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Signaling pathways involved in tooth development: why use zebrafish in dental research?

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Abstract

Introduction: Zebrafish (*Danio rerio*, formerly named *Brachydanio rerio*) is an established model organism used in health research for a long time. In the past decades, some research groups have been using zebrafish model to understand the genes involved in tooth development. **Objectives:** To introduce zebrafish as an animal model for tooth development researchers and to highlight the advantages and limitations of this model. **Literature review:** Tooth development (also known as odontogenesis) is a complex process that relies on precise control of several critical regulatory pathways. Because of the complex development and morphogenesis of teeth, many human developmental dental anomalies arise due to disruptions during tooth development. The knowledge regarding signaling pathways and genes expressed during tooth development has mainly been discovered using mice (not modified and genetically modified) and other rodents. Zebrafish (*Danio rerio*, formerly named *Brachydanio rerio*) has also been used by some recent researchers. **Results:** Zebrafish as a model organism has several advantages, and for which a large and diverse set of genetic and molecular tools are already available. Zebrafish has an easy husbandry system and a relatively fast embryonic development. **Conclusion:** This review provides some insights for the use of zebrafish in understanding tooth development and developmental dental alterations for clinical and basic researchers alike.

Introduction

Tooth development (or dental development) is a complex process also known as odontogenesis. This process relies on the precise control of several critical regulatory pathways. It is regulated by sequential and reciprocal epithelial-mesenchymal interactions. In mammals, the involved mesenchyme derives mostly from neural crest cells (NCCs), whereas the epithelium has ectodermal origin [14, 64]. In bony fish, by contrast, the epithelium of pharyngeal teeth has endodermal origin instead [48].

Teeth are organs for which genes determine the development from initiation to final morphology, including shape, size, and structure, and environmental factors play only a minor role in the final phenotype [69]. Mutations and genetic polymorphisms in many genes involved in tooth development can lead to congenital developmental dental anomalies, which are common in humans and can be categorized in alteration of tooth number, shape, structure, and position.

In the past decades, active research in many groups worldwide has led to a better understanding of tooth development stages and morphogenesis [31, 69]. These studies unraveled some hundred genes involved in epithelial-mesenchymal interactions and regulation of tooth development [70]. The knowledge regarding the genes expressed during tooth development and their functions in odontogenesis has been mainly discovered using mice (not modified and genetically modified) and other rodents. Despite their evolutionary proximity to humans, rodents, however, also have their disadvantages for investigating embryonic tooth development, for example their dentition does not present lateral incisors, canines, or premolars. Further, the embryos develop inside the mother, and it is difficult to monitor odontogenesis during embryonic development, and the mother must be killed in order to analyze the embryonic phenotype. Therefore, other animal models than rodents have been discussed, such as zebrafish (*Danio rerio*, formerly named *Brachydanio rerio*), which has already been established as a model organism and used in research for a long time with tested methods and established husbandry available.

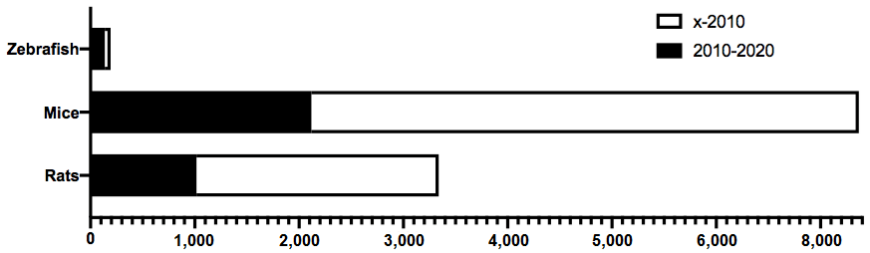
The zebrafish model and its suitability to understand the genes involved in tooth development have been explored by some research groups during the past decade [7, 92]. Therefore, in this review we for the first time summarize these finding introducing zebrafish as an animal model for tooth development alterations and highlight the advantages and limitations of it. Firstly, we give a brief overview about the importance of animal studies to human dental research and tooth development in general.

Animal to human: using animal models to identify genes driving tooth development

Studies with non-human species can be used to understand some specific biological processes and to gain insight into the inner workings of other organisms. Animal models provide a fundamental platform for understanding key processes involved in human dental development, including genes expressed during crucial stages of odontogenesis. Historically, animal research in this regard started with the search for spontaneous mutations that appeared in various animal breeds and nowadays includes direct genome editing methods.

Loss of function of many known genes arrests the process of tooth development in genetically modified mice, and their mutations cause tooth agenesis in humans [31]. Other studies using animal models identified candidate genes for supernumerary teeth [30, 83], and enamel defects [5, 82].

Therefore, it is important to highlight that animal studies have proven to be a valuable tool to identify candidate genes for congenital dental anomalies in human with a plethora of respective studies available, as a systematic literature search in MEDLINE database revealed (Figure 1).



*Rats: 1946-2020; mice: 1946-2020; zebrafish: 1996-2020

Figure 1 – Historical perspective of studies using animal models to understand dental development. We performed the literature search in June 2020 using the search strings "Dental development" AND "rats" / "mice" / "zebrafish"*

Zebrafish as a model

Zebrafish is a relatively recent, but prominent, model organism in biological, health, and dental research. They are tropical freshwater fish belonging to the family of Cyprinidae (carp-like fish) in the infraclass of Teleost (bony fish) of the class of Actinopterygii (ray-finned fish). Although actinopterygian and mammalian lineages have evolved separately for approximately 420 million years, resemblances between zebrafish and tetrapod tooth development were conserved (see further ahead).

As in all vertebrates, the anterior-posterior axis is defined early in embryonic development. The head develops at the anterior end of the larvae, what is established by late gastrulation. Along the dorsal anterior-posterior axis, the notochord and the neural tube develop in vertebrates. The beginning of craniofacial development is similar in all vertebrates: NCCs from the most dorsal part of the neural tube start to migrate into the ventral parts, in which their predetermined destinations in the head region are [12, 55]. The first stream splits and the posterior part migrates ventrally to populate the first seven pharyngeal arches [12, 68, 77]. Arches 2-7 are subsequently populated from other streams, mainly from the rhombomeres of the hindbrain. Upon arrival, all NCCs surround a mesoderm core [44].

Methods in zebrafish

The zebrafish larvae develop outside of the parent and are transparent. These characteristics allow in-vivo visualization of biological events, such as the development of the skull. They can be easily embryonically manipulated, allowing several important observations by Laale [38], as reviewed by Lieschke and Currie [40]. Several methods are used to manipulate zebrafish gene activity.

Mutations can occur naturally or be induced exposing male fish to the chemical mutagen Ethylnitrosourea (ENU) [13, 79]. Another method for targeted mutagenesis (reverse genetics) is the targeted induced local lesions in genomes (TILLING) method, in which the mutation itself is induced by a chemical compound, and then polymerase chain reaction (PCR)-based methods are used to screen for the mutation [79].

Further tools for targeted mutagenesis were developed, including zinc-finger nucleases (ZFNs), as reviewed by Porteus and Carroll [52], transcription-activator-like effectors fused with a nuclease (TALEN) [8], and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) [10]. In all three cases, mutagenesis is sequence specific. ZFN and TALEN consist of sequence-specific DNA-binding domains and DNA endonucleases. Cas9 is an endonuclease that requires an RNA guiding sequence to activate and target the nuclease to the complementary DNA sequence. All three methods work by causing double-strand breaks that are repaired by the cell. These repairs of non-homologous ends can cause inserts and deletions leading to frameshift mutations, as reviewed by Gaj *et al*. [15]. Because of its alleged higher efficiency, most labs prefer CRISPR/Cas9 nowadays. This method can also be used for genetic engineering, as reviewed by Ceasar *et al*. [6].

The creation of transgenic zebrafish lines is also established. It is used to create genetically modified animals, but also to introduce reporter genes as green fluorescent protein (GFP) or to put the gene of interest under control of a heat shock (HS) promoter. Using the HS promoter enables the researcher to control the expression of the gene of interest temporally [39]. The mostly used method to generate transgenic animal lines is the Tol2 transposon system, based on the Tol2-mediated transgenesis protocol, as reviewed by Kawakami [34]. Another method is based on the meganuclease I-Sce-1 enzyme [18]. This enzyme is co-injected with a plasmid containing the gene of interest flanked by the recognition sequence of I-Sce-1. Thus, the gene can be introduced into the genome via homologous recombination.

The described methods so far can only be used to generate zebrafish lines, meaning that the genetically manipulated fish can be used after at least six months, because the F1-generation must be screened for positive embryos. One approach to investigate the F0-generation is the knockdown of a target gene using morpholinos (MO). These are synthetic molecules having a similar structure to nucleic acids. The desoxyribose is replaced by a morphine ring (hence the name), and instead of phosphates the backbone of a MO contains phosphodiamide molecules. These replacements make structure uncharged, and the MO is neither recognized nor degraded by the cell. Depending on the type of MO, either the translation or the splicing is disturbed. In either case, no functional protein is formed [46, 67].

The embryos developed from the injected zygote are called morphants. However, they can also cause non-specific artefacts due to toxicity or off-target effects, related to the higher injected amount of injected MO, as reviewed by Schulte-Merker

and Stainier [60]. To deal with these obstacles, various controls were introduced to validate the findings: co-injecting a MO against a p53-MO to block apoptosis [54], rescuing the phenotype by co-injection wildtype mRNA that does not bind to the MO, or injecting another bath of embryos from the same parents with a control MO that does not bind to any endogenous RNA [36].

The use of MO was introduced because siRNA did not sufficiently work in zebrafish [93, 94]. An RNA-based alternative to MO and siRNA could be pri-miRNA, by introducing the required sequence into the genome [11, 53].

Another method to manipulate F0-embryos is the usage of small molecule, which is relatively simple. Small molecules have a low molecular weight (< 900 Da) and are organic compounds that regulate biological processes. This is because proteins can be inhibited or activated by small molecules binding to the active or allosteric site. Thus, the activity of enzymes, transcription factors or proteins involved in signaling pathways can be regulated by adding the compound to the solution, which the larvae or embryo reside in. By changing the solution, a temporal control of compound activity in the embryo is possible.

Established methods to manipulate gene expression during development are summarized in Table 1. Staining methods such as in-situ hybridization or immunohistochemistry can be used to visualize tissue changes caused by manipulation of gene expression. They can be applied on sections, such as dental arches (upper and lower jaws), or whole embryos.

Tooth development in zebrafish

During evolution of gnathostomes, structural characteristics of teeth are conserved [23, 66]. Further, mammalian teeth have derived from a polyphodont (teeth are replaced throughout life) and homophodont (teeth are shaped similarly) ancestor [23].

The zebrafish is a polyphodont, and their dentition consists of 22 pharyngeal teeth [4]. The pharyngeal teeth are located at the fifth ceratobranchials, which are the only teeth-bearing elements in the whole buccal and pharyngeal region and show a distinct pattern (Figure 2). This pattern consists of three rows that extend rostro-caudally: ventral, mediodorsal, and dorsal, with five, four, and two teeth, respectively [25, 74].

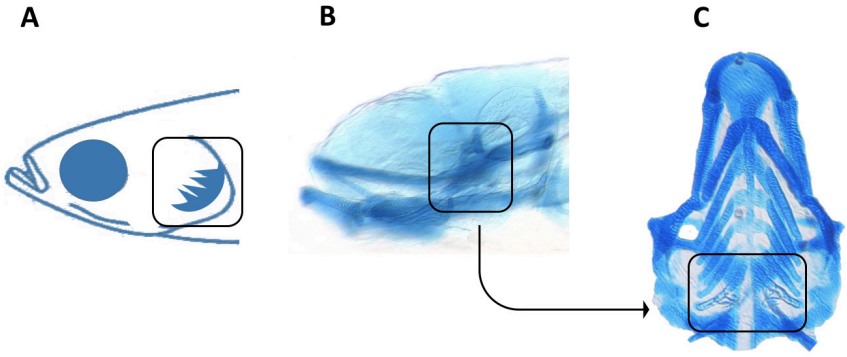


Figure 2 – Zebrafish larvae seven days post-fertilization. **(A)** Tooth location in a lateral view of the head. **(B)** Lateral view of the head stained with alcian blue. **(C)** Ventral view of the head stained with alcian blue

The ceratobranchial development starts at two days post-fertilization (dpf), when there is evidence of chondroblast condensation. At three dpf, a small dentigerous area can be seen. The initiation of new tooth germs follows a regular pattern during the first 14 days. Huysseune *et al.* [25] described three stages for zebrafish first-generation tooth development:

- initiation and morphogenesis;
- cytodifferentiation;
- attachment.

The first phase – initiation and morphogenesis – starts with the commitment of odontogenic tissues. At the beginning, the pharyngeal epithelium consists of three layers: the basal layer, the intermediate layer, and the superficial layer. These layers undergo a change, which already starts before the onset of epithelial invagination forming a disc that develops into the tooth. The first phase ends with the deposition of the initial matrix. The second phase – cytodifferentiation – is characterized by dentinogenesis. During the third phase –

attachment –, the developing tooth will be attached to the cartilage which will be the bone later. The mineralization of the bone matrix does not start before the mineralization of the first-generation tooth has reached the base [25].

There are a few differences between first-generation and replacement teeth. First replacement teeth are bigger, and within the already described phases further sub-phases (leading to five steps) can be distinguished [74]. Secondly, they develop from an epithelial thickening at the transition zone between the dental organ of the functional tooth and the epithelium proper. There is neither an independent development from the epithelium nor a permanent or continuous dental lamina, from which the new germ buds form. The development of a replacement tooth starts close to the already existing tooth, which is also involved in the onset of the formation of its successor. This suggests that the initiation takes place in an already committed epithelium during the first initiation event in the larvae stage. Furthermore, maybe because of the bigger size of replacement teeth, they and first-generation teeth show structural differences. Before the previous tooth is replaced, the attachment bone and part of the supporting bone are reabsorbed. The area of the reabsorption is close to the developing germ of the successor, suggesting that the developing germ might act as a trigger for resorption [75].

The molecular aspects of the development of zebrafish teeth and how they can be compared to tooth development in mammals were also investigated. As indicated, differences, but also similarities, exist between tooth development in zebrafish and mice. This also relates to genes and signaling pathways.

Jackman and Draper [27] showed that fibroblast growth factor (FGF)-signaling is required in zebrafish and in mice. Firstly, they searched for tooth markers based on already known markers from mice. They found several good candidates as *pitx2, dlx2a*, *lhx*6 and *lhx7*, but not *pax9*. They also marked different parts of the developing tooth: *dlx2* (both zebrafish duplicates) are expressed in the tooth germ from 48 hours post-fertilization (hpf) onwards, similar to *dlx2* expression in mice. The two *lhx* genes (*lhx6* and *lhx7*) are expressed in the mesoderm prior to tooth formation. Since *dlx2*, *lhx6*, *lhx7*, and *pitx2* are regulated by FGF-signaling, they also analyzed how the inhibition of FGF-signaling influences the expression of those genes in zebrafish. The expressions of *zfgf3* and *zfgf4* are similar to their orthologs in mice.

Jackman and Draper [27] used the small molecule SU5402, which can inhibit FGF-signaling by binding to its receptor [43]. When SU5402 was applied at 32 hpf, the epithelial morphogenesis of pharyngeal teeth was completely inhibited, but the expression of *pitx2* did not change. This was only observed for *pitx2*, but the expressions of *dlx2*, *lhx6*, and *lhx7* require FGF-signaling. Interestingly, an up-regulation of FGF-signaling and a down-regulation of bone morphogenic protein (BMP)-signaling lead to multicuspid teeth and can be therefore an indicator for their evolution [28].

All six mouse *dlx* genes involved odontogenesis, and eight *dlx* genes are known in zebrafish. Borday-Birraux *et al.* [2] investigated the similarities and differences between *mdlx* and *zdlx* orthologs. They found that two genes involved in murine tooth development are not expressed during zebrafish tooth development. The remaining six *dlx* genes involved in zebrafish tooth development cover both the period and domains of the eight orthologous *dlx* genes in mice, but they do not showed an identical expression pattern when considering the developmental phases and compartments.

The Wnt/β-catenin signaling pathway also plays an important role during tooth development [41]. O'Connell *et al.* [47] showed that Wnt/β-catenin signaling is necessary for the regulation of reciprocal epithelial-mesenchymal signaling interactions shifting the tooth-inductive potential. This signaling expands from the dental epithelium at the initiation stage to the dental mesenchyme at early morphogenesis. The Wnt/β-catenin signaling activity changes between the epithelium and the mesenchyme and affects sequential tooth development [62]. There are critical time points, in which Wnt/β-catenin is crucial for normal tooth development. Morphogenesis arises from cell proliferation during organogenesis [89]. Shim *et al.* [62] showed that maintaining Wnt/-β-catenin signaling in the dental epithelium during initiation stage is crucial for the activation of Wnt/β-catenin signaling in the mesenchyme to achieve initiation potential for sequential teeth. This suggests that Wnt/β-catenin signaling supports the regulation of the number of teeth in zebrafish as well, as it was already shown for mammals [32].

Further, Wnt/β-catenin also plays a role during the development of replacement teeth. It is active in a time window between late cytodifferentiation and morphogenesis of its successor [26]. In mice, it was shown that Wnt10a is one candidate for tooth regulation [86], and Yuan *et al.* [90] showed

that Wnt10a function is conserved by proving its function in zebrafish. In mammals and zebrafish, a knockdown of Wnt10a leads to reduced activity of tooth development genes such as *msx1*, *dlx2b*, *eda*, and *axin2*, causing an arrest of tooth development.

In mice, it was shown that BMP signaling also plays an important role in tooth development regarding the determination of the tooth type [73]. It was noticed that BMP signaling is also required during tooth development in zebrafish. An inhibition of BMP signaling caused by a mutation of the Alk8 receptor or by an overexpression of constitutively active, and dominant-negative versions of the encoded protein leads to a change in tooth number and/or shape [49, 50]. Mutations in the BMP antagonist Ogon, and the ligand Bmp7a also caused a delay or arrest in tooth formation [50, 87]. It was also found that *bmp2a*, *bmp2b*, and *bmp4* were expressed during initiation and morphogenesis stages of pharyngeal teeth in mice [81]. On the other hand, Wise and Stock [80] found that neither Bmp2b nor Bmp4 are required for tooth formation in zebrafish, even though they play a crucial role in murine tooth development. One reason could be redundancy, because several *bmps* share the same temporal and spatial expression during zebrafish tooth development. Another reason could be that one ligand is required in buccal and another in pharyngeal tooth development.

Three hedgehog proteins are known: Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh). Due to genome duplication, zebrafish has five reported hedgehog ligands and two patched (Ptch) receptors, but only one semi-ortholog *shha* and its receptors *ptch1* and *ptch2* were found to be expressed in developing dental tissue in teleost [9, 29, 37]. The expression of Ptch is widespread during dental development [29, 45, 71]. Jackman *et al.* [29] showed that, when Shha signaling is inhibited during the initiation stage, *pitx2* expression is missing, indicating that Shha signaling is already crucial during very early stages. When inhibited at 36 hpf, further development of the dental mesenchyme and papilla is arrested with Shha inhibition at 48 hpf leader to dental hypoplasia [88].

As shown, tooth development in zebrafish is already well studied, but there are still several open questions. Answering these questions could in the future give a better understanding of odontogenesis in human.

Further consideration

In this review article, we briefly present some of the strengths and weaknesses of the zebrafish as a model to study tooth development. Some of these characteristics are summarized in Tables 2 and 3. It is also important to emphasize that some of these aspects should be taken into consideration in dental development research, as described ahead.

Table 1 – Summary of methods to modify zebrafish gene activity*

Method	Line[a]	F0[b]	Specificity	Application	Function	Reference
Ethylnitrosourea (ENU)	Yes	Yes	None	High amounts are required to achieve high locus hit rates. It is diluted in the medium that the larvae live in	Causes point mutations in the genome	[13, 21, 58, 79]
Targeted induced local lesions in genomes (TILLING)	Yes	No	None	Depends on the chemical used for mutation induction	The chemically induced mutation is screened for via sensitive polymerase chain reaction (PCR) methods	[79]
Zinc-Finger nucleases (ZFN)	Yes	No	DNA sequence specific	Must be injected into the zygote	A nuclease is bound to a zinc-finger construct which binds a specific DNA-sequence. After binding, the DNA is cut and repaired by the cell in a non-homologous manner	[15, 52]

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Continuation of the table I

Method	Line[a]	F0[b]	Specificity	Application	Function	Reference
Transcription activator-like effectors fused with a nuclease (TALEN)	Yes	No	DNA sequence specific	Must be injected into the zygote	The molecules consist of peptides that recognize a certain DNA sequence and are similar to the binding sequence of transcription factors. The other part is a nuclease that cuts the DNA, and repairs occur by the cell in a non-homologous manner	[8, 15, 19]
Clustered regularly interspaced short palindromic repeats (CRISPR/Cas9)	Yes	No	DNA sequence specific	Must be injected into the zygote. The endonuclease needs a guiding RNA. The plasmid containing the guiding RNA must be co-injected	Cas9 is the endonuclease. This method can be used for more genetic engineering than just deletions	[6, 10, 15]
Morpholinos (MO)	No	Yes	RNA sequence specific	Must be injected into the zygote.	Depending on the MO-type, either the translation or the splicing of mRNA is affected, and no proper protein is formed. They can have a toxic effect, which can be avoided by co-injection with a p53-MO. Also, off-target effects were reported, what makes controls necessary	[46, 60, 67]
pri-miRNAs	Yes	No	Yes	Must be injected into the zygote	Function similar to siRNA, in which the mRNA by small RNA-constructs and then is degraded. Because of signals in the cell, all of the mRNA carrying the gene of interest is degraded. The coding sequence for the pri-miRNA is introduced into the genome	[11, 16, 53]

Continue...

Continuation of the table I

Method	Line[a]	F0[b]	Specificity	Application	Function	Reference
Transposon	Yes	No	DNA sequence specific	Tol2 transposase mRNA and plasmid are co-injected. Kits for this method are available	Transgenesis. It is based on transposable elements and similar to retroviral vectors. The gene of interest is flanked by sites similar to the ones transposable elements are flanked by. The transposase opens the DNA at a site, in which a mobile element could be introduced. There, the gene of interest is introduced	[34]
I-Sce1	Yes	No	DNA sequence specific	Enzyme and plasmid must be injected into the zygote	Transgenesis. The plasmid contains the gene of interest flanked by the I-Sece1 recognition site. The gene is introduced into the DNA via homologous recombination	[18]
Small molecule	No	Yes	Affects protein. Binding depends on many factors	Diluted into the medium the larvae live in	They have a molecular weight < 900 Da and are organic compounds. Biological processes are regulated when the molecule binds to the protein and inhibits or activates it. Temporal control of protein function is also possible by applying the molecule during different stages	

*The protocols for these methods can be found at: https://wiki.zfin.org/display/prot [91]; athis method is used to generate a genetically modified line. The earliest screening of the effect can only be done in the F1-generation; bthe effect of this method can be seen in the embryos, whose zygotes were treated. No genetic line required or possible.

Table 2 – Dental characteristics of each model

Model	Description
Zebrafish 	polyphodont organism; 22 pharyngeal teeth (11 on each lateral body side); no buccal teeth.
Mouse/rat 	monophodont dentition; four continuously growing incisors and 12 molars; canine teeth and anterior premolars are absent – the toothless space between the incisors and molar teeth is known as diastema.

Zebrafish as a model organism has several advantages. It is a model organism with a large and diverse set of genetic and molecular tools that is already readily available. Zebrafish has an easy husbandry system and relatively fast embryonic development. They develop rapidly, and at 24 hpf most of the embryos' organ primordia has formed [20] with the whole development described by Kimmel *et al*. [35]. An overview of the development and established methods can also be found on the community website zFIN (https://zfin.org).

Another big advantage of zebrafish as a model organism is that the embryos develop outside of the maternal body and can easily be manipulated. As an aquatic animal, its development can be partially influenced by adding small molecules into their watery environment to test if these target molecules impact on dental development. Due to their relatively high number of offsprings, which supercedes that of rodents, they can also be used in screening experiments. In-vitro fertilization protocols were established leading to a better control, when eggs are fertilized, and thus allow proper staging of the embryo. The respective protocols can be found on the community website (https://zfin.org/zf_info/zfbook/chapt2/2.8.html).

Some differences between zebrafish and mammals, however, must be considered:

It should be mentioned that teleost fish duplicated their whole genome during evolution, as reviewed by Braasch and Postlethwait [3]. Many of these paralogs might have diverged in expression pattern and/or function throughout evolution. This process is called subfunctionalization [3, 17] and has two implications: it can lead to redundancy in mutants or morphants, in which cases a phenotype would only appear when both paralogs are silenced; and before comparing the gene expression in zebrafish and mammals, in which the degree of homology must be determined. It could very well be that the gene found in zebrafish is not expressed in the homologue part of the mammal or has receive a different function there;

Fishes develop ossified pharyngeals deriving from arch 7 via the fifth pair of ceratobranchials. These pharyngeal teeth are unique, as reviewed by Mork and Crump [44]. In contrast to other fish species, zebrafish lacks teeth in the buccal cavity. This makes the pharyngeal teeth the only ones which can be studied regarding their development. Because the pharyngeal teeth are located posterior of the buccal cavity, they develop in an endoderm covered pharynx, thus making the early development more complex than in mammals, in which the teeth derived only from ectodermal epithelium [57]. Oralova *et al*. [48] showed that multiple epithelia are required for tooth development inside the pharynx. In vertebrates, the endodermal epithelium of the developing pharynx produces pouches, that contact the skin ectoderm at corresponding clefts [19]. Pouch 6 plays a role in pharyngeal tooth development. Periderm (the initial epithelial covering of the embryo) partially invades the pouches and endogenous cells that resemble periderm cells phenotypically, spread over the endoderm along the midline [56]. Thus, at the time of tooth differentiation, the pharynx epithelium is composed of a double layer: a basal endoderm beneath a layer with periderm-like characteristics. Only after this bilayer has formed, tooth development can start, meaning that endoderm and periderm are required for tooth development. [48]. Further, Oralova *et al*. [48] showed that the enamel organ is derived from medial endoderm posterior to pouch 6, that dental morphogenesis starts only after pouch 6 made contact with the skin ectoderm, and that pouch 6 contact and the presence of midline cells are required, but not alone, sufficient, for tooth development. The involvement of different germ layers causes a more complex regulation before tooth development initiation. That leads to a higher cell communication, and so the involvement of signaling pathways and regulatory genes is not to be found in buccal tooth development;

One consideration has also to be about tooth eruption, what is defined as the movement of the tooth from its site of development within the jaw to its functional position in the mouth [59]. There are two possible tooth locations in vertebrates: extraosseous (extramedullary) or intraosseous (intramedullary). In case of teleost, both can be in the oral and in the pharyngeal region, as reviewed by Trapani [72]. Zebrafish teeth develop in an extraosseous position. Its eruption is defined as piercing of the tip through the pharyngeal epithelium, irrespective of whether the tip of the tooth is still hidden by folds of the epithelium, or distinctly protrudes into the pharyngeal cavity. During its eruption of first-generation teeth, firstly a lumen is formed in the area of the pharyngeal tooth. The ventral epithelium first firms a shallow depression, which becomes a gutter-like depression. This depression is called crypt [24]. Teeth erupt when their tips emerge through the lateral wall of these crypts. At the beginning, the tooth lies within the crypt. Later, the epithelium folds more elaborately, and the tooth tip protrudes the surface. The crypts form in association with the development of tooth germs, what might lead to correct positioning of

the crypt [24]. One obstacle in this kind of eruption is that the enamel organ in zebrafish remains broadly connected to the epithelium throughout tooth development. This makes it more difficult to identify the different epithelial layers during the eruption process. In mammals, teeth develop intraosseously, what means that they have to move from their site of development within the jaw to their functional site. It includes several phases and only one is mucosal penetration [42]. This difference also causes different cell behavior and makes it more difficult to figure out which genes are involved in both vertebrate classes;

Sire *et al.* [63] showed that first-generation teeth are a good model which can be compared between different vertebrate species, but they are not sure about replacement teeth, because they can differ from first-generation teeth in several ways. They are bigger and show a finer substructure. Often, they are also adapted to their function, what is not to be seen in first-generation teeth.

In summary, since mammalian ancestors and the ancestors of zebrafish diverged about 450 million year ago [1], there are genetic and cell behavioral differences between mammals and zebrafish that must be taken into consideration during tooth development research.

Table 3 shows the main differences between dentition in zebrafish and mouse, while Table 4 indicates the main advantages using zebrafish or mouse in dental research. Both facts should be considered while deciding what animal model should be chosen.

Conclusion

We showed in this review that zebrafish is a good model organism to study odontogenesis. In Figure 3, structural similarities and differences in tooth development in zebrafish and mice are demonstrated, what indicates that the early steps can be compared. It is a fair assumption that, because of these similarities, tooth development in zebrafish and mouse can be compared as long as the differences are kept in mind as well.

Furthermore, already established methods were described here. There is a large community, which keeps improving respective methods and knowledge, and there are also facilities, where certain fish lines can be ordered for research. Every region has their own facility, which is mentioned on the community website.

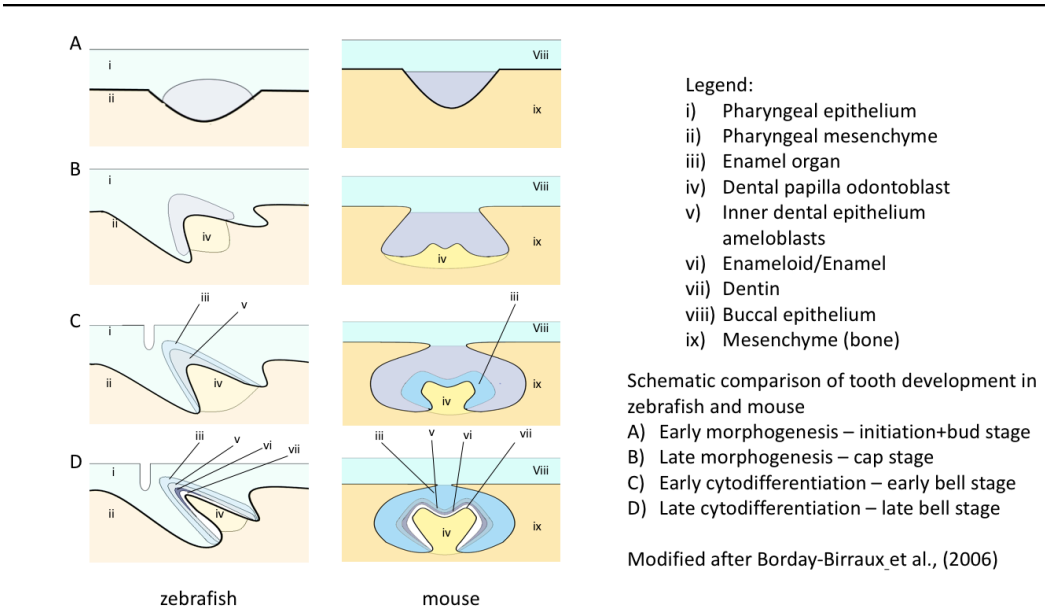

Figure 3 – Comparison of early tooth development in zebrafish and mouse: structural similarities and differences in tooth development in zebrafish and mice are demonstrated, indicating that the early steps can be compared

Table 3 – Some attributes of the models used to study dental development

Attribute	Model	
	Zebrafish 	**Mouse/rats** 
Husbandry infrastructure	$	$$$
Cost per animal per year	$	$$$
Dental morphology similarity	+	++
Genetic similarity	+	++

$: cost; + strength

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