930; 1932; Gerhardt 1932) did not bring in similar conclusions. Only precise fate-mapping of tissues labelled by a stable marker is a tool for seeking the embryonic origin of developing organs (Stern & Fraser 2001).

5.1. Development of Mouth in Urodeles and in Other Vertebrates

Fate-mapping of the ectoderm and the endoderm enabled me to visualize both of the crucial germ-layers playing role during the development of mouth in the axolotl. Therefore, it was possible to compare different modes of oral formation that take place in urodeles and in other vertebrates (Fig. 17).

Generally, it is understood that vertebrate oral development advance through several characteristic steps (Balinsky 1975; Kardong 1995; Dickinson & Sive 2006). Early manifestation of oral development is a massive invagination of oral ectoderm that forms a blind pocket-like structure, the stomodeum. On the account of the ectodermal invagination, the stomodeum at its posterior limits contacts foregut endoderm, so that these tissues together form a transient double-layered oropharyngeal membrane. Thinning of the membrane and its eventual perforation finally causes opening of the stomodeal (oral) cavity into the foregut.

In the axolotl, and also in other urodeles (*Pleurodeles*, Chibon 1970; *Hynobius*, Takahama et al. 1988), the developing mouth displays different tissue dynamics compared to the general scheme of oral formation (Fig. 17). No distinct stomodeum is visible; however, invagination of the oral ectoderm nevertheless occurs (Fig. 5). Thus, whereas the outer layer of oral ectoderm stays intact covering the whole oral area, the inner layer inflexes to form the

stomodeal collar (Fig. 5A, B). The cells of the collar gradually populate the surface of the solid oral endoderm. Finally, mouth opens due to formation of a cleft that appears among the endodermal cells (Fig. 6).

Although oral development in urodeles results in the same feature, i.e. opened mouth, the way, in which this is achieved, is dissimilar from the situation in other vertebrates. Moreover, the epithelial lining of the mouth cavity also seems to be different. Generally, it is assumed that the oral cavity is lined by the ectodermal epithelium anteriorly and endodermal epithelium posteriorly with a sharp border in between them. However, in urodeles, the alternative mode of mouth development clearly leads to the alternative distribution of cells from different germ-layers. Interestingly, whereas the posterior portion of the urodele oral cavity is lined by endoderm, the anterior part is composed of cells of dual germ-layer origin: the ectodermal basal layer (formerly stomodeal collar) and the endodermal apical layer (formerly solid oral endoderm) (Fig. 5D, 17). The ecto-/ endodermal border is thus situated in between the apical and basal layer of oral epithelium. This is the first reliable demonstration of epithelium where the apical layer has different germ-layer origin than the basal layer.

Similar double-germ-layer epithelium was, however, based on histology, reported also from the pharyngeal cavity in carp (Edwards 1929; 1930). Such an epithelium was supposed to be an outcome of translocation of superficial ectodermal cells on the surface of pharyngeal endoderm that was observed to take place during the perforation of pharyngeal pouches (Edwards 1929). Interestingly, the apical layer of this epithelium was proposed to be of ectodermal origin, whereas the basal layer was assumed to be an endodermal derivative (Edwards 1929; 1930), i.e. reciprocal condition according to double-germ-layer epithelium in the axolotl (Fig. 5D, 17). Therefore, such epithelium demonstrates a considerable plasticity of both ectoderm and endoderm while generating various types of epithelia.

5.2. Oral Development via the Stomodeal Collar

The alternative mode of mouth development in axolotl, most notably the presence of oral endoderm, might not be solely an autapomorphy of urodeles. Kerr (1902; 1910) observed absence of stomodeum and presence of (supposedly endodermal) cluster of cells in lungfish *Lepidosiren paradoxa* and paralleled this situation to axolotl oral development. According to his observations, during later stages of *Lepidosiren* development, no ingrowth of ectodermal layer was, however, detected, but a gradual transition from “ectodermal” to “endodermal” cells in terms of cell compartments was observed. Developing teeth were found deep within

the cluster of cells long before any signs of oral cavity formation. Also according to recent authors working with *Neoceratodus* embryos, the oral development in dipnoans clearly resembles that of urodeles (J. Ziermann pers. comm.).

Ingrowth of ectodermal cells compared to that found in urodeles was found also in the frog *Ascaphus truei* (Reiss 1997). This ingrowth is however not represented by a collar ensheathing the anterior portion of foregut endoderm in a ring-like manner (Fig. 17), but it is formed by ectodermal bands running back along the gut wall from the corners of the mouth. It was speculated that such alternative mode of oral development might be somehow correlated with the ventral position of mouth in *Ascaphus* compared to other anurans (Reiss 1997).

Comparable oral development in urodeles, dipnoans and *Ascaphus* suggests that this feature could be more common within vertebrates. Because from positions of these groups on the cladogram it is not probable that this feature might represent a character once achieved from a common ancestor, it seems to be more probable that this feature was achieved several times separately, i.e. via analogical evolution. We can therefore ask whether these groups do have any particular features in common that might serve as a prerequisite of such a special type of oral development. As pointed out by Olsson (2003), one of the most characteristic features of all these groups is the evolutionary tendency to the biphasic life style, i.e. aquatic larvae, whereas more or less terrestrial adults. Such an amphibic tendency clearly corresponds to potential changes in developmental pathways and evolutionary remodelling of tissues that, indeed, have to respond to perpetual circulative changes of surrounding environment. These developmental potencies are visible during metamorphosis into an adult animal (e.g. loss of outer gills or closure of pharyngeal slits in amphibians), but, interestingly, are found also within the larval period (e.g. the specialized feeding apparatus of extant frogs). No wonder that many craniofacial features and developmental processes are conserved within these groups as a potential result of external environmental cues (Olsson 2003).

A prerequisite for the alternative mode of mouth development might be a type of egg, particularly the amount of yolk. Urodeles, anurans as well as dipnoans have mesolecithal eggs and, thus, holoblastic cleavage (Gilbert 2000) and have a very similar embryonal and larval development (cf. Semon 1893; Nieuwkoop & Faber 1967; Kemp 1982; Bordzilovskaya et al.1989). Whether mouth development via stomodeal collar is caused by the amount of yolk within the egg is, however, a pure speculation. On the other hand, it seems that a majority of anurans as well as caecilians, which have mesoolecital eggs, too, develop their mouths via classical oropharyngeal membrane (Laubman 1925; Teipel 1932; Watanabe et al. 1984; Dickinson & Sive 2006).

Similarly, whether mouth development via the collar is the basis for development of teeth from endoderm, i.e. whether endodermal teeth could be found also in Dipnoi, remains to be elucidated. Dentition in dipnoans consists of large tooth plates situated on the palate and on the inner surface of the lower jaw (Kemp 2002), i.e. positions, where teeth develop from endoderm or ecto-/ endoderm in the axolotl (Fig. 15). Lungfishes thus represent promising model where the direct endodermal influence on the dental development would be interesting to demonstrate as in the axolotl.

5.3. On the Presence and Homology of the Oropharyngeal Membrane

None of the past authors that studied the oral development in urodeles, have observed a structure that could be homologized to double-layered oropharyngeal membrane (Kingsley & Thyng 1904; Johnston 1910; Landacre 1921; Adams 1924; 1931; Marcus 1930; Reisinger 1933; Ströer 1933; Balinsky 1947; de Beer 1947; Chibon 1970). Takahama et al. (1988) on the other hand proposed that the structure homologous to the oropharyngeal membrane is represented by both the outer layer of oral ectoderm together with the solid oral endoderm. The process of the horizontal cleft formation within the oral endoderm was then claimed as comparable to the rupture of such proposed "oropharyngeal membrane".

Careful staging and high number of studied axolotl specimens enabled me, however, to observe a structure comparable to the oropharyngeal membrane of other vertebrates. Homology of this structure to the oropharyngeal membrane of other vertebrates was determined according to following features: (1) It was found to be a double layered structure, anteriorly composed of a layer of the oral ectoderm and posteriorly formed by a layer of endodermal cells directly adjacent to the ectoderm (Fig. 7A, B). (2) It was physically connected with the surrounding oral epithelia. (3) It was situated at the anterior-most limit of the alimentary canal as a transient structure present at the end of cleft formation (st. 42 and 42-43, Fig. 7A, B). (4) Its perforation enabled continuation of the alimentary canal with the external environment (Fig. 7C, D; Fig. 6). Accordingly, from the double-layered structure, tissue composition, position according to other epithelia and functional cues, this structure can be homologized to the oropharyngeal membrane found in other vertebrates.

The oropharyngeal membrane of the axolotl can, on the other hand, be seen as a rather unique structure. It is represented by a continuation of the upper lip epithelium and connects the lower jaw in the area anteriorly to the lower lip (Fig. 6A, D; Fig. 7E, F). It was not studied

whether the process of the membrane perforation was caused by increased apoptosis within the membrane or by increased growth of surrounding epithelia. The latter mechanism, however, seems to be more possible, since from the sections it looks like if increased growth within surrounding epithelia or within adjacent mesenchyme of developing lips caused tension leading to perforation of the membrane. This mechanism could explain presence of endodermal layer within the lip epithelia and its extent up to the outer head surface (Fig. 5D).

5.4. Relation of the Oropharyngeal Membrane to Teeth

In the majority of vertebrates, mouth develops throughout an ectodermal stomodeum (Romer 1962; Kardong 1995). The oropharyngeal membrane is seen as the posterior-most structure of the stomodeal invagination, while teeth are considered to develop anteriorly within the ectodermal epithelium. The situation in the axolotl, as shown by my research, is, however, completely different (Fig. 17). All teeth develop posteriorly to the membrane (as do pharyngeal teeth in fishes) from either ectodermal, or endodermal or even mixed epithelia.

We might speculate that the stomodeal collar evolved as a response to the increasing amount of yolk. Accordingly, increasing number of endodermal yolk-laden cells in the mouth would result in the shift of ecto-/ endodermal border (oropharyngeal membrane) from its central position up to the anterior-most tip of the mouth[7]. In such a case, increasing amount of yolk would block the formation of teeth, as it is the ectoderm which is considered to be a central tissue for tooth initiation (Mina & Kollar 1987; Lumsden 1988). Then, the movement of the oral ectoderm inwards on the surface of the oral endoderm could be interpreted as a need of presence of ectodermal tissue at exact places, i.e. where teeth have to be initiated. The odontogenic potential for tooth induction and development would then shift posteriorly into the endodermal portion of mouth. The result of this hypothetical scenario is the initiation and development of teeth from the ectoderm, endoderm and from the mixture of both.

Altogether, the ectodermal invagination in urodeles (and maybe also in lungfishes and some frogs) is an odd structure. Why does the development of mouth in these taxa advance through such complicated tissue rearrangements and not via classical stomodeum? Why should there be any deep ectodermal invagination, either during the mouth development as in urodeles, or during the perforation of pharyngeal slits as in the carp (Edwards 1929; 1930)? Maybe the deep ectodermal invagination is just a remnant of past generations. Maybe it is a

[7] The presence of considerable cluster of endodermal cells nearly until the opening of the mouth is why the oropharyngeal membrane can be observed only in a short time-window and why it was shown only as late as in my study (see above), i.e. after a hundred years of research on urodele oral development (since Kingsley & Thyng 1904).

result of the crucial need of presence of this tissue for initiation and development of certain structures (teeth) at certain places. Further research on the development of mouth in urodeles as well as other taxa is needed to decipher why the oral formation in different animals advances via completely different modes.

5.5. Tooth Origin in Evolutionary Contexts

Indubitable evidence that tooth enamel epithelia can be derived from the ectoderm, endoderm or from the mixture of both is important from developmental as well as evolutionary point of view. The axolotl data, however, cannot apparently indicate anything about the plesiomorphic germ-layer derivation of teeth. Still, the evidence of endodermal teeth in axolotl has significant influence on tooth evolutionary scenarios and it can tell us something about the origin of teeth.

So, how can my data contribute to recent opinions on tooth evolution?

Outside-in theory proposes evolutionary origin of teeth from outer dermal denticles captured into the stomodeum during gradual ingrowth of external ectodermal epithelium. According to the logic of this process, teeth are always proposed as derivatives of ectoderm and neural crest (Reif 1982). Moreover, this theory does not take into account any endodermal influence on tooth development or initiation. Therefore, teeth developing at the interface of neural crest and oral endoderm refute the scheme of purely ectodermal derivation of tooth enamel epithelium. The outside-in theory should therefore, according to my data, take into account a shift from ectodermal oral teeth into endodermal oral teeth. Whether derivation of enamel epithelium from endoderm is, or once was, somehow dependent on ectodermal signals, or whether the tooth forming capacity was co-opted for endodermal areas, remains to be elucidated.

Alternatively, the inside-out theory suggests that, although teeth of recent vertebrates are probably found in the ectodermal areas, the place of their evolutionary origin is primarily restricted to the endodermal pharyngeal cavity (Smith & Coates 1998). Evidence of the axolotl teeth developing in the ectodermal as well as endodermal areas could support this scenario of the tooth evolution. Hence, the axolotl could represent a transitional stage, where endoderm (as well as ectoderm) has a direct contribution to tooth enamel epithelia. The situation in mammals, where the enamel of teeth is developing from the ectoderm directly adjacent to endoderm, but where the endoderm probably does not directly contribute to the cells of enamel organ (Imai et al. 1998), would represent a derived stage.

These tooth evolutionary scenarios offer schemes of gradual shift from denticles to teeth either in the outside-in or inside-out manner. Moreover, it was also suggested that tooth evolution might be explained by a combination of both of these theories. Combinatorial derivation of teeth from external ectodermal as well as pharyngeal endodermal areas and, most notably, their different patterning and developmental mechanisms could account for the diversity of dentitions within the wide variety of vertebrates (Tucker & Sharpe 2004). In the mouse, expression of several "pharyngeal" genes was found in the molar (but not incisor) region leading to an assumption that molars develop from (possibly ectodermal) epithelium that shares molecular characteristics with pharyngeal endoderm (Ohazama et al. unpublished). Mammalian multicuspid teeth (premolars and molars) were thus proposed to have evolved in an inside-out manner and unicuspid teeth (incisors and canines) in an outside-in manner. Tooth shapes and complexity would thus reflex different genetic mechanisms that evolved separately. The proposal of simultaneous outside-in and inside-out scenarios, however, implies that teeth within vertebrates would represent a convergently achieved, non-homologous character with separate evolutionary histories.

All teeth in axolotl larvae, however, look identically as single-cone shaped unicuspid elements no matter of their position. It is only later, when some differences in the shape and structure occur within axolotl teeth. The tendency to develop bicuspid pedicelate teeth takes place gradually only from the late larval period onwards (Kerr 1960; Wistuba et al. 2002). Differences in axolotl tooth structure are, therefore, correlated rather with the transition to pedomorphic or adult life than with their germ-layer origin. Moreover, all teeth in the axolotl, no matter of their germ-layer origin, show similarities in the mode of development, timing, position, shape and complexity (Adams 1924; Wistuba et al. 2002; and also this study), which suggests that they probably represent homologous elements with shared evolutionary history. Similarly, studies on the molecular control of oral (presumably ectodermal) and pharyngeal (presumably endodermal) teeth in fishes already revealed shared developmental mechanisms within these dentitions (Fraser et al. 2004; 2006a; 2006b; Debiais-Thibaud et al. 2007).

Both developmental data from the axolotl (this study) and molecular data from fishes (Fraser et al. 2004; 2006a; 2006b; Debiais-Thibaud et al. 2007) support the suggestion that teeth should not be considered as denticles mechanistically transferred into oral areas either in the outside-in or inside-out fashion. Teeth should rather be seen as derivatives of the interplay between the epithelium and adjacent mesenchyme and driven by reciprocal interactions of odontogenic signalling pathways. Teeth can thus be generated at all positions, where these

mechanisms take place, i.e. at the most desired areas of mouth and pharynx, no matter of the distribution of different germ-layers.

Hence, an alternative evolutionary explanation on the origin of teeth can be put forward here. Since axolotl teeth can be derived from ectoderm, endoderm or from a combination of both and still look identically, the epithelial germ-layer origin of teeth probably does not matter at all. One can easily imagine that external or pharyngeal denticles have always been part of the exoskeleton and have constantly been developing at the interface of neural crest mesenchyme and epithelium of either ectodermal or endodermal origin. Teeth might simply evolve from denticles found within the oropharyngeal cavity no matter of the germ-layer distribution, as their need to be produced at exactly defined places could not be tied to a single germ-layer (neither ectoderm nor endoderm). Therefore, details about the germ-layer derivation of respective epithelium might not be an essential component of both tooth evolution and development at all.

5.6. Ecto-/ Endodermal Boundary as the Site for Tooth Initiation

In vitro studies in urodeles demonstrated that tooth anlagen can develop only when cranial neural crest cells are co-cultured with both oral ectoderm and foregut endoderm (Wilde 1955; Graveson et al. 1997), while cultures of cranial neural crest cells together with either foregut endoderm or oral ectoderm were not able to produce teeth (Wilde 1955; Takata 1960). The boundary of ectoderm and endoderm was thus suggested to be the place necessary for the initiation of vertebrate teeth (Smith & Coates 2001; Johanson & Smith 2003; Smith 2003). Teeth or specialized patterned denticles could thus develop at any place alongside this boundary, whether situated at the margins of jaws, palate, or in the pharyngeal region and would be under control of inductive influence of ecto-/ endoderm. The supposed presence of ecto-/ endodermal boundary between pharyngeal pouches and at the gill arches was claimed to be the key prerequisite for patterned pharyngeal denticles of *Loganellia*, placoderms and actinopterygians (Johanson & Smith 2003). The supposed ecto-/ endodermal border along the jaw margins was even proposed to be a plesiomorphic feature of tooth initiation of all extant gnathostomes (Smith 2003).

In mouse, endodermal epithelium was found directly posteriorly to developing incisors as well as molars, leading to a proposal of ecto-/ endodermal boundary as the possible place of origin of teeth (Imai et al. 1998; 2004). My study demonstrated development of axolotl teeth anteriorly to and posteriorly to the ecto-/ endodermal boundary (Fig. 11, Fig. 12).

Moreover, a considerable number of tooth germs was found also directly at the ecto-/ endodermal contact (Fig. 14). The position of initial developing tooth germ, which could support the tooth inducing ability of the ecto-/ endodermal border, has not, however, been found yet. The supposed tooth inducing ability of the ecto-/ endodermal boundary is a hot question for further research, in which axolotl represents the most suitable model organism, since the distribution of epithelia can be quite easily demonstrated (as was shown in my study).

5.7. Tooth Induction: Epithelium or Mesenchyme?

Tissue recombination experiments in the mouse have identified the oral ectodermal epithelium as the central tissue in tooth development, in that it is the source of the first instructing signal for odontogenesis (Mina & Kollar 1987; Lumsden 1988). Signalling from the oral ectoderm to the underlying mesenchyme leads to the mesenchymal expression of variable homeobox-containing factors, which subsequently specify the type of developing tooth (reviewed in Jernvall & Thesleff 2000). In the mouse, the ectodermal epithelium is thus seen as the key inducing tissue, while adjacent mesenchyme is proposed to govern the type and shape of the tooth.

On the other hand, neural crest mesenchyme possessing the first inducing signal was proposed based on research on recombination experiments between chick oral epithelium and mouse cranial neural crest cells (Mitsiadis et al. 2003; 2006). Although extant birds do not possess any teeth, these experiments resulted in their formation. Aside from marginal positions, tooth germs were induced also at ectopic places suggesting that the mouse neural crest mesenchyme likely induced expression of signalling factors within the chick epithelium (Mitsiadis et al. 2003). Mouse neural crest thus may play a primary role in initiation of odontogenic programme in the chick oral epithelium by inducing or maintaining the expression of odontogenic signalling factors (like *Bmp4*, *Shh* and *Fgf8*) by yet unknown signal (Mitsiadis et al. 2006).

Similarly, primary instructing signals emanating from the mesenchyme are believed to govern also development of other integumental derivatives, such as hair and feathers, which, as the “first dermal message”, directs the overlying epithelium to form an appendage (Pispa & Thesleff 2003). The identity of this signal however remains unknown.

Presented evidence that teeth in the axolotl are generated by ectoderm, endoderm as well as by a combination of both germ-layers could support the leading role of neural crest

mesenchyme in tooth development. However, the possible primary epithelial signal cannot be excluded. The different germ-layer origin of oral epithelia does not reject the presence of their potential inducing ability. Since teeth are generated at exact positions along the jaw, it is not the distribution of germ-layers, but the interplay of signalling factors within the oral epithelium that govern the polarities of developing jaw primordia and specify the sites of prospective teeth. Whether neural crest mesenchyme somehow initiates or directs these epithelial interplays is not known. The question on tooth induction thus remains open for further research.

5.8. Are Axolotl Teeth Homologous Despite Different Embryonic Development?

The demonstration that teeth in the axolotl can apparently develop from ectoderm, endoderm as well as from a mixture of both germ-layers could suggest that these structures are not homologous because of their derivation from non-homologous germ-layers. Indeed, since it is the embryonic development which produces various structures within the body, it is natural to look upon embryology as the source of homologous organs, either within or between species. We might then ask a question: Can axolotl teeth be claimed as homologous organs despite their different germ-layer origin?

Actually, axolotl teeth might not be so unique in terms of derivation from non-homologous germ-layers, because also several other vertebrate organs are reported to develop from different germ-layers or different cell populations. Comparable epithelial origin as axolotl teeth is reported for vertebrate taste buds. Taste buds develop from the local epithelium lining the oropharyngeal cavity (Stone et al. 1995a; Barlow & Northcutt 1995). Based on their distribution in the oral area it was suggested that taste buds in the mouse develop from both ectodermal and endodermal epithelia (Stone et al. 1995b). Similarly, appearance of taste buds within the pharynx of carp, *Ameiurus* and shark and on the outer body surface of *Ameiurus* suggested their endodermal as well as ectodermal derivation (Landacre, 1907; Cook & Neal 1921; Edwards 1930). In the axolotl, taste buds were demonstrated, by means of fate-mapping, to be derivatives of endodermal epithelium (Barlow & Northcutt 1995). However, it was later proposed, based on assumed border between ectoderm and endoderm that they arise also in the ectodermal epithelia, and are induced by signals from endoderm (Barlow 2000). The tissue derivation of taste buds probably reflects the place of their need within the oral areas, pharyngeal cavity or the outer body surface, so

that they cannot be tied to one population of cells or one germ-layer. Similarly, although adenohypophysis has been demonstrated as an ectodermal derivative within several vertebrates (chick, Couly & Le Douarin 1985; *Xenopus*, Eagleson et al. 1986; mouse, Kawamura & Kikuyama 1992; Kouki et al. 2001; axolotl, this study), it was proposed to originate from endodermal epithelium in hagfish (Gorbman 1983).

Trabecula cranii[8] also represents a structure which might be generated by different cell populations. Although it is generally considered, together with other viscerocranial elements, to be a derivative of the neural crest mesenchyme in all vertebrates (Kardong 1995; Le Douarin & Kalcheim 1999), research in lamprey proposed rather mesodermal origin of structure called "trabecula" (Kuratani et al. 2004). Therefore, the lamprey trabecula was, based on possible mesodermal origin, homogized to parachordals (i.e. mesodermal elements) of jawed vertebrates. The true homologue of gnathostome trabecula was assumed, according to the neural crest origin, to be the cartilage of the upper lip (Kuratani et al. 2004). Similarly, one of the bones situated at the skull vault, namely the parietal bone, can also have different origins in different animals. Whereas in mouse, the parietal bone was demonstrated to arise from a cephalic paraxial mesoderm (Morris-Kay 2001; Jiang et al. 2002), chick parietal was claimed to have a neural crest origin (Couly et al. 1992; 1993; reviewed in Cerny et al. 2006 and Gross & Hanken 2008). These are just several examples where structures proposed to be homologous are derived from different embryonic tissues, but more examples like that can be found (e.g. de Beer 1947; Wagner 1994).

The proposal that homologous structures must be derivatives of homologous germ-layers stems already from the 19th century and results from the then influential evolutionary embryology and widely accepted germ-layer theory dogma (for discussion see Hall 1998 and literature therein). However, since then, it has been found many times that a considerable number of organs can develop via different developmental processes (Spemann 1915; de Beer 1947; 1971; Wagner 1994). The most classical example is the salamander lens, which is normally a derivative of external ectoderm, but which can be regenerated, after a surgical removal, from the iris mesoderm (Spemann 1915). Is this regenerated lens really homologous to the original one arisen from ectoderm? Probably is, because it has the same structure, tissue composition, position within the eye and relationship to other organs (Spemann 1915).

Axolotl teeth display identical characteristics no matter of their germ-layer origin as well: they are initiated at the same place (epithelial/ mesenchymal interface; Adams 1924; de

[8] Trabecula cranii is a cartilaginous element situated at the anterior portion of the vertebrate head, which constitutes the cranial base and physically separates the brain from the oropharyngeal cavity (Cerny et al. 2006).

Beer 1947; this study), advance through the same developmental stages (Wistuba et al. 2002; this study), are morphologically identical (cone-shaped structures covered by epithelial layer and filled with mesenchymal cells; this study and many others) and have the same tissue composition (Smith & Miles 1974; Wistuba et al. 2002). Therefore, although axolotl teeth can be derived from different, non-homologous germ-layer epithelia (having different embryonic histories), they are homologous in other aspects (position, structure, morphodifferentiation, etc.) and can thus be assumed to represent homologous structures.

Clearly, it has long been known that homologous organs can develop via non-homologous processes, from different tissues or parts of these tissues, or even from different germ-layers (e.g. Spemann 1915; de Beer 1947; 1971; Wagner 1994). It was therefore stated that: “It does not seem to matter where in the egg of the embryo the living substance out of which homologous organs are formed comes from” (de Beer 1971). I am of that opinion that although the germ-layer origin might not matter for the development of the organ, it might matter, however, for its history and evolution. It is not hard to imagine that if we knew the germ-layer origin of e.g. parietal bone (and its homologues) within the major clades of extant vertebrates, we would be able to restore its evolution (e.g. whether it was formerly a mesodermal derivative, when the potential for its production shifted into neural crest/ or mesoderm, or what mechanisms caused this shift, etc.). All the above mentioned examples, where structurally homologous organs are generated by cells of different germ-layer origin either within a single, or among different organisms, account for the great plasticity of respective tissues. It is, therefore, not striking that all these structures develop at places of contact of different tissues (mesoderm/ neural crest for parietale and possibly also trabecula, ectoderm/ endoderm for adenohypophysis, taste buds and teeth) as it is primarily the place (morphospace), where these structures have to be generated and function as morphological units no matter of the germ-layer derivation.

6. CONCLUSIONS

This project was meant to study development of mouth and teeth in the Mexican axolotl from the germ-layer dynamics point of view. By utilizing GFP (Green Fluorescent Protein) transgenic embryos and experimental-embryological techniques, it was possible to follow the fate of ectoderm and endoderm, the two germ-layers that line the oral cavity, during the course of embryonal and larval development. Such an approach enabled observations of the direct contribution of these germ-layers to tooth enamel epithelia.

Transplantation of oral ectoderm from the GFP embryo into the white host enabled visualization of this germ-layer during the process of oral development. This approach allowed reconstruction of the mode of mouth formation in the axolotl and its comparison to other vertebrates. Although in vertebrates, the mouth development generally advances through stages of a stomodeal invagination and formation of an oropharyngeal membrane, in the axolotl it is only the inner layer of oral ectoderm which invaginates to form a stomodeal collar, whereas the outer ectodermal layer covers the whole oral area. It is only later, when the formation of a cleft among the endodermal cells starts a process of mouth opening. An oropharyngeal membrane comparable to that of other vertebrates (which was demonstrated for the first time in urodeles) is the last obstacle before the complete oral opening. On the account of such an alternative mode of mouth formation, the oral epithelium in the anterior portion of the oral cavity is of dual germ-layer origin. Whereas the apical layer is the endodermal derivative, the basal layer originates from the ectoderm. Endodermal cells can be found, as a continuation of the apical layer, even outside of the mouth.

Tooth germs were found to develop within the stomodeal (ectodermal) collar as well as more posteriorly in the non-ectodermal epithelia. To substantiate the possible endodermal origin of these non-ectodermal tooth anlagen, a double germ-layer labelling approach was performed. Aside from the transplantation of GFP oral ectoderm into the white host, the endodermal layer directly beneath the transplanted ectoderm was focally tagged by a red fluorescent lipophilic cell tracer DiI. Such an approach enabled unequivocal labelling of all ectodermal cells and some of the endodermal cells. The DiI was found directly within the enamel epithelia of posteriorly developing tooth germs demonstrating their endodermal origin. Moreover, at the posterior limit of the stomodeal collar, there were found tooth germs developing directly at the contact zone of ectoderm and endoderm, so that these tooth germs possessed the enamel epithelium of a dual germ-layer origin. The tooth enamel epithelia in the axolotl are, therefore, of ectodermal (premaxillary/ maxillary, vomeropalatal and dentary

teeth), endodermal (vomeropalatal and splenial teeth) or of mixed ecto-/ endodermal origin (vomeropalatal, dentary and splenial teeth).

Obtained results are consistent with some of the preceding studies using classical histological approaches, but were achieved by the precise fate-mapping of ectoderm and endoderm, which enabled indelible labelling and following the fate of these tissues. Present study represents a milestone in the comparative-embryological research on tooth development in that it represents the first indubitable evidence on direct endodermal contribution to oral tooth germs. Such evidence, however, does not indicate where the tooth forming potential evolved from. Although it has long been suggested that teeth evolved from external dermal denticles as the denticle-producing ectoderm invaginated to form the stomodeum (outside-in theory), an opposing evolutionary scenario (inside-out theory), based on denticles found in the pharyngeal region of some ancient fishes, proposed rather endodermal origin of teeth. A combination of these theories was also brought in to account for the diversity of dentitions in extant vertebrates. Some teeth would, thus, have evolved according to the inside-out manner and some according to the outside-in manner. The dual evolutionary origin of teeth would, however, imply that teeth within vertebrates would not be homologous structures as they would not share the same evolutionary history. Axolotl teeth, however, look identically and share different aspects of their formation and structure, which implies that teeth within axolotl dentition are homologous structures despite the different germ-layer origin of their enamel epithelia. Moreover, tooth germs developing at the contact zone between ectoderm and endoderm indicate that the germ-layer origin of tooth enamel epithelia does not matter, because mesenchymal cells can apparently easily interact with both epithelial germ-layers forming teeth when in the oral cavity, pharyngeal cavity or on the skin surface.

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SUPPLEMENTS

Figures

List of Figures

Abbreviations used in Figures

a, adenohypophysis

b, brain

den, dentary tooth field

e, eye

ECT, ectoderm or ectodermal teeth

ECT/END, ecto-endodermal teeth

END, endoderm or endodermal teeth

h, heart

ha, hyoid arch

ll, lower lip

m, future position of mouth opening or opened mouth

ma, mandibular arch

Mc, Meckel's cartilage

n, nasal epithelium or nasal cavity

nc, notochord

oc, otic capsule

oe, oral endoderm

pal, palatal tooth field

pc, parachordalium

ph, pharynx

pmx/mx, premaxillary/maxillary tooth field

pq, palatoquadrate

spl, splenial tooth field

tr, trabecula

ul, upper lip

vom, vomeral tooth field

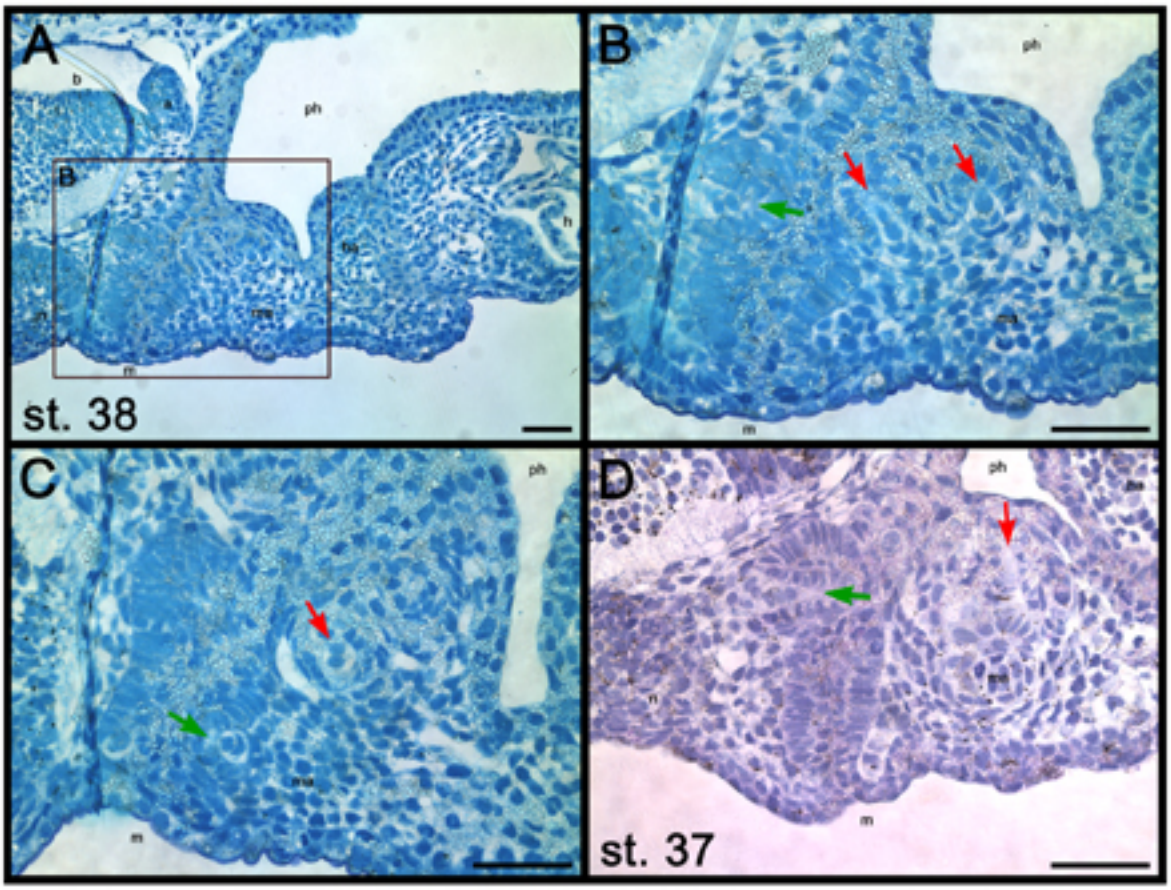

Fig. 1. **Histological observations on the development of the mouth in the Mexican axolotl.** Plastic resin sagittal sections, head to the left. Sections show yolk free cells on the surface of the yolk-laden oral endoderm. Tooth germs seem to be generated by cells both possessing (anteriorly, green arrows) and not possessing yolk granules (posteriorly, red arrows). (A-C) Azure B/ Eosin, (D) Hematoxylin/ Eosin. Scale bars = 100 μm.

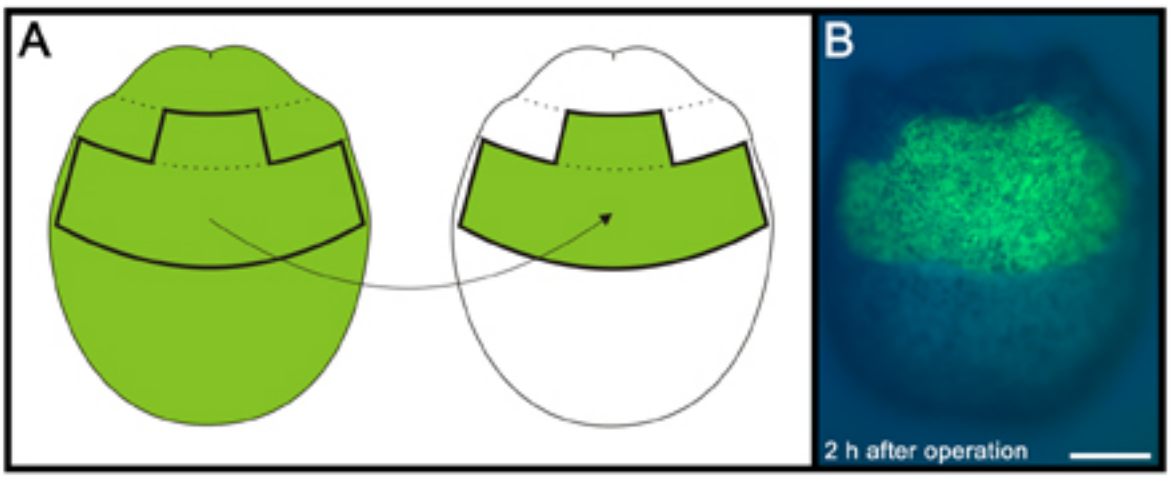

Fig. 2. **GFP oral ectoderm transplantation.** (A) A scheme of a transplantation of prospective oral ectoderm form the GFP embryo (green) into the white host. (B) The extent of the GFP graft two hours after the operation (stage 14). Scale bar = 500 m.

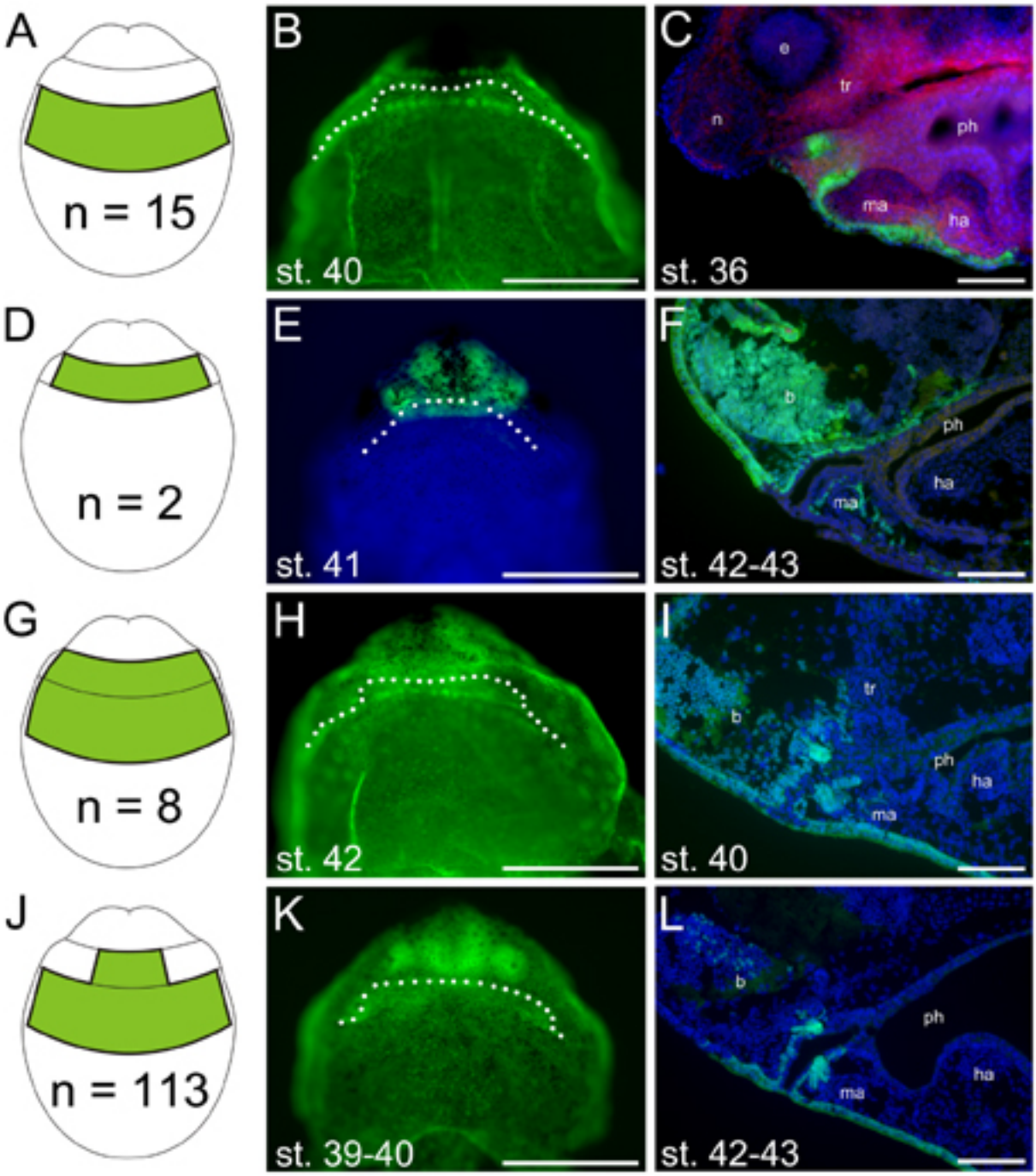

Fig. 3. **Types of transplantations performed in order to identify tissues contributing to mouth development.** (A, D, G, J) Schemes of transplanted areas; (B, E, H, K) ventral views of operated animals; (C, F, I, L) sagittal sections (head to the left) demonstrating contribution of transplanted tissues to mouth development; dots indicate the position of the future mouth opening. The area situated directly ventrally to the transverse neural fold (A, *n*=15) does not include all of the cells contributing to the mouth (B, C). Neural fold grafting (D, *n*=2) reveals that some of these cells participate in oral development (E, F). Transplantation of both of these areas together (G, *n*=8) ensure labelling of all the cells contributing to mouth (H); however, lateral parts of grafted neural fold possibly give rise also to neural crest cells (F, I). The most satisfactorily results were obtained by transplantation of a "T" shaped area including the ventral epithelium together with the median transverse neural fold (J, *n*=113). This area includes the ectoderm of the prospective oral epithelium as well as surrounding tissues (K, L), so that none of the non-labelled ectodermal cells could contribute to the mouth. Scale bars (B, E, H, K) = 1 mm, (C, F, I, L) = 200 μm.

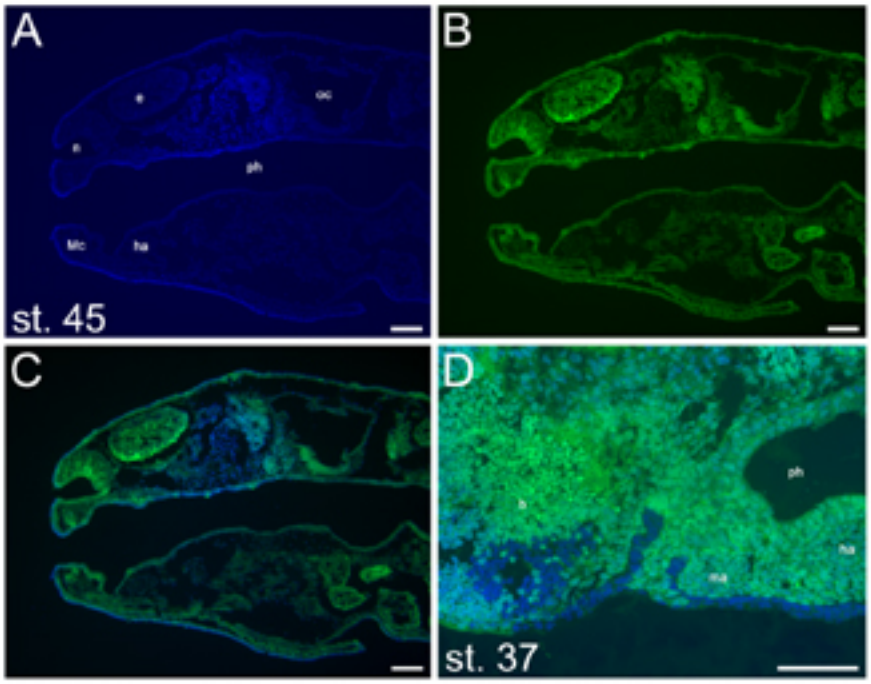

Fig. 4. **Controls.** (A-C) Sagittal section (head to the left) of a GFP larva demonstrating that there is no loss of GFP signal during the course of development. (A) DAPI, (B) GFP, (C) merged image. (D) Sagittal section (head to the left) of a GFP embryo with a white graft (reciprocal transplantation according to Fig. 2A) showing the same results as obtained by GFP to white grafting. Scale bars = 200 µm.

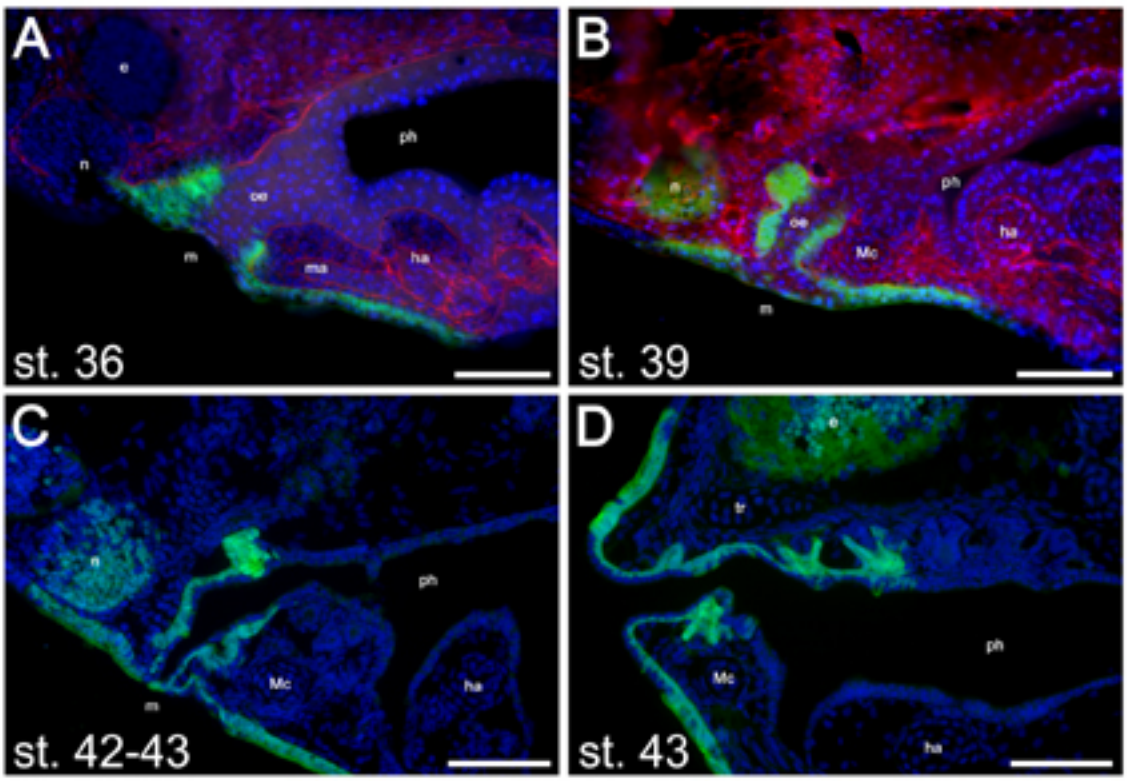

Fig. 5. **Oral development in the axolotl.** The development of the mouth begins by an inflexion of the oral ectoderm, which forms the stomodeal collar (A). The collar translocates on the surface of solid oral endoderm as a "sleeve" (B). The appearance of an horizontal cleft in the middle of this oral endoderm (C) eventually leads to opening of the mouth (D). Scale bar = 200 µm.

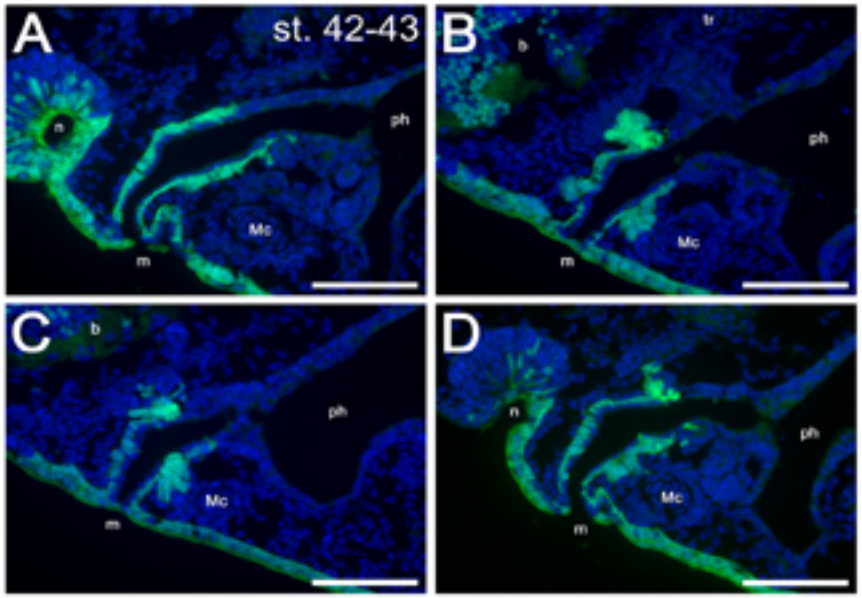

Fig. 6. **Opening of the mouth.** Sagittal sections, taken from a single specimen, show formation of the cleft, which directly precedes opening of the mouth. Whereas, at some positions, the mouth is completely opened (A), at other places, the upper and lower jaws are still contacting each other either anteriorly via oral membrane (B), or posteriorly at the posterior limit of the mandibular arch (D), or even in both of these areas (C). Scale bars = 200 µm.

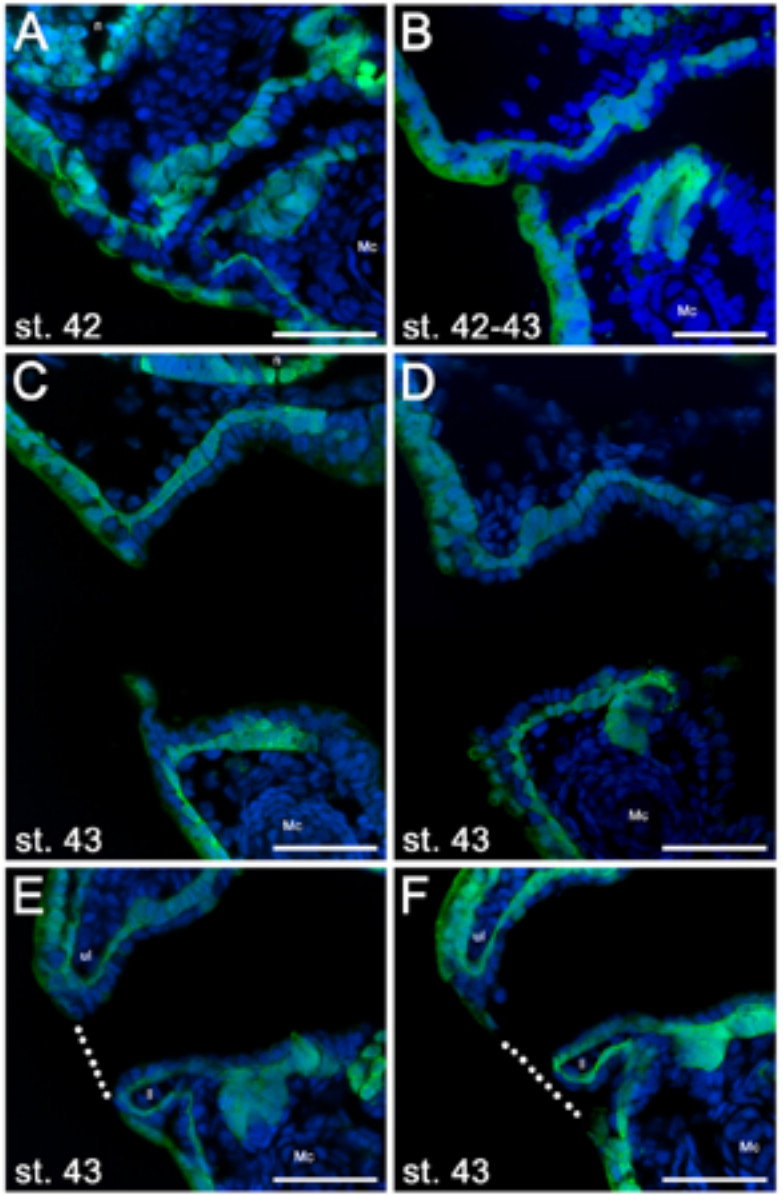

Fig. 7. **Oral membrane.** Oral membrane is first visible at the end of cleft formation as a double layered structure (A, B). However, it is subsequently ruptured (C), which causes opening of the mouth (D). (E, F) Lateral sagittal sections show remnants of oral membrane situated even anteriorly to lips. Scale bars = 100 µm.

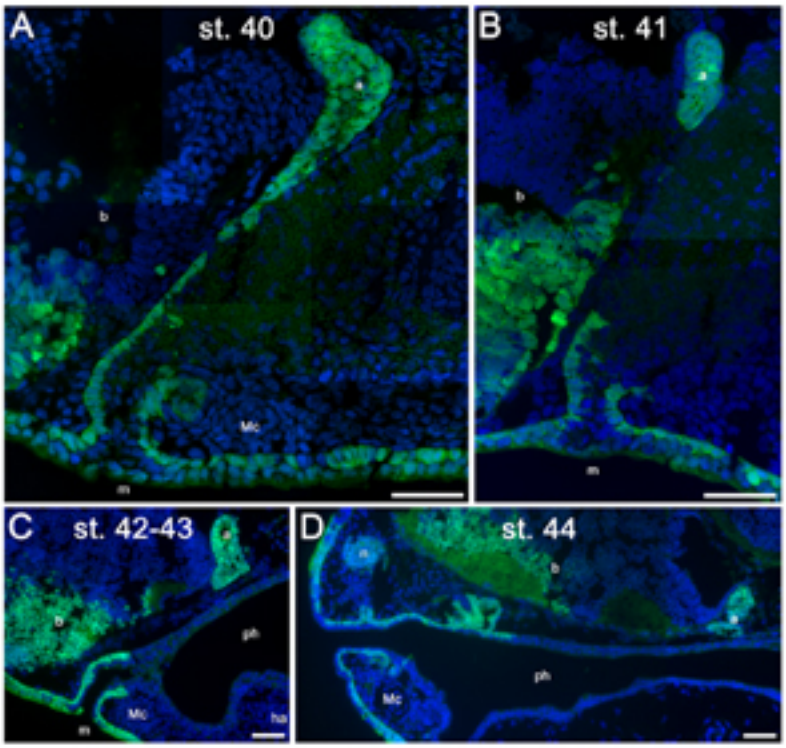

Fig. 8. **Development of the adenohypophysis.** Prospective adenohypophysis is situated at the leading edge of the anterior portion of the stomodeal collar, which later migrates to the level of the infundibulum, interestingly however, without losing the contact with the collar (A). The contact is lost only later (B) and, since then, adenohypophysis develops independently on the collar (C, D). Scale bars = 100 μm.

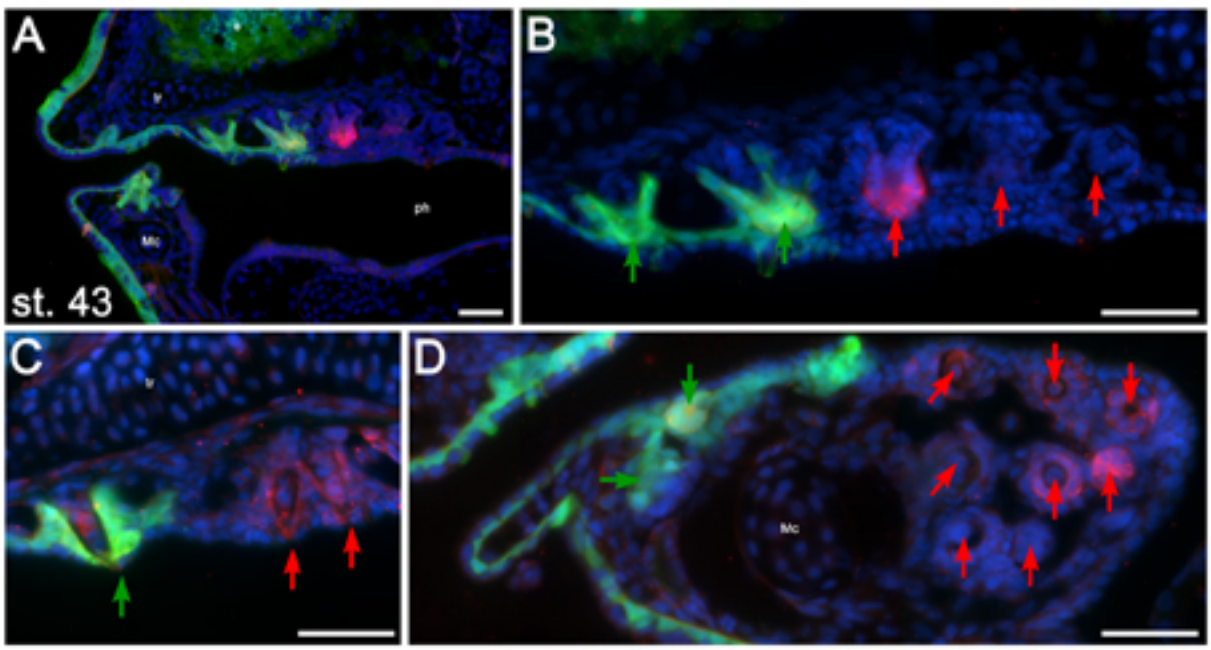

Fig. 9. **Non-ectodermal teeth.** Monoclonal mouse anti-calbindin antibody (Sigma) (red channel), which was shown to specifically label tooth germs in the axolotl (Barlow & Northcutt 1997), reveals developing teeth from GFP ectodermal (green channel) as well as from non-GFP epithelia. Non-ectodermal teeth are found posteriorly directly behind the limit of the stomodeal collar on the palate (A-C) and on posterior parts of the lower jaw (D). Scale bars = 100 μm.

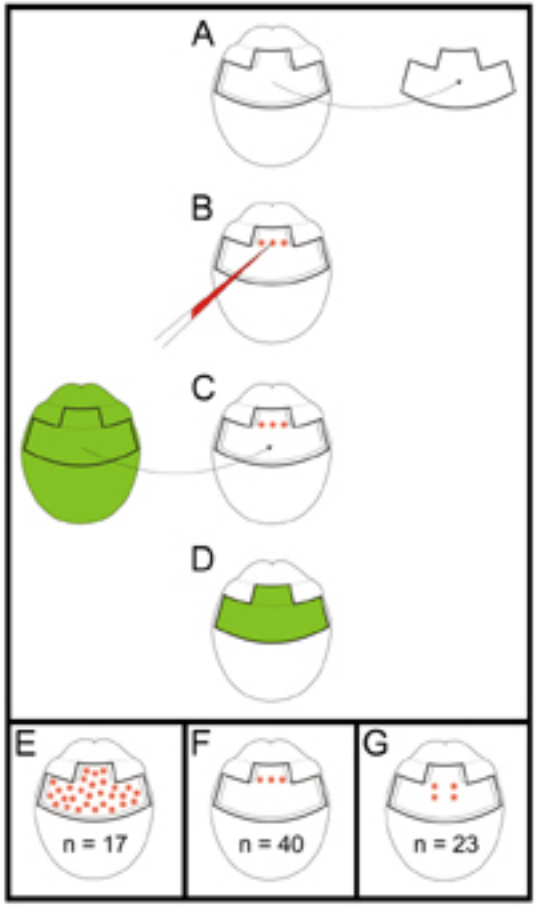

Fig. 10. **Dual labelling of oral epithelia.** An approach ensuring labelling of both ectoderm and endoderm was performed (A-D) to indelibly demonstrate that teeth in the axolotl do develop within endodermal areas. After removal of the ectoderm contributing to mouth from a white embryo (A), the exposed endoderm was injected by DiI (B). The wound was subsequently covered by the GFP ectoderm (C, D). Thanks to this aproach, all of the ectodermal cells contributing to mouth and teeth were GFP positive and some of the endodermal cells were tagged by DiI. Mainly three types of injections were performed (E-G). Either the exposed endoderm was injected many times (E, *n*=17), or only three times directly below the transverse neural fold (F, *n*=40), or four injections were made directly ventrally to the neural fold (G, *n*=23).

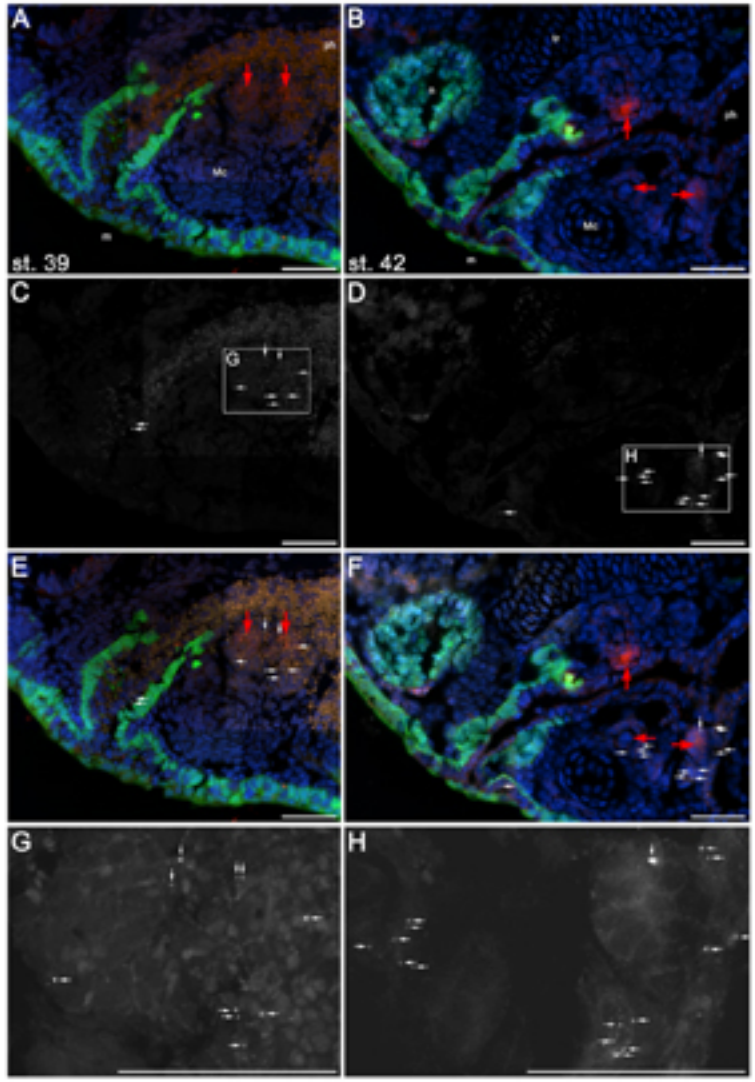

Fig. 11. **Detection of DiI injected into the oral endoderm.** (A, B) Sagittal sections, head to the left, showing the extent of GFP stomodeal collar (green channel) and developing teeth labelled by anti-calbinding (red channel, red arrows). (C, D) DiI granules (white arrows), enlarged in (G, H). (E, F) Merged images. DiI can be found in the endodermal mass as well as in the tooth forming region of the lower jaw. Scale bars = 100 µm.

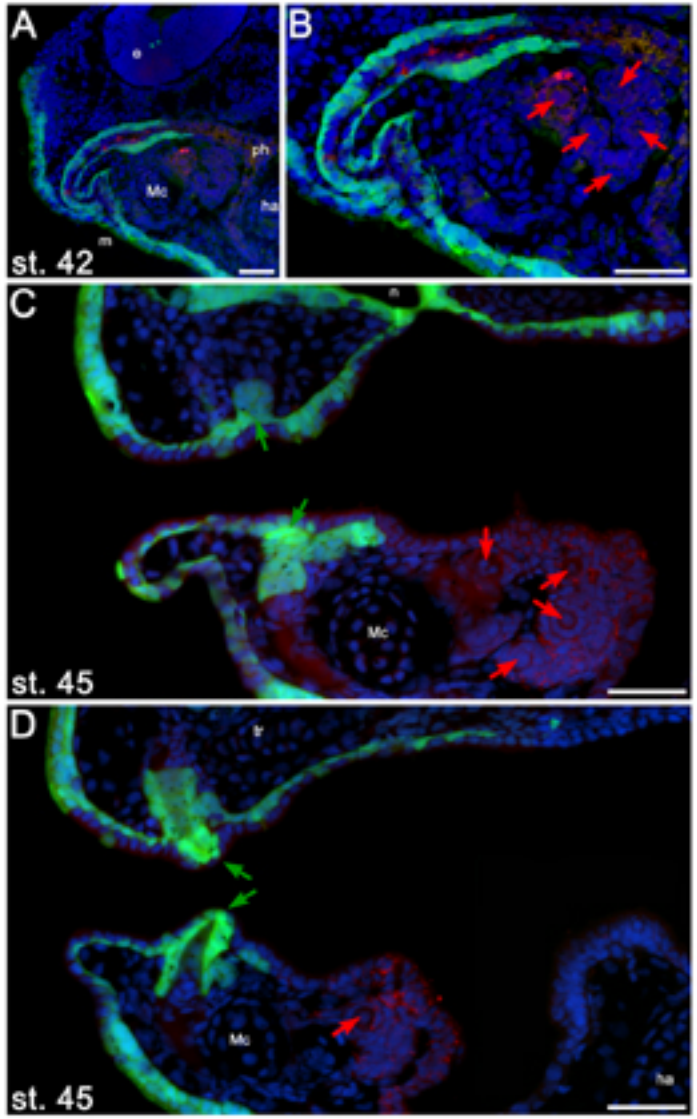

Fig. 12. **Detection of DiI injected into the oral endoderm.** Sagittal sections show DiI (red channel) situated in the oral endoderm between the dorsal and ventral portion of the stomodeal collar (A, enlarged in B), and in endodermal teeth of the lower jaw (red arrows) localized posteriorly to ectodermal teeth (arrows). Scale bars = 100 µm.

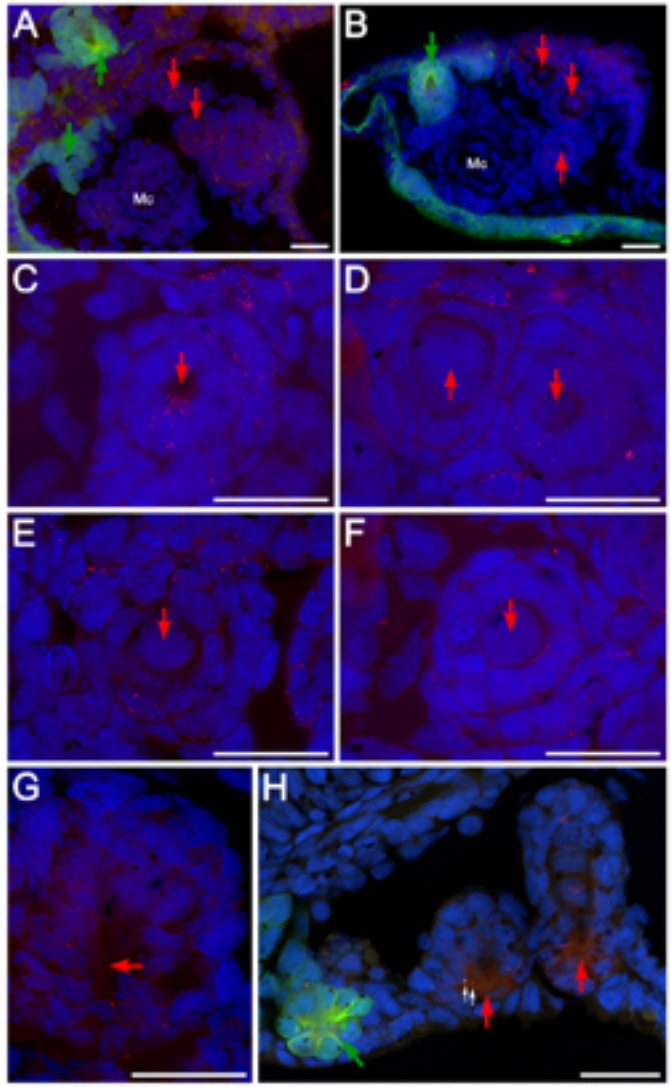

Fig 13. **DiI-endodermal teeth.** DiI granules (red channel in A-G, white channel and arrows in H) are found in the outer as well as inner enamel epithelia of some teeth, which demonstrates their endodermal derivation (red arrows). Endodermal teeth are found posteriorly to GFP ectodermal teeth (green arrows) on the lower jaw (A-F) and on the palate (G, H). Scale bars = 50 µm.

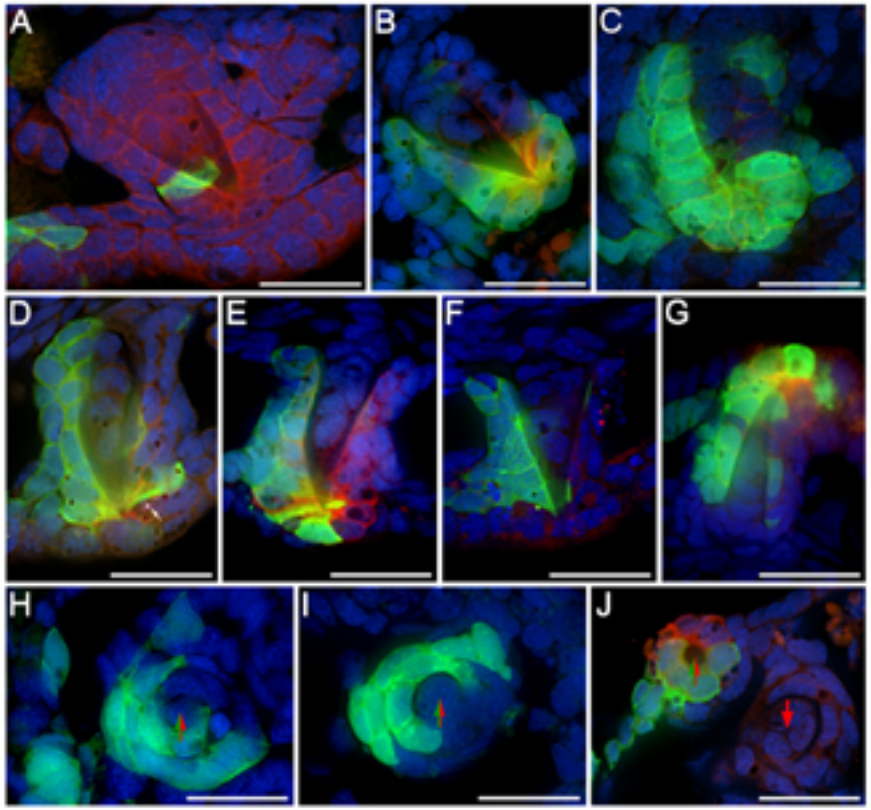

Fig. 14. **Dual origin of tooth enamel epithelia.** Teeth possessing the inner enamel epithelium of dual germ-layer origin (green/ red arrows) develop at the posterior-most limit of the stomodeal collar. The extent of the germ-layer contribution to enamel epithelia can vary from one ectodermal cell surrounded by endodermal cells (A) up to many ectodermal cells covering the tooth nearly completely with a little endodermal contribution (D, G). Ecto-endodermal teeth can be found on the palate (A-F), in the dentary tooth series (G) as well as in the splenial area (H-J). Red channel is anti-albindin, white channel and arrow in D indicates DiI granule, red arrow in J points to endodermal tooth. Scale bars = 50 m.

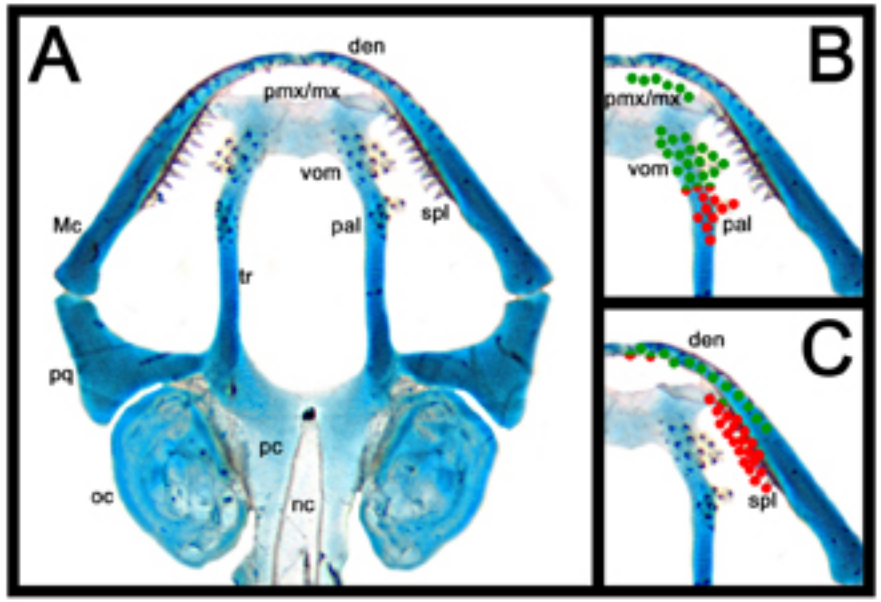

Fig. 15. **A scheme of the germ-layer derivation of axolotl teeth.** (A) A chondrocranium of axolotl larva (visualized by alcian blue staining) demonstrates the distribution of teeth into five tooth fields: premaxillary/ maxillary, vomeral and palatal on the upper jaw and palate; and dentary and splenial on the lower jaw. (B) Teeth on the upper jaw and palate. Premaxillary/ maxillary tooth field is of ectodermal origin (green dots). It was not possible, from the sections, to precisely distinguish between vomeral and palatal tooth fields and their germ-layer derivation. Nevertheless, these vomeropalatal teeth are derived from ectoderm (anteriorly, green dots), endoderm (posteriorly, red dots) as well as from both germ-layers (in between, green/ red dots). (C) Teeth on the lower jaw. Dentary tooth field is of ectodermal origin with several ecto-endodermal teeth medially. Splenial teeth are on the other hand endodermal in origin with few ecto-endodermal teeth.

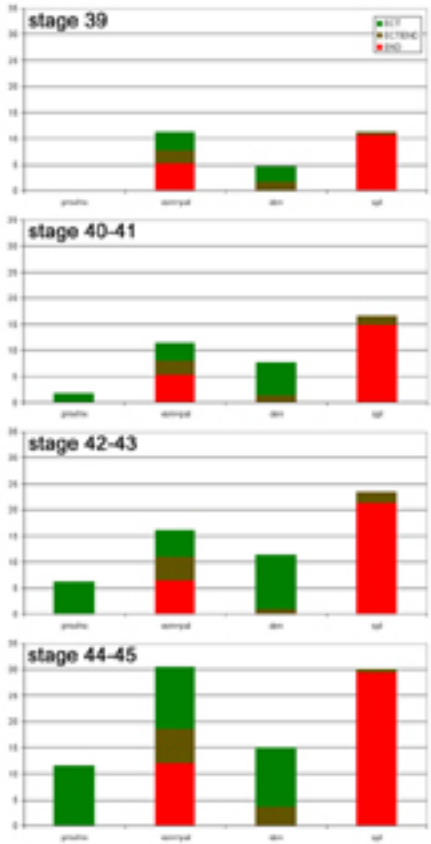

Fig. 16. **Germ-layer derivation of axolotl teeth.** Graph shows the number and germ-layer origin of teeth within tooth fields in four stages of axolotl development. Based on Table 1.

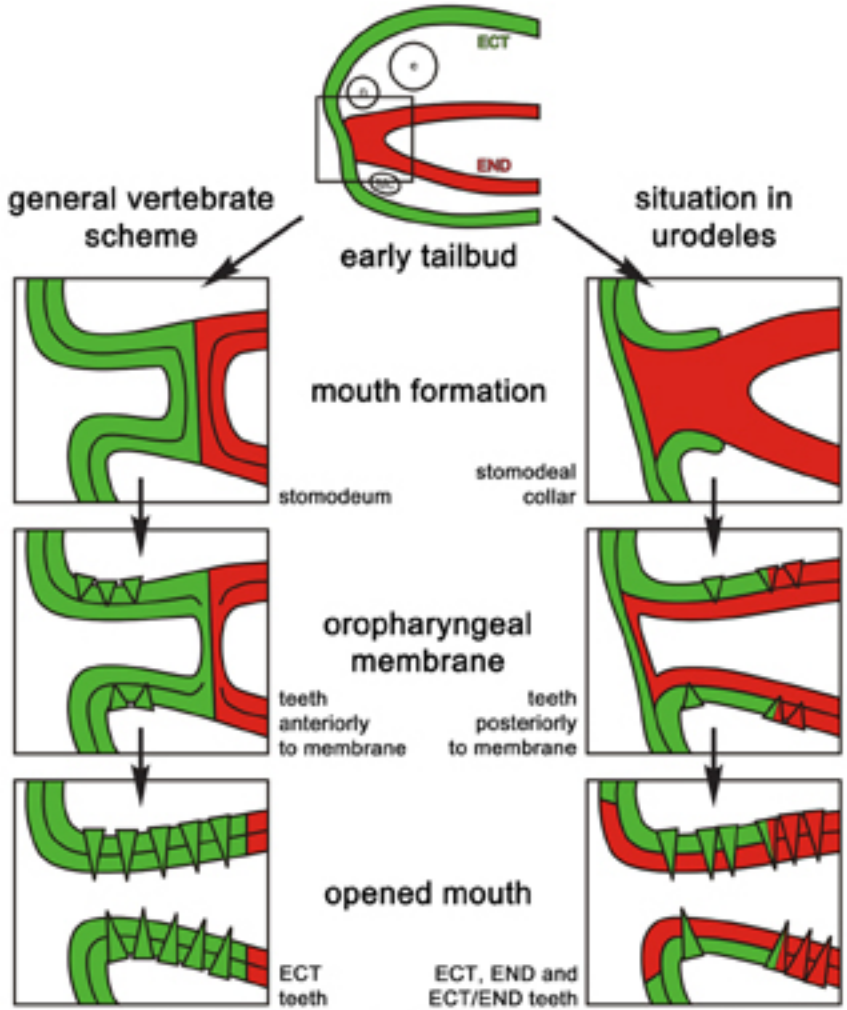

Fig. 17. **A scheme of oral development in urodeles and in other vertebrates.** Generally, mouth develops via the deep ectodermal stomodeal invagination, which, together with the foregut endoderm forms an oropharyngeal membrane. Perforation of the membrane causes opening of the mouth. Teeth are suggested to develop anteriorly to the membrane in the ectodermal areas. In urodeles, the mouth develops in a different way. Only the inner layer of the oral ectoderm invaginates and migrates on the surface of the oral endoderm, whereas the outer layer stays intact. The morphodynamics of the ectodermal layer and subsequent appearance of the cleft within the endoderm results in the formation of a double-germ-layer oral epithelium. The oropharyngeal membrane is situated at the anterior-most end of prospective oral cavity. Teeth develop posteriorly to the membrane from ectodermal, endodermal as well as ecto-endodermal epithelia. Perforation of the membrane eventually leads to the opening of the mouth.

Histological Techniques

JB-4 Embedding

Embedding for histology was performed by plastic resin JB-4 Embedding Kit (Polysciences) consisting of A, B and C solutions.

1) cut out a tail from specimen prepared for histology to ensure faster and better infiltration of solutions
2) specimen fixed in 4% PFA wash in distilled water overnight
3) process through a successive series of ethanol

25% ethanol	15 min
50% ethanol	15 min
75% ethanol	15 min
75% ethanol	15 min
75% ethanol	15 min
100% ethanol	15 min

4) wash in A + C solution (100 ml A solution + 1.25 g B solution)
5) put into A + C solution overnight

6) embed in A + B + C solution (add 1 drop of B solution into 1 ml A + C solution) in embedding plate (Polysciences) covered by parafilm overnight
    - anaerobic conditions cause embedding solution to form a hard resin block

7) cut the resin block and embed again (according to step 6) (together with a blockholder) overnight to ensure exact positioning of specimen for subsequent sectioning)

8) section the resin block by Leica RM 2155 microtome sagittally (thickness 8 μm, knife angle 6°)
9) put the sections onto a drop of water on Poly-L-Lysine (Sigma-Aldridge) coated slides
10) dry the sections at 35°C

Histological Staining

Haematoxylin/ Eosin staining

Staining was performed on sections embedded in JB-4 synthetic resin; therefore, steps applying xylens or alcohols are not necessary.

Mayer haematoxylin 1000 ml dH_2O
1 g haematoxylin (Sigma)
50 g aluminium ammonium sulphate
0.2 g sodium iodate
1 g citric acid
50 g chloral hydrate

eosin staining solution 0.5 g eosin
100 ml dH_2O

1) slides with adhered sections wash in tap water and dry out
2) stain by Mayer haematoxylin for 3 min
3) wash in tap water several times, haematoxylin is strongly destaining
4) differentiate in 2% $NaHCO_3$ for 30 s
5) wash in tap water several times
6) stain by eosin staining solution for 2.5 min
7) wash in tap water several times and dry out
8) mount the sections in DPX (Fluka) and coverslip overnight

9) remove redundant DPX
10) isolate the coverslip by nail lacquer to prevent drying of the mounting medium

Azure B/Eosin staining

staining solution 400 µl azure-B
100 µl eosin
12.5 ml dH_2O

1) slides with adhered sections wash in tap water and dry out
2) stain by Azure B/Eosin staining solution for 3 min
3) wash in distilled water and dry out
4) mount the sections in DPX (Fluka) and coverslip overnight

5) remove redundant DPX
6) isolate the coverslip by nail lacquer to prevent drying of the mounting medium

Presentations of the Project

Main results of this project have been presented at a number of Czech as well as international conferences and, moreover, have been successfully published:

Scientific Talks

Soukup V, Epperlein HH, Horácek I & Cerny R

Oral teeth in extant vertebrates can be derived from ectoderm, endoderm and/or even from both germ-layers: implications for development and evolution
EED 2 (Euro Evo Devo 2) Ghent 2008
July 29-August 1 2008

Soukup V

Development of tooth germs: origin of vertebrate teeth in evolutionary contexts
Development and Regeneration in Vertebrates, Whitsun meeting, Zastler Hütte, Rinken, Black Forest, Germany
May 13-16, 2008

Soukup V, Epperlein HH, Horácek I & Cerny R

Oral morphogenesis in axolotl and the first evidence of (oral) endodermal teeth for gnathostomes
TMD-9 (Tooth Morphogenesis and Differentiation 9) Zurich 2007
September 4-8, 2007
Abstract in: European Cells & Materials Journal 14 (Suppl. 2), 5

Soukup V, Epperlein HH, Horácek I & Cerny R

Oral morphogenesis in axolotl and the first evidence of endodermal teeth for gnathostomes
ICVM 8 (International Congress of Vertebrate Morphology 8) Paris 2007
July 16-21, 2007
Abstract in: Journal of Morphology, 268: 421.

Soukup V

First evidence of endodermal teeth in gnathostomes
Early Developmental Processes in Vertebrates, Whitsun meeting, Amrum Island, Germany
May 31-June 4, 2007

Černý R & Soukup V

První evidence entodermálních zubů u obojživelníků
22. konference České herpetologické společnosti (Heřmanovice)
May 23-25, 2007
Abstract in: Herpetologické informace 6: 19.

Soukup V & Černý R

Orální morfogeneze axolotla a první evidence vzniku zubů z entodermu u čelistnatců
Zoologické dny Brno 2007
February 8-9, 2007

Posters

Soukup V, Epperlein HH, Horácek I & Cerny R

Development of oral teeth of endodermal origin in axolotl
Gordon Research Conference "Craniofacial Morphogenesis & Tissue Regeneration" Lucca (Barga), Italy 2008
February 10-15, 2008

This poster was awarded the Prize for the best poster in the Postdoctoral series in the Gordon Conference on "Craniofacial Morphogenesis and Tissue Regeneration".

Soukup V, Epperlein HH, Horáček I & Černý R

Evidence of tooth germs derived from ectoderm and endoderm in axolotl argues for a single evolutionary origin of vertebrate teeth
Zoologické dny České Budějovice 2008
February 14-15, 2008

Research Article

Soukup V, Epperlein HH, Horácek I & Cerny R (in press)
Dual epithelial origin of vertebrate oral teeth.
Nature.

doi:10.1038/nature07304

LETTERS

Dual epithelial origin of vertebrate oral teeth

Vladimír Soukup[1], Hans-Henning Epperlein[2], Ivan Horácek[1] & Robert Cerny[1]

The oral cavity of vertebrates is generally thought to arise as an ectodermal invagination[1,2]. Consistent with this, oral teeth are proposed to arise exclusively from ectoderm, contributing to tooth enamel epithelium, and from neural crest derived mesenchyme, contributing to dentin and pulp[3–5]. Yet in many vertebrate groups, teeth are not restricted only to the oral cavity[6–9], but extend posteriorly as pharyngeal teeth that could be derived either directly from the endodermal epithelium, or from the ectodermal epithelium that reached this location through the mouth or through the pharyngeal slits[6]. However, when the oropharyngeal membrane, which forms a sharp ecto/endodermal border[10], is broken, the fate of these cells is poorly known. Here, using transgenic axolotls with a combination of fate-mapping approaches, we present reliable evidence of oral teeth derived from both the ectoderm and endoderm and, moreover, demonstrate teeth with a mixed ecto/endodermal origin. Despite the enamel epithelia having a different embryonic source, oral teeth in the axolotl display striking developmental uniformities and are otherwise identical. This suggests a dominant role for the neural crest mesenchyme over epithelia in tooth initiation and, from an evolutionary point of view, that an essential factor in teeth evolution was the odontogenic capacity of neural crest cells, regardless of possible 'outside-in'[11] or 'inside-out'[12] influx of the epithelium.

Teeth are one of the key vertebrate innovations, but their evolutionary origins are still a matter of debate. It is widely accepted that teeth initially evolved from outer skin denticles captured in the stomodeum (the odontode theory)[11] and modified there specifically in the context of newly developed jaws ('outside-in' theory). However, as there is good evidence of teeth/denticles inside the pharyngeal regions of many fossil jawless groups[7,12], they must have evolved with a great degree of independence from the stomodeal cavity and the jaw elements. An alternative scenario reflecting these facts has been suggested, in which oral teeth arose by the progression of ancient denticles from the endodermal pharynx towards the stomodeum ('inside-out' theory)[12]. More recently, however, it was argued on the basis of fossil evidence that teeth may have evolved independently through a convergent evolution and, thus, are not homologous among jawed vertebrates[13]. A new, appealing hypothesis was then proposed, namely that the diversity and complexity of dentitions can be explained by combinatorial derivation of teeth from both external (ectodermal), and internal (pharyngeal) denticles[4].

Teeth are commonly ranked among ectodermal organs[5], although they are composite structures of dual embryonic origin. The dental mesenchyme has been shown, using a fate-mapping approach, to be derived from neural crest cells in mammals[3], urodele amphibians[14] (also this study; Supplementary Fig. 3) and fish[15], and this is generally assumed to be the case in other vertebrates as well[16]. The germ-layer origin of the epithelium, however, is far less clear. Because tooth development is most completely understood in mouse embryos[4], it is often generalized accordingly that teeth develop exclusively in the region of the oral ectoderm, which invaginates to form a stomodeum[2]. The accepted view is that the presence of teeth in any region is an indubitable criterion for the existence of the ectodermal germ layer in this region at some time of development[1]. However, in various vertebrate lineages, so-called pharyngeal teeth, or even a second set of toothed jaws, are commonly found posterior to the stomodeum in areas that are presumably lined by endoderm rather than ectodermal epithelium[6,8]. Convincing developmental evidence for an endodermal origin of teeth situated in the pharyngeal cavity is lacking, and uncertainties arise also from the fact that some structures situated within the pharyngeal cavity of bony fishes are apparently derived from the ectoderm (for example gills or opercular bones). Apart from the facts that the endoderm was suggested, on the basis of histology, to contribute to tooth formation in some lower vertebrates during the first half of the twentieth century[17,18] and that such a role has been questioned even in mammals[19,20], our understanding of the germ-layer origin of tooth epithelia is fundamentally limited by the difficulties in distinguishing between ectoderm and endoderm during critical stages of later mouth development. Hence, after the breaking of the oropharyngeal membrane, which constitutes the border between the oral (ectodermal) and pharyngeal (endodermal) epithelia, the fate of these cells is not known, owing to a lack of reliable fate-mapping studies even for model vertebrate species like mouse, chick or zebrafish.

Urodele amphibians are an interesting group for the analysis of the germ-layer origin of teeth because the presumptive border between the oral ectoderm and endoderm is substantially more anterior than in mammals[17,18,21]. To study mouth development and the germ-layer origin of dental tissues in details, we took advantage of recently developed transgenic axolotls[22]. We designed a novel experimental procedure that enables us reliably to mark the ectoderm of the entire prospective mouth area and to follow its fate during the course of development.

First we performed transplantations of four different areas of double-layered ventral epithelia using axolotl GFP-positive neurulae (Supplementary Fig 1a; GFP, green fluorescent protein) and found conclusively that for reliable marking of the ectodermal layer of the prospective mouth, it is necessary to graft both prospective oral ectoderm and transverse neural folds (in total, $n = 113$; Fig. 1a, b). Moreover, we always ascertained that these GFP-positive grafts comprised the entire mouth area, so that no GFP-negative cells could contribute to mouth formation (Supplementary Fig. 1b).

Next we used this experimental system to trace the accurate contribution of ectodermal cells to mouth and tooth formation. In the axolotl, the epidermis in the prospective mouth region initially consists of a double-layered ectoderm but subsequently becomes reduced to a single outer layer when the inner layer bends inwards over non-ectodermal mouth tissue as an 'ectodermal collar'[17,18] (Fig. 1c). This oral ectodermal lining deepens (compare Supplementary Fig. 1c, d) and during later tail-bud stages contributes to prominent buds (Fig. 1d, arrowhead). However, morphologically identical budding structures also appear in the non-ectodermal area (Fig. 1d, arrow; notice the proximity to the lower-jaw cartilage, MC). Later, still before hatching, buds are easily identified as developing teeth, which are regularly distributed in both ectodermal (Fig. 1e, arrowheads) and non-ectodermal areas (Fig. 1e,

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arrows). To confirm the identity of these structures as tooth buds, we used an antibody directed against calbindin (Sigma), a calcium-binding protein that specifically recognizes ameloblasts[23]. From sections where both GFP and immunostaining is visualized (Fig. 1f, g and Supplementary Fig. 2a, b), it is evident that the tooth primordia are developing from both ectodermal and non-ectodermal epithelia.

To substantiate our finding that in the Mexican axolotl some oral teeth develop from non-ectodermal epithelia, we invented a double-labelling approach using which cells of both oral ectoderm and foregut endoderm can be reliably marked and mapped (Fig. 2a). First, at a neurula stage, the double-layered prospective oral epithelium (from the same area as in the previous experiment) was extirpated. The exposed endodermal layer was then focally injected using the lipophilic dye DiI (Molecular Probes). Next a GFP-positive graft comprising the entire prospective oral ectoderm (as above) was transplanted orthotopically to wild-type host embryos. In this approach ($n = 91$), the entire prospective ectoderm of the oral area was marked with a green fluorescent dye and some of the foregut endodermal cells, expected to contribute to tooth buds, were labelled with a red fluorescent dye.

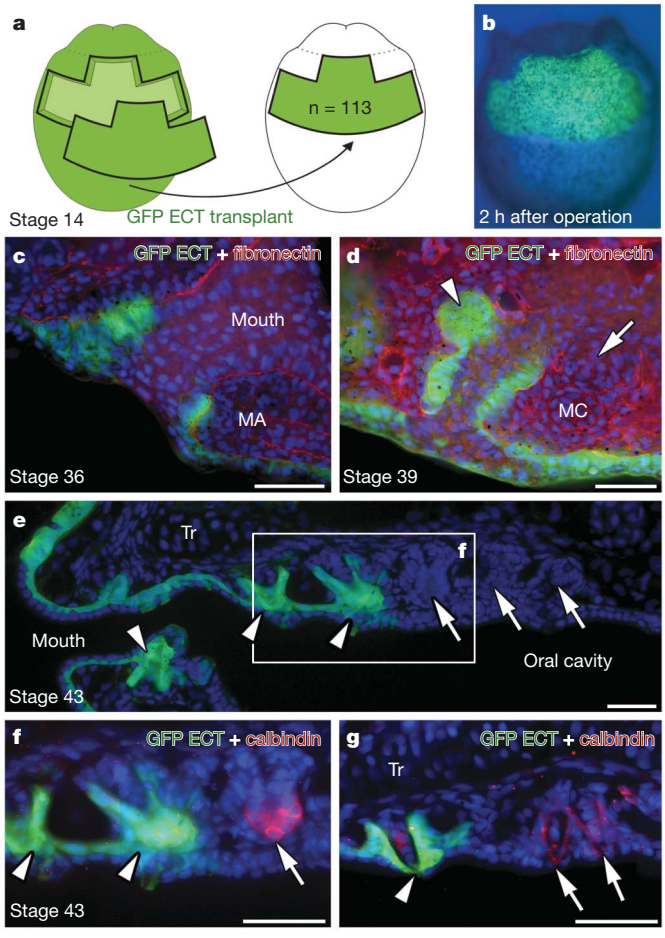

Figure 1 | Ectoderm contribution to mouth and tooth formation in the Mexican axolotl. **a**, An experimental scheme with the prospective oral ectoderm (ECT) transplanted from a GFP-positive donor to a host embryo, ventral view (END, endoderm). **b**, An embryo 2 h after operation. **c**–**g**, Paramedial sections, head to the left, showing a contribution of the oral ECT (green) to mouth and tooth formation. DAPI (blue) stains cell nuclei; fibronectin (red in **c**, **d**) marks cell and tissue borders. Initially the oral ECT (green) inflexes as a stomodeal collar (**c**). Then prominent tooth buds develop in ECT areas (arrowheads) as well as in non-ECT areas (arrows; **d**, **e**). Tooth buds, identified using anti-calbindin (red), develop within ECT areas (arrowheads) as well as non-ECT areas (arrows; **f**, **g**). Tr, trabecula; MA, mandibular arch; MC, Meckel's cartilage. **c**, **d**, Vibratome 100-μm sections; **e**–**g**, cryostat 20-μm sections. Scale bars, 50 μm.

Using this double-fate-mapping approach, we obtained strong support for our previous conclusions that the axolotl possesses oral teeth with an epithelial lining of non-ectodermal origin. Specifically, dye injected into the foregut endoderm at the neurula stage was found in oral tooth germs and later in developing teeth (Fig. 2b–d, h and Supplementary Fig. 4), as well as, notably, in the epithelium situated between GFP-positive ectodermal epithelia (Fig. 2c). Moreover, alongside the contact zone between the ecto- and endodermal oral epithelia, we found tooth germs that consistently demonstrate a mixed contribution from both ecto- and endodermal cells to their enamel epithelia (Fig. 2e–g and Supplementary Fig. 5). On the basis of our combined tracing approaches, we conclude that on the upper jaw the enamel epithelia of the premaxillary/maxillary teeth are always ectodermal, whereas the enamel epithelia of the vomero-palatal teeth are derived from the ectoderm, endoderm or

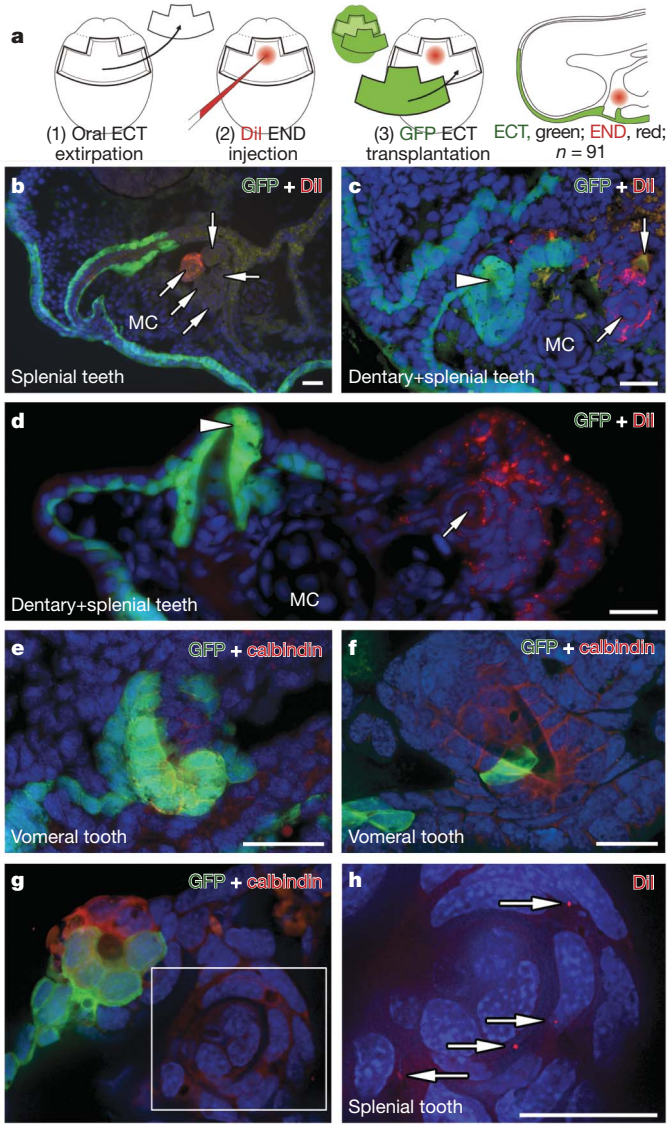

Figure 2 | Endoderm contribution to tooth formation. **a**, Sketch of double-fate-mapping experiment: following extirpation of the prospective oral ECT (1), DiI was injected into foregut END (2) and the prospective oral ECT (GFP-positive) was transplanted orthotopically (3). **b**–**h**, Paramedial (cryostat 20-μm) sections, head to the left, showing a contribution of the oral ECT (green) and mouth END (red in **b**–**d**, **h**) to tooth formation. DAPI (blue) stains cell nuclei, calbindin (red in **e**–**g**) marks tooth buds. Arrows point to END teeth; arrowheads to ECT teeth. **e**–**g**, Details of teeth of mixed origin. **h**, A confocal image, inset in **g**, showing the END (DiI, red) contribution to splenial tooth germ. Scale bars, 25 μm.

from a mixed source, according to their position (Fig. 3a). On the lower jaw, dentary teeth are basically ectodermal and splenial endodermal; however, there are teeth of mixed origin situated on the anterior parts of these fields (Fig. 3a).

Next a quantitative screening was performed in which all teeth were counted and their respective germ-layer origins determined at four different stages and based on 26 embryos from the double-labelling experiment (Fig. 4). This analysis revealed that of 1,137 teeth, 374 were derived from ectoderm, 598 from endoderm, and 155 were of mixed ecto/endodermal origin. We note that during the course of development, the proportion of ectoderm-derived teeth slightly increases as teeth located on the premaxillary and maxillary bones, which are purely ectodermal, develop very late. Thus, in the average embryo (analysed at stage 45, when the mouth opens and animals start to eat), of 82 teeth 29 were of ectoderm, 42 were of endoderm and 11 were of mixed epithelial origin (Supplementary Tables 1–4). Non-epithelial derivatives, such as tooth dentin and papillae, were derived from neural crest mesenchyme (from the trigeminal neural crest stream; Supplementary Fig. 3). All quantitative and statistical analyses were strongly significant (Supplementary Tables 1–4) and constitute robust support that our data are not biased by any technical problems.

Previous theories have identified the ectodermal border in the mouth as being central to tooth positioning[11] (Fig. 3b, upper row). However, in the Mexican axolotl, the oral ectoderm does not form a true stomodeum[17,18]. Instead, only an inner ectodermal layer bends inwards as a stomodeal collar over the dense endodermal rod, which blocks the prospective mouth at early stages of development (Fig. 3b, lower row). Because of this positioning, the collar cells develop into the basal cells, and the outer cells of the endodermal rod develop into the apical cells of the oral epithelium during the course of mouth opening (Fig. 1e and Supplementary Fig. 2a–d; summarized in Fig. 3b, lower row). The endodermal cells of the mouth, as part of the epithelial lining, are consequently found also on the outer surface of the mouth (Fig. 1e and Supplementary Fig. 2b–d, arrowheads; summarized in Fig. 3b, lower row). Thus, in the axolotl, the posterior part of the oral cavity is lined with the endodermal epithelium, whereas the anterior part is lined with an epithelium of double origin (Fig. 3b and Supplementary Fig. 2b). This provides reliable documentation of an oral endodermal epithelial lining that reaches outside the mouth, and, also, of an oral epithelium originating from two germ layers. A considerable number of reports on mouth development have been published, but, as underlined by our results, there is still a need for detailed fate-mapping approaches in studies of dynamic interactions of cells and tissues derived from different germ layers.

Progressing from recent vivid discussions on the subject[4,6,8,24], our data present reliable evidence of oral teeth of endodermal origin in vertebrates. We speculate that oral teeth of endodermal origin might form in all animals with oral endoderm, that is, in urodele and probably also lungfish species, and maybe even in some frogs[25], where the mouth develops from a structure similar to the stomodeal collar. However, as a possible interdigitation of cells from both epithelial tissue layers during mouth formation has not been fate-mapped for any vertebrate species, and some reports indicate that foregut endoderm may stretch more to

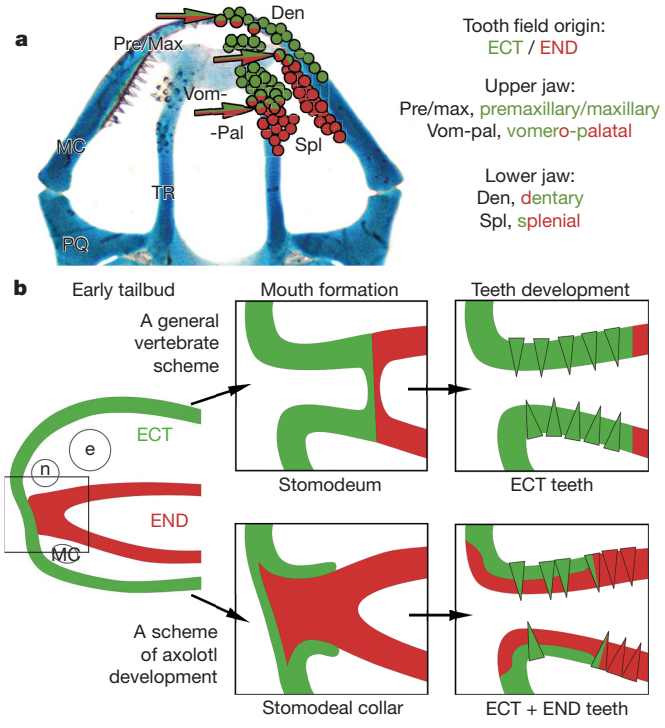

Figure 3 | Germ-layer origin and morphogenesis of teeth of the Mexican axolotl. a, A sketch of the germ-layer origin of teeth in the Mexican axolotl. ECT teeth, green; END teeth, red; teeth of mixed origin, red–green (the colouring in the key is a qualitative guide to the ratio of the components in each tooth field). Cartilages visualized using alcian blue. **b**, Comparative developmental morphogenesis of the mouth region and the germ-layer origin of teeth of vertebrate (upper row) and an axolotl (lower row) embryo. In the majority of vertebrates, the mouth develops from a stomodeum with teeth distributed in invaginated ECT. In contrast, in urodeles the mouth develops from a stomodeal collar with an oral epithelium either of a dual origin, with teeth of ECT or END, or of a mixed origin. PQ, palatoquadrate; TR, trabecula cranii; n, nose; e, eye.

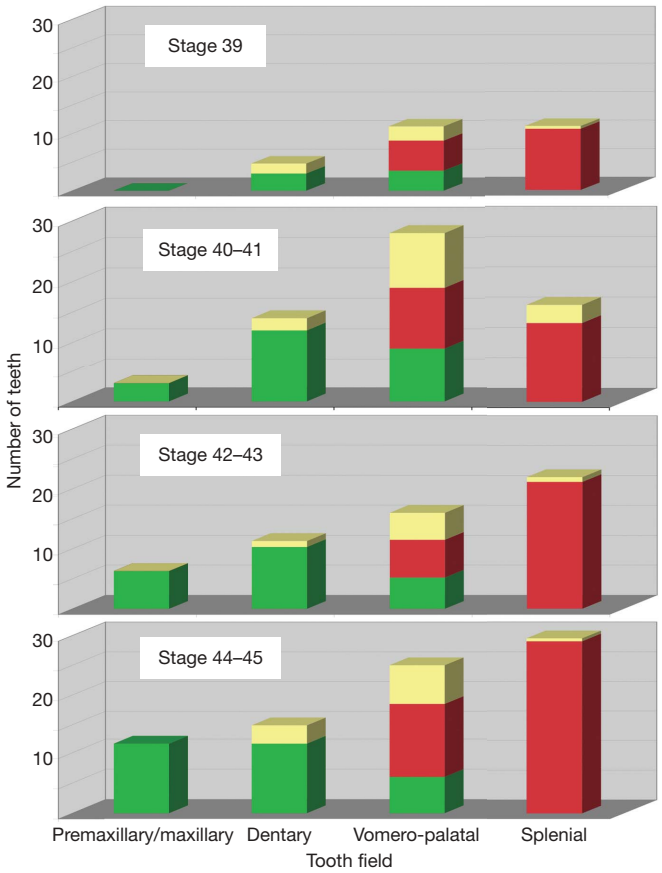

Figure 4 | A quantitative screening of teeth from different tooth fields with respect to their germ-layer origin. $n = 1{,}137$ teeth: 374 ECT (green), 598 END (red), 155 ECT–END mixture (yellow). Data based on 26 animals, visualized at four stages: four animals at stage 39, 14 animals at stage 40–41, six animals at stage 42–43, two animals at stage 44–45. For details and original data, see Supplementary Tables 1–4.

the anterior than hitherto believed[8,19–21], we speculate that oral teeth of endodermal origin might present a more common feature in vertebrate oral development than previously assumed.

Whereas the classical 'outside-in' theory implies that teeth were initially derived from the oral ectodermal layer[11], the 'inside-out' theory strongly suggests that they were derived from the endodermal layer[12], and this derivation is believed to impart differences to denticles, teeth or dentition in terms of shape and complexity[4,12,24]. However, the dual origin of enamel epithelia in otherwise morphologically identical axolotl oral tooth primordia (as regards complexity, shape, position, timing and morpho-differentiation of teeth), together with studies illustrating deep shared molecular similarities between oral (supposedly ectodermal) and pharyngeal (supposedly endodermal) teeth[15,26,27] imply that 'ectodermal' and 'endodermal' teeth do not differ essentially. It is beyond the scope of this study to identify the plesiomorphic germ-layer origin of tooth epithelium. However, our results clearly demonstrate that the germ-layer origin of epithelium into which the mesenchyme cells come into contact does not affect the final product of the odontogenic cascade. Mesenchyme cells can thus apparently interact with a host of epithelial cells, forming teeth/denticles when in the stomodeum, in the pharyngeal cavity or on the skin surface. All this suggests that the major agent of dental development is the neural crest mesenchyme rather than the epithelium, the role of which in tooth patterning[5,12,28] and even in tooth initiation[29] may be less fundamental than commonly believed. It therefore seems most likely that all teeth of extant vertebrates—or, more precisely, the developmental machinery producing them—have evolved only once, somewhere in the oropharynx, driven by a neural crest signal.

METHODS SUMMARY

Embryos. Embryos of the Mexican axolotl (*Ambystoma mexicanum*) were obtained, reared and staged as previously described[30]. GFP embryos were obtained from the Max-Planck-Institute of Molecular Cell Biology and Genetics in Dresden, Germany, and were developed in the laboratory of E. Tanaka[22].

Operations and injections. GFP ectodermal transplantations were performed as sketched in Fig. 1a ($n = 113$). At first, however, transplantations of four different areas of double-layered ventral epithelia were performed (Supplementary Fig. 1a) to define the entire ectodermal layer of the prospective mouth.

The double-labelling approach by which cells of both oral ectoderm and foregut endoderm were marked and mapped (as sketched in Fig. 2a ($n = 91$)) includes extirpation of the double-layered prospective oral epithelia (from the same area as in the previous experiment), focal injection of a CellTracker CM-DiI (Molecular Probes) into the exposed endodermal layer and, lastly, the orthotopic transplantation of a GFP-positive prospective oral ectoderm (as above) into wild-type host embryos.

Sectioning and immunostaining. Axolotl embryos were anaesthetized using MS-222 (Sigma), fixed in 4% paraformaldehyde in phosphate buffered saline and sectioned using a Vibratome 1000 sectioning system (Ted Pella) or a CM3050 cryostat (Leica). Sections were counterstained using anti-fibronectin antibody (Dako) to visualize tissue borders, with 4,6-diamidino-2-phenylindole (DAPI) to mark cell nuclei, or with anti-calbindin antibody (Sigma), which specifically recognizes ameloblasts[23].

Image acquisition. Separate fluorescence images were captured using an Olympus BX51 microscope with a SPOT RT camera, or the Olympus Cell^R IX81 with a Hamamatsu Photonics Orca camera, merged and optimized using Spot and Adobe Photoshop software.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Embryos. Embryos of the Mexican axolotl (*A. mexicanum*) were obtained, reared and staged as previously described[30,31]. GFP embryos were obtained from the Max-Planck-Institute of Molecular Cell Biology and Genetics in Dresden, Germany, and were spawned from a β-actin promoter-driven GFP germ-line transgenic animal that had been produced by plasmid injection[22]. Embryos were kept in tap water, and before being used for transplantations and injections, embryos were washed thoroughly with tap water and sterile Steinberg solution containing antibiotics (Antibiotic-Antimycotic, Gibco) and then decapsulated manually.

Operations and injections. GFP ectodermal transplantations were performed as sketched in Fig. 1a ($n = 113$). Operations were performed under sterile conditions using tungsten needles in an agar dish containing 1 M Steinberg solution plus antibiotics. We designed an experimental procedure that enabled us to mark the ectoderm of the entire prospective mouth area reliably and to follow its fate during the course of development. First we performed transplantations of four different areas of double-layered ventral epithelia from GFP-positive to host neurulae (Supplementary Fig. 1a; numbers of animals used for each operation are indicated there) and found conclusively that for reliable marking of the ectodermal layer of the prospective mouth it is necessary to graft both prospective oral ectoderm and a transverse neural fold (Fig. 1a, b). Using this type of transplantation, we were able to follow the fate of the entire ectodermal layer that translocates into the mouth, and, therefore, in this way all ectoderm-derived teeth became GFP-positive. We always ascertained, however, that these GFP grafts comprised the entire mouth area, so that no GFP-negative cells could contribute to mouth formation (Supplementary Fig. 1b).

Next we invented a double-labelling approach by which cells of both oral ectoderm and foregut endoderm can be reliably marked and mapped (Fig. 2a). First, at a neurula stage, double-layered prospective oral epithelia (from the same area as in the previous experiment) were extirpated. The exposed endodermal layer was then focally injected using the lipophilic dye DiI (Molecular Probes), dissolved in absolute ethanol to a concentration of 1 mg ml^{-1} and further diluted in nine parts of 10% sucrose in water just before injection. Then a graft from a GFP-positive neurula comprising the entire prospective oral ectoderm (as above) was transplanted orthotopically to wild-type host embryos. In this approach ($n = 91$), the entire prospective ectoderm of the oral area was marked green (GFP) and some of foregut endodermal cells, expected to contribute to tooth buds, were labelled red (DiI).

Neural crest transplantations. Trigeminal neural crest cells were transplanted from GFP-positive to wild-type embryos at the neurula stage as described in detail elswhere[32].

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