How to Replace a Tooth: Fish(ing) for Answers

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Summary: The dentition of vertebrates evolved from a condition with simple conical teeth, showing continuous replacement, to a condition with teeth of a highly complex shape, and - in mammals - replaced only once, at the most. The zebrafish (*Danio rerio*) has become a model organism for genetic, molecular and developmental studies and is used in our laboratories to analyse the mechanism responsible for such continuous tooth replacement. This review addresses three issues. Firstly, we show that understanding the tooth replacement pattern is vital for a correct interpretation of experimental and molecular data regarding odontogenesis in this zebrafish model. Secondly, recent morphological evidence suggests that tooth replacement in the zebrafish is a biphasic process, possibly regulated by independent mechanisms: the formation of an epithelial down growth (called successional dental lamina), and the development of a tooth germ from this lamina. Thirdly, within the frame of testing the hypothesis where adult epithelial stem cells underlie the process of continuous tooth renewal in the zebrafish (and other polyphyodont vertebrates); we focus on the Wnt signaling pathway as a possible molecular control mechanism.

Conclusion: Future studies can take advantage from the wide array of tools available for zebrafish research to test the hypothesis of stem cell involvement and to examine the potential role of Wnt signaling in the process of tooth renewal. Eventually, answering the question of why zebrafish (and most other vertebrates) are capable of renewing their teeth throughout life can help to solve the riddle why this process is arrested in mammals.

Key words: tooth replacement, stem cells, zebrafish, Wnt

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INTRODUCTION

The dentition of vertebrates (including mammals) evolved from a condition with simple conical teeth, showing continuous replacement, to a condition with teeth of a highly complex shape, and - in mammals - replaced only once, at the most. Despite different tooth shapes and different numbers of tooth generations, the basic process of generating a tooth is highly conserved among vertebrates (Huysseune and Sire, 1998). To understand the mechanism responsible for continuous tooth replacement, the study of an organism which is still capable of life-long tooth renewal, and for which a wide array of research tools is available, presents unquestionable advantages.

The zebrafish (*Danio rerio*) has become such a model organism for genetic, molecular and developmental studies. Although the zebrafish has pharyngeal teeth only, they are homologous to other vertebrate teeth (Huysseune and Sire, 1998). Like all other tooth-possessing non-mammalians, the zebrafish has the capacity to replace its teeth throughout life, making this animal an extremely attractive model. Below, we will briefly review, first, how zebrafish teeth are patterned and why this knowledge is important, and second, when and how replacement teeth are formed, highlighting resemblances and differences to mammalian replacement tooth formation. Finally we will discuss a recent hypothesis on stem cell involvement in continuous tooth replacement, and the possible molecular control of the process.

THE ZEBRAFISH DENTITION: KNOWING THE PATTERN IS ALL IMPORTANT

The zebrafish is a small freshwater fish (adult size is approx. 3cm standard length (SL)), related to carps (fam-

ily Cyprinidae) and thus belonging to the dominating group of modern bony fish called the teleosts. Whereas more primitive members of the teleosts often have teeth distributed over the mandibular, hyoid and all of the five branchial (gill-bearing) arches, the zebrafish dentition is restricted to the last (fifth) branchial arch. Here the teeth (called pharyngeal teeth) are associated with the ventral elements of the arch, the paired fifth ceratobranchials. Like in other members of the cyprinid family, the teeth on the mandibular arch got lost during evolution. In adults, each ossified fifth ceratobranchial carries three rows of teeth, a ventral (V), mediodorsal (MD) and dorsal (D) row, with five, four and two teeth, respectively (Van der heyden and Huysseune, 2000; Van der heyden et al, 2001) (Fig 1). The ventral tooth row is the first to form during early development, and is completed at 16 days post-fertilization (dPF), prior to the formation of the mediodorsal row, which itself is completed by the time the dorsal row starts to form. Embryonic development in the zebrafish proceeds very quickly. Under standard conditions the embryo's hatch at 48 hrs after fertilization (48 hPF), at which time the very first tooth is being formed (the fourth tooth in the ventral row, called $4V^1$, the superscript indicating that this tooth belongs to the first tooth generation). When the animal reaches 80 hPF, this tooth is fully formed, attached to the underlying ceratobranchial, and flanked by the two tooth germs that develop next, $3V^1$ and $5V^1$. These teeth form from an invagination of the pharyngeal epithelium that delimits the prospective pharyngeal cavity along its ventral side (Figs 2-3).

Tooth replacement in zebrafish starts at an extremely precocious stage: even before the germ of the next tooth, $2V^1$, becomes visible, a replacement tooth germ, called $4V^2$, is initiated in association with $4V^1$, followed by the initiation of replacement teeth ($3V^2$ and $5V^2$) for the two adjacent teeth, $3V^1$ and $5V^1$ (Figs 1, 4). From that moment on, the addition of new, first-generation, teeth along the ventral tooth row coincides with the formation of replacement teeth in the "older" positions. Finally, a pattern is established in which teeth are replaced simultaneously, first in the odd positions (1V-3V-5V) followed by those in the even positions. At about 28 dPF, the full complement of eleven teeth is formed and, except for the last ones formed, all have gone already through cycles of replacement.

The first-generation teeth, and even the first replacement teeth (or second-generation teeth) are extremely small (approximately 60µm tall and 12µm across at the tooth base), and closely packed. Yet, the developmental processes involved in odontogenesis are remarkably similar to those involved in mammalian tooth formation: the tooth develops through a concerted action of an epithelial invagination, the enamel organ, and a mesenchymal cell condensation, the dental papilla, leading to a conical structure with a hypermineralized cap, and a dentine cone encircling the pulp cavity (Huysseune et al, 1998; Van der heyden et al, 2000). However, the cyprinids are part of an evolutionary lineage of their own (they are not at all to be considered the ancestors of mammals, cf. Metscher and Ahlberg, 1999; Witten et al, 2001), and so they possess both primitive and derived dental characters. A primitive character of zebrafish teeth is the lack of root structures; instead the tooth attaches directly to the underlying bone via a collar of attachment bone. Almost immediately after attaching to the pharyngeal jawbone the tooth erupts, largely through remodeling of the overlying epithelial surface (Huysseune and Sire, 2004). Attached and erupted teeth constitute the functional teeth of the dentition. Different from mammals and more advanced

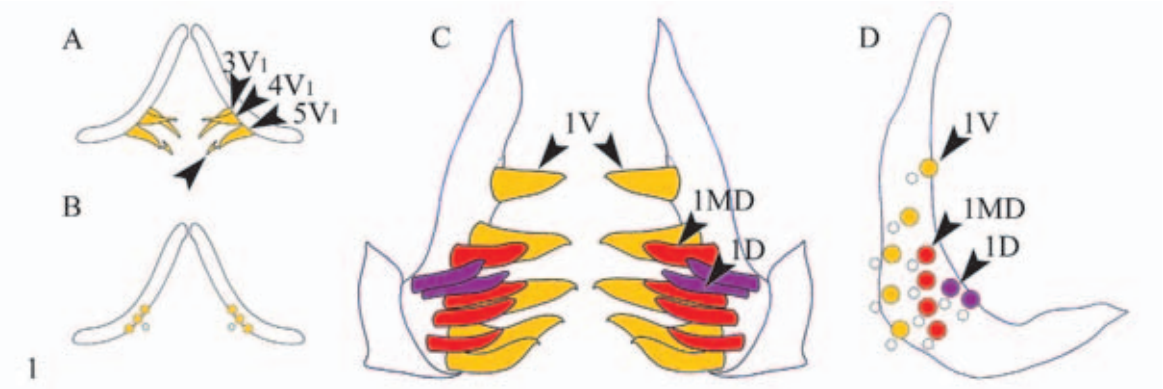
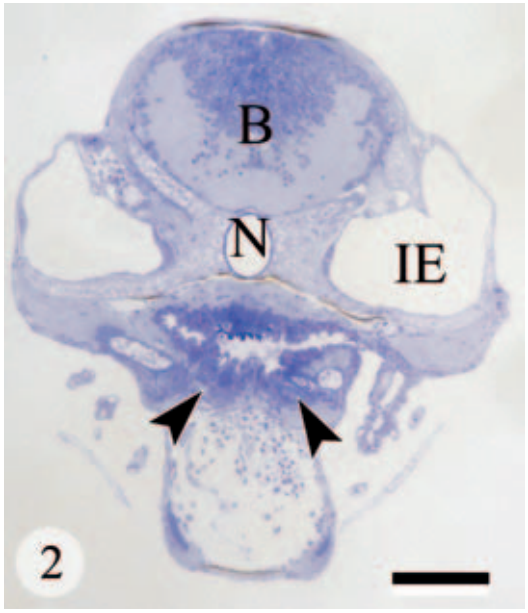


Fig 1 (a, c) Dorsal and slightly posterior view on the pharyngeal jaws of a six-day-old larva **(a)** and a one month old juvenile **(c)** zebrafish; anterior to the top of the figures. In **(a)**, teeth $3V^1$, $4V^1$ and $5V^1$ are attached and functional, the tooth germ in cytodifferentiation stage is the replacement tooth for $4V^1$ ($4V^2$), and is indicated by an arrow. In **(c)**, only functional (attached) teeth are shown: the teeth of the ventral row (1V-5V) in orange, those of the mediodorsal row (1MD-4MD) in red, and the two dorsal teeth (1D-2D) in purple.
Fig 1 (b, d) Schematized drawings from the situation in **(a)** and **(c)**, indicating the position of each functional tooth (large circles) and their associated replacement teeth (small circles). The replacement teeth develop ventral and posterior to each functional tooth. The drawing in **(d)** is obtained by tilting the right pharyngeal jaw on **(c)** over 90°.



Figs 2-7 Transverse, 1µm thick, toluidine blue stained plastic sections through the pharyngeal region of a six-day-old larva (Figs 2-4) and one month old juvenile (Figs 5-7) zebrafish.
Fig 2 Larva of 6 dPF, cross section through the head, showing the position of the brain (B), inner ear (IE), notochord (N) and pharyngeal dentition (black arrowheads). Bar = 100µm.

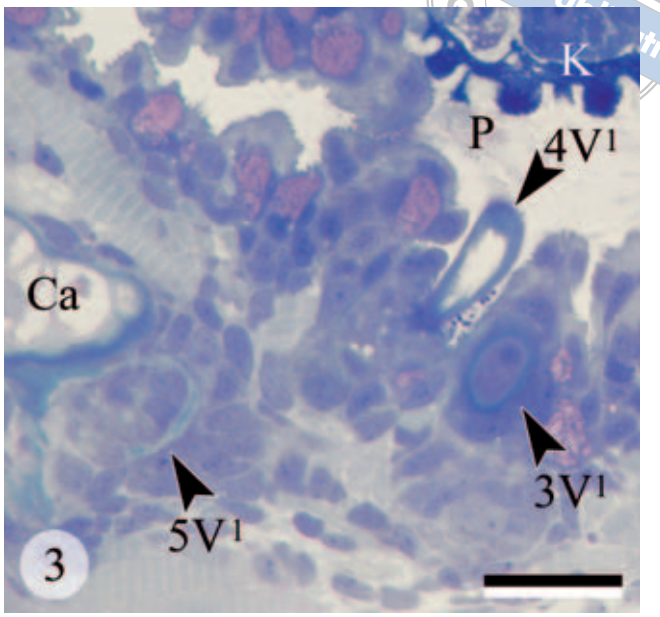


Fig 3 Detail of the left pharyngeal jaw. Because the section was cut slightly obliquely, all three first-generation teeth present are visible in one section (the tip of tooth $3V^1$ and $4V^1$, and the base of tooth $5V^1$). Ca: ceratobranchial cartilage; K: keratinized pad in the roof of the pharyngeal cavity (P). Bar = 20µm.

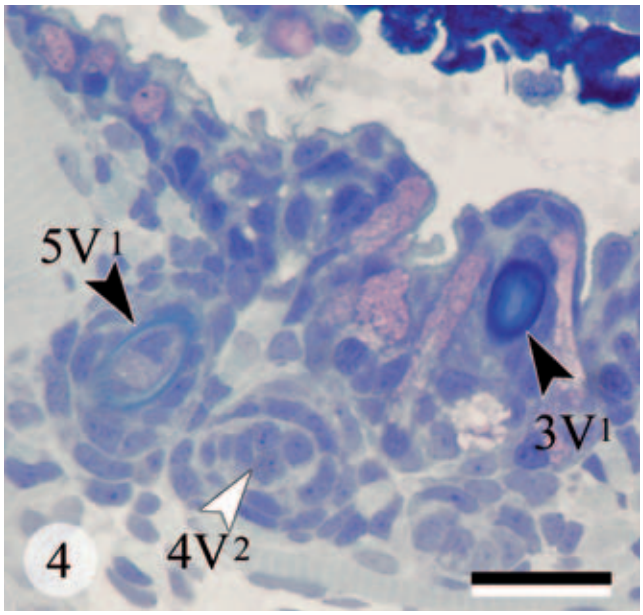


Fig 4 Same specimen, level slightly posterior to that shown in Fig 3. Below tooth $4V^1$, the anlage of the first replacement tooth in the dentition, $4V^2$ is visible. Still visible are the tip of tooth $3V^1$ and a mid-level section through tooth $5V^1$. Bar = 20µm.

bony fish with intramedullary tooth development (see below), eruption does not involve the resorption of the dentigerous bone to allow passage of the tooth, but some smoothening of the future tooth attachment site by osteoclasts appears to be required cf. Witten et al, 2001. On the other hand, a likely derived character is the nature of the hypermineralised cap (an admixture of epithelial and mesenchymal products, laid down before the dentine, and called enameloid) (see discussions by Smith, 1995; Huysseune and Sire, 1998).

Because of the small size and close packing of the teeth, and given that replacement starts long before the full complement of teeth is formed, it is easy to confound first-generation and replacement teeth, at least in early larval development. Making this distinction is nevertheless crucial if we want to know in what way replacement teeth differ from first-generation teeth, i.e. if we wish to answer the question whether similar cascades of gene activation are involved in making the first tooth at a given locus, or its replacement tooth. Thus, inevitably, interpreting patterns of gene expression

requires a detailed knowledge of the pattern of initiation and replacement of the teeth, and a detailed analysis of expression patterns at the microscopic level. Meeting these requirements, it has been possible to identify genes expressed either in the germs of first-generation teeth, of replacement teeth, or of both (Jackman et al, 2004; Laurenti et al, 2004; Borday-Birraux et al, submitted). Similarly, detailed knowledge of the pattern has been essential to establish the failure of replacement teeth to form under certain *in vitro* conditions (Van der heyden et al, 2005), opening perspectives for experimental approaches to dissect the replacement process.

THE SUCCESSIONAL DENTAL LAMINA IN ZEBRAFISH: RENEWAL SECURED

In a first attempt to trace the pattern of tooth replacement in zebrafish from larvae up to the adult stage, Van der heyden (2001) used cleared and stained specimens. From this study, the impression was gained that not every functional tooth had a replacement tooth ready to succeed it. However, detailed examination of a number of serially (1µm) sectioned specimens (Huysseune, unpublished results) revealed two important new findings (Fig 5). First, every functional tooth is associated with an epithelial down growth, preceding the formation of the replacement tooth bud proper. This down growth is similar to a dental lamina, and called a successional lamina (Fig 6). Second, whereas young functional teeth are associated with young replacement teeth only, old functional teeth can have a replacement tooth still in a stage of successional dental lamina. Thus, it appears that the successional lamina can be quiescent for a while, and start with the development of a replacement tooth only upon the stimulation by so far unknown factors (Fig 7). This suggests that the formation of the successional lamina and the replacement tooth bud arising from it could be regulated independently. Further support for the notion of two independently regulated events comes from advanced teleosts which, unlike zebrafish, develop their teeth within the medullary cavity of the bone: here the formation of a deep epithelial down growth, penetrating into the bone marrow cavity via the creation of a resorption channel, precedes by far the development of a tooth bud at its deep end.

The most widespread model in tooth research is the mouse, which unfortunately makes only one tooth generation and lacks evidence of the anlage of a second generation tooth. Humans however, being diphyodont mammals, produce a second generation of teeth (the permanent incisors, canines and premolars). Given the scarcity of the material, few microscopic studies are available on the developing human dentition. According to Mjör and Fejerskov (1979), the successors of the deciduous teeth are preceded by an increased growth of the free end of the dental lamina, lingual to the enamel organ of each deciduous tooth. This extension is designated the successional lamina and occurs from the fifth month in utero (permanent central incisor) to 10 months of age (second premolar). In enamel organs of

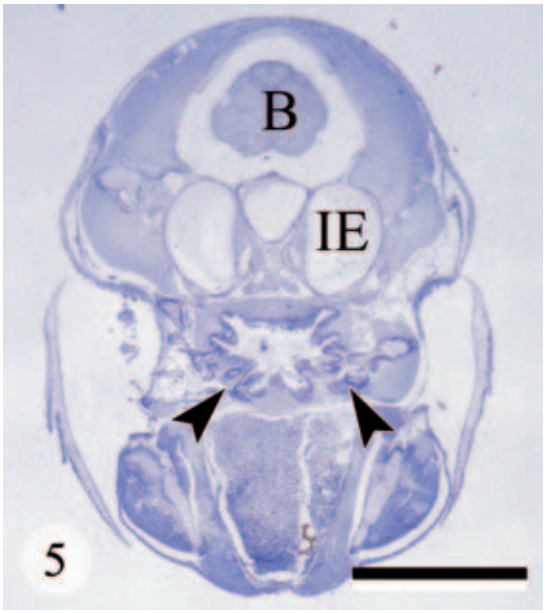


Fig 5 Juvenile of one month of age (9mm SL), cross section through the head, showing the position of the brain (B), inner ear (IE), and pharyngeal dentition (black arrowheads). Bar = 500µm.

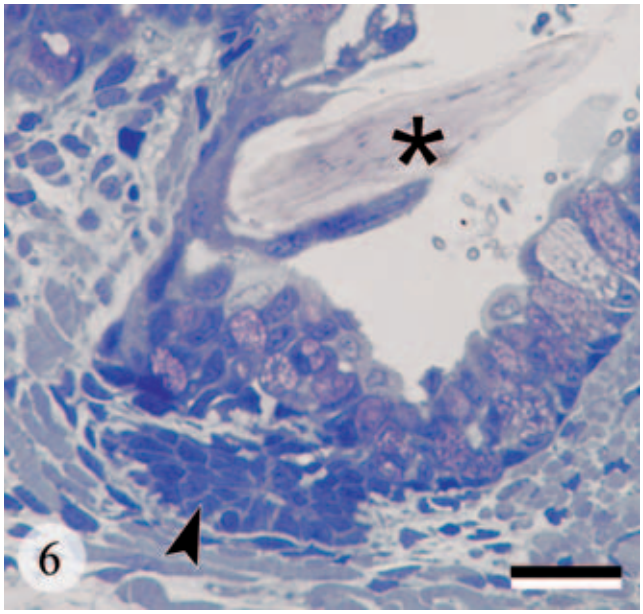


Fig 6 Same specimen, successional dental lamina (arrowhead) associated with the functional tooth in position 2V. The rear margin of the predecessor (asterisk) is still visible in the picture. Bar = 20µm.

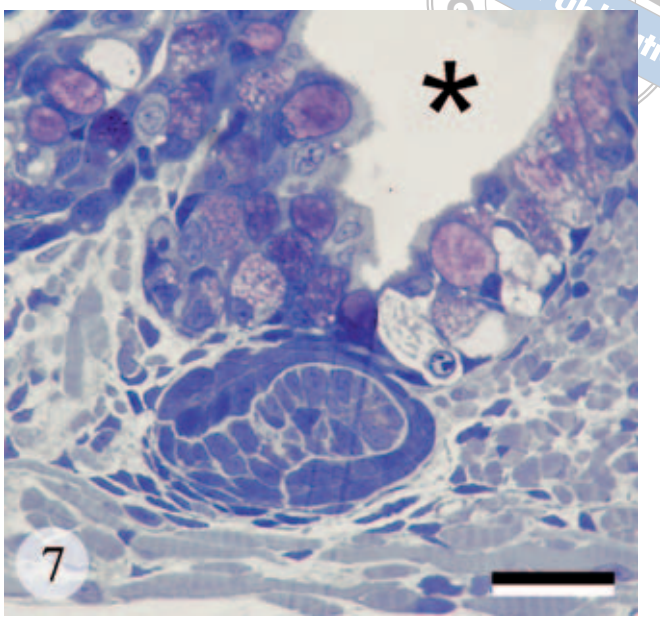


Fig 7 Same specimen. Morphogenesis stage replacement tooth (cf. Van der heyden et al, 2000), associated with the functional tooth in position 3V. An asterisk marks the position of the epithelial crypt surrounding the erupted part of the predecessor (not visible in the section). Bar = 20µm.

permanent teeth, such lingual down growths eventually disappear (Berkovitz et al, 1992).

It is tantalizing to speculate that organisms that develop but two tooth generations, like humans, still possess the source of cells that could make a replacement tooth, but that activation fails, or that activation is prevented by some inhibitory mechanism. Recently, Wang et al (2005) suggested that Runx2 might have just this sort of function, to prevent cycling of teeth. Their evidence comes from the formation of an unusual epithelial down growth, resembling a successional lamina, in Runx2 and Runx2/Runx3 knockout mice, as well as in Runx2 heterozygotes, and from the observation that humans suffering from cleidocranial dysplasia (CCD), a syndrome caused by lack of function of one allele of RUNX2, develop a tertiary dentition.

What could be the nature, in the zebrafish, of the source of cells securing the formation of new teeth, and what could be the factors that activate them? In the final section, we will consider the possibility that stem cells could be involved in the process of continuous replacement.

DEVELOPMENT OF REPLACEMENT TEETH THROUGH STEM CELLS AND WNT SIGNALING?

Mammals possess several continuously cycling structures, like the skin, the hairs, or the intestinal crypts. In these systems, adult epithelial stem cells have been proposed to underlie the cycling process. The classical view is that stem cells undergo division, whereby one daughter cell proliferates and its descendants enter a differentiation pathway, whereas the other daughter cell perpetuates the stem cell. Recently, Huysseune and Thesleff (2004) have proposed the hypothesis that adult epithelial stem cells might also be involved in the generation of new replacement teeth in continuously cycling dentitions, like in the zebrafish. Some of the assumptions on which this hypothesis is based are currently being tested. For example, we are currently mapping the spatial and temporal patterns of proliferation of epithelial (and mesenchymal) cells during the formation of replacement teeth in one month old zebrafish (i.e., when the full complement of teeth is present). However, independent of the nature of the source of cells (whether they are true stem cells or not), the formation of a successional lamina, and the subsequent (and sometimes delayed) development of a tooth bud from it, calls for a mechanism (or perhaps two separate mechanisms) that activates the cells involved.

The so-called canonical Wnt signaling pathway has been shown to be involved in stem cell activation in other cycling epithelial structures like hairs and intestinal crypts (Alonso and Fuchs, 2003; Pinto and Clevers, 2005), and is possibly also implicated in the development of replacement teeth (Huysseune and Thesleff, 2004). When a Wnt signal factor binds to its receptor, β–catenin is no longer subjected to cytoplasmic breakdown; instead it translocates to the nucleus, associates with transcription factors and regulates the transcription of target genes. Jamora et al (2003) showed that, as a result of Wnt signaling, E-cadherin is down regulated in the stem cells of the hair, allowing rearrangement of the cells to make an epithelial bud. Can we collect evidence to support a similar scenario during the process of continuous tooth replacement?

Here is where the extensive toolbox available to zebrafish researchers comes into play again. Gene knockdown techniques using antisense, morpholino-modified oligonucleotides (morpholinos) (Nasevicius and Ekker, 2000) have become a widespread tool to analyse gene function. Large-scale methods have been developed to reveal live gene expression in zebrafish using fluorescent proteins as markers, and hundreds of transgenic lines have been isolated with specific expression of fluorescent protein in tissue-specific patterns. Somewhat longer ago, large scale mutagenesis has yielded many hundreds of mutants (Haffter et al, 1996), and the mutated gene is being identified in an ever increasing number of mutants, some of which are very useful to analyse the role of Wnt in the dentition. E.g., the masterblind (mbl) mutant carries a mutation in axin 1, a scaffolding protein of the degeneration complex of β–catenin (Heisenberg et al, 2001). Loss of function of this gene results in stabilisation of β–catenin, thus phenocopying overactivation of Wnt signaling. In ongoing experiments we test whether overexpression of Wnt using the Wnt activator LiCl can rescue replacement tooth formation *in vitro*. Together, these observations should give us indications on the possible involvement of Wnt in tooth replacement in this model.

The task is daunting, nevertheless. The picture that emerges from the numerous studies on the role of Wnt signaling in cell proliferation, embryonic development and tumorigenesis (see reviews by, for example, Pires-daSilva and Sommer, 2003; Behrens and Lustig, 2004) is that of a highly complex pathway, with multiple components and interacting proteins which can either activate or inhibit Wnt signaling, as well as the likely occurrence of cross-talk with other signal transduction pathways. In addition, β–catenin is also involved in control of cell-cell adhesion, both directly by binding to E-cadherin (Perez-Moreno et al, 2003) and via the Wnt pathway, with E-cadherin as a target gene of canonical Wnt signalling (Jamora et al, 2003). Moreover, it has become increasingly clear that Wnt signaling seems to control the differentiation program of adult stem cells in a dosage dependent way (Gaspar and Fodde, 2004). Not surprisingly, studies examining the involvement of Wnt signaling often yield contradictory results. de la Fuente and Helms (2005) recently highlighted such conflicting results in the light of skeletogenesis. More important in the context of continuous renewal are the contradictory phenotypes observed in the dentitions of human patients affected by mutations in the Wnt signaling pathway. Loss of AXIN2 leads to hypodontia (Lammi et al, 2004), whereas loss of APC, another scaffolding protein of the β–catenin degradation complex, can lead to hyperdontia (Wang et al, 1998). Obviously, taking into account timing, duration and dose of Wnt signaling will be all important to understand its potential role in tooth cycling. We can take advantage of the many technologies available to zebrafish research in order to get more insight into the role of Wnt signaling on possible stem cell activation and replacement tooth formation. Eventually, answering the question why zebrafish (and most other vertebrates) are capable to renew their teeth throughout life can shed light on the question why this process was arrested in mammalian evolution.

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