Morphological features of tooth development and replacement in the rabbit Oryctolagus cuniculus

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Title page

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Highlights

- Tooth replacement is initiated at the lingual side of the deciduous tooth
- Permanent tooth morphogenesis can occur at the vestibular side of the deciduous tooth
- Rabbit first molars present a rudimentary successional dental lamina
- Newborn rabbits present dentin holes in their incisors opening the pulp cavity
- The rabbit is a suitable model to study mammalian tooth replacement

Abstract

Dental development mechanisms in mammals are highly studied using the mouse as a biological model. However, the mouse has a single, unreplaced, set of teeth. Features of mammalian tooth replacement are thus poorly known. In this paper, we study mammalian tooth development and replacement using the European rabbit, *Oryctolagus cuniculus*, as a

new model. Using 3D-reconstructions associated with histological sections, we obtained the complete description of the histo-morphological chronology of dental development and replacement in rabbit. We also describe the presence of holes in the dentin opening the pulpal cavity in newborns. These holes are quickly repaired with a new and fast apposition of dentin from the pre-existing odontoblasts. The detailed dental morphogenesis chronology presented allows us to propose the rabbit *Oryctolagus cuniculus* as a suitable model to study mammalian tooth replacement

Keywords

Tooth development, mammalian tooth replacement, diphyodonty, 3D-reconstructions, rabbit teeth

Main text

Introduction

The signaling pathways involved in tooth development and morphogenesis are highly studied in mice (Balic & Thesleff, 2015), fishes (Rasch et al., 2016), or reptiles (Whitlock & Richman, 2013). Regarding tooth replacement (Bertin, Thivichon-Prince, LeBlanc, Caldwell, & Viriot, 2018), a majority of fish and reptiles have the ability to replace their teeth continuously throughout their life (polyphyodonty) contrary to the majority of mammals that replace their teeth only once (diphyodonty). In mammals, most odontogenesis studies use the mouse as a model species and mouse tooth development is well described from its morphogenesis to the genetic mechanisms involved. Tooth development is commonly divided into five stages: the placode, the bud, the cap, the bell, and finally the maturation stages. At first, a dental placode appears from the oral epithelium by thickening. Next, at the bud stage, the dental epithelium invaginates, followed by a condensation of the dental mesenchyme. Then, the epithelium starts to fold, forming the cap stage; this stage marks the beginning of

histo-differentiation. The tooth cuspidogenesis starts at the bell stage. Cells begin to secrete dentin and enamel layers at the late bell stage. In the maturation stage, the tooth gets fully mineralized. Because the mouse has no dental replacement, the mechanisms of tooth replacement in mammals are poorly known. Some studies have been done in the ferret (Järvinen, Tummers, & Thesleff, 2009; Jussila, Crespo Yanez, & Thesleff, 2014), the shrew (Järvinen, Välimäki, Pummila, Thesleff, & Jernvall, 2008), the fruit bat (Popa, Anthwal, & Tucker, 2016) and the minipig (Wang et al., 2014; Wang et al., 2019). Altogether, these studies have characterized the morphological changes involved in tooth replacement: the initiation of tooth replacement begins by a budding of the replacement dental lamina at the lingual part of the tooth stalk. Then the replacement dental lamina plunges into the mesenchyme and the tooth morphogenesis begins. Moreover, the first molecular observations of tooth replacement mechanisms in mammals have been done in the ferret *Mustela putorius furo* (Järvinen et al., 2009; Jussila et al., 2014). However, few developmental stages were studied due to the difficulty to obtain ferret embryos at the correct stage and the presence of seasonal estrus. The shrew *Sorex araneus* is limited as a model because their deciduous teeth never erupt. In the fruit bat *Eidolon helvum*, 3D reconstructions and histological sections are good illustrations of mammalian tooth replacement but authors indicated that this species is no longer available for experimental purposes (Popa et al., 2016). Tooth development and replacement has also been described in the minipig *Sus scrofa,* whose teeth size and morphology are pretty similar to human teeth (Wang et al. 2014). However, at least 100 days are necessary from the appearance of the replacement dental lamina to the mineralization of the replacement tooth in the minipig. So, the slow development, the size of the animal and ethical concerns are significant obstacles to study tooth development in the minipig. For now, any model can answer both morphological and molecular questions about mammalian tooth replacement, and previous studies are restricted to some parts of dental development. We thus

decided to use the European rabbit (*Oryctolagus cuniculus*) as a new model to study tooth development and replacement. The rabbit is already an important model for biomedical research (Bosze & Houdebine, 2006) and the whole genome has been sequenced (OryCun2.0, 2009). The rabbit dentition is diphyodont with a replacement of some incisors and all premolars (Horowitz, Weisbroth, & Scher, 1973). They possess two upper and one lower incisors, three upper and two lower premolars and three upper and lower molars on each side. Deciduous premolars have a limited growth with root formation whereas permanent teeth are continuously growing (Sych & Reade, 1987). Associated with this continuous growth, each permanent tooth in the rabbit is supposed to possesses a pool of mesenchymal and epithelial stem cells in its growing part, as in all hypselodont species (Tummers & Thesleff, 2003). Rabbit dental development has been studied during the 70's and 80's, Horowitz and collaborators (1973) completing a chronology of deciduous tooth eruption using radiography, giving information on the mineralized part of the teeth. Navarro carried out histological studies of the postnatal development in maxillary (1975) and mandibular cheek teeth (1976). However, the deciduous incisors and premolars are completely mineralized at birth and thus Navarro et al. do not document their development. Hirschfeld et al. (1973) and Ooë (1980) have studied the incisor development in embryos, but nothing has been reported for the cheek teeth development. Michaeli et al., (1980) gave a simplified chronology of rabbit cheek tooth development during embryogenesis without separating each tooth and with histological illustrations only for the post-natal stages. All these studies result in a composite and fragmental chronology of the tooth development in rabbit. The purpose of our work is to supply a complete report of the development of all deciduous and permanent teeth in the rabbit. We decided to study rabbits from 12 days post fertilization to 4 days post-natal in order to obtain a complete chronology of tooth development from the initiation of the deciduous tooth to the mineralization of the replacement teeth. Using histological studies and 3D-

reconstruction of soft tissues, we present the developmental and morphological characteristics of rabbit teeth.

Material & Method

Samples

Embryos and newborn rabbit samples used for histology and X-Ray microtomography were obtained through collaboration with a standard production for feeding recorded at the Biology Departmental livestock establishment (EDE) n°38044102. We worked on common rabbit breeding regularly crossed with wild rabbits to ensure genetic mixing. The gestation period for a rabbit is about 31 days. We collected embryos at 12, 14, 16, 18, 20, 22, 24, 26 and 28 days post fertilization (dpf) and rabbits at 0, 1, 2, 3 and 4 days post-natal (dpn). 3 to 21 embryos were analyzed for each developmental stage. For juvenile and adult rabbits, observations were made on 230 specimens of *Oryctolagus cuniculus* from the Muséum National d'Histoire Naturelle (MNHN, Paris, France). Age of the specimens were not indicated so we used the skull size as proxy, our sampling contained skull size from 36.65 to 108.8mm. In the 230 specimens, only four still had their deciduous teeth. Each tooth is specifically named: we use 'I', 'P' and 'M' for incisor, premolar and molar respectively; the deciduous teeth are indicated with a 'd'; the tooth number is in exponent for the upper teeth and in index for the lower teeth.

Histology

After dissection of the upper and lower jaws, samples were fixed overnight in 4% PFA. Samples were then rehydrated and fixed in Holland Bouin solution during 1 day. Jaws were demineralized for 1 to 7 days in Morse's solution (10% sodium citrate + 22% formic acid) or in RNase-free 12.5% EDTA + 2.5% PFA during 6 weeks, according to the

subsequent experiment to be performed. Then, jaws were embedded in paraffin and serially sectioned with a Leica microtome (7 µm thick slices). Sections were carried out in parasagittal or frontal plane. For histological purpose, samples were dewaxed in xylene, and then colored via an optimized Masson's trichrome protocol with aniline blue, hemalum and fuschin.

In situ hybridization

Specific probes for Osteocalcin and K14 have been designed using the rabbit genome available online (OryCun2.0, 2009). Each probe has been sequenced before use, and then probes were synthesized with RNA-dig labeling nucleotides. Classical mouse in situ protocol has been optimized to be compatible with rabbit tissues. Tissues were treated with HCl 0.2M, with proteinase K 5µg/ml and then incubated with TEA-Acetic anhydride. Hybridization step were done at 62°C. Sections were whashed in a warm washing buffer (1X SSC and 50% formamide) and in MABT solution. After 2 hours in a blocking solution, slides were covered of anti-Dig-AP-antibody solution at 4°C overnight. Sections were washed with MABT and NTMT with teramisol. Sections were stained with BM purple solution and mounted in Aquatex.

X-ray microtomography and 3D reconstruction

Conventional X-ray microtomography allows reconstructing 3D models of mineralized tissues such as bony head and teeth, thus providing a non-invasive access to internal structures. Using phosphotungstic acid (PTA) staining, that binds heavily to various proteins and connective tissue (Metscher, 2009); we were able to detect soft tissues by X-Ray microtomography. After fixation, half-head samples were stained in a solution 0.3% PTA in 70% ethanol between 1 week and 8 months according to the size of the sample. Oldest specimens were demineralized to better detect the soft tissue staining. Once the PTA contrast

staining was complete, samples were embedded in paraffin and radiographed using a Phoenix Nanotom S (GE Measurement and Control), which was set up with a tungsten source X-ray tube operating at 100 kV and 70 µA for the mineralized tissues and 60kV and 70µA for the soft tissues. The Phoenix datosx2CT software was applied to gather radiographies in a reconstructed 3D volume, in which the voxel size ranged from 1 to 12 µm depending on the size of the specimen. Post-treatment of 3D volumes, including virtual sectioning as well as sub-volume extractions, was performed using VG-studio max software. Surface smoothing was done with Meshlab software. Incisors hole positions were detected using VG-studio max software and frontal sections were done at each hole using a rotation section plane to respect the natural curvature plane of the incisors. We studied 59 holes from ten incisors (6 individuals); the two remaining incisors were used for histology purposes. The centroid of the incisor section was drawn using ImageJ software and mesio-distal and linguo-vestibular axes were positioned in the incisor section. An angle was calculated to determine the hole position, with the mesial position at 0° (and 360°), the vestibular at 90°, the distal at 180° and the lingual position at 270°. Plots were made using R software and the ggplot2 library.

Results

Chronology of incisor development and replacement

We have observed the establishment of the incisors on the upper and lower jaws. The dI1 and the dI2 incisors in the upper and lower jaws appear synchronous in their development. Rabbits have another incisor in the maxillary, named the dI^3, which is replaced during ontogeny. We will focus here on the description of the upper incisors (Fig.1), since we do not observe dental replacement in the lower ones. All our 3D reconstructions of the epithelial parts of the incisors are available in Morphomuseum (3D Dataset will remain locked until publication of the main article, Bertonnier-Brouty, Viriot, Joly, & Charles, 2019).

At 12 dpf, we observed no morphological structure of incisor development. Then, at 14 dpf, we detect the initiation of the placode formation at the upper and lower jaws (Fig.1). The epithelium is thicker and the mesenchyme begins to condensate around it. At this stage, right and left placodes are not connected to each other. Two days later, it is still impossible to name the incisors, the lower and upper incisors are at the bud stage, the left and right buds seem now connected lingualy. At 18 dpf, all the upper incisors are linked to each other and to the oral epithelium by an epithelium bond, supposed to be a dental lamina rest. As the three incisors follow each other in a mesio-distal plane they are identifiable. From this stage, we can follow the development of each tooth individually.

Vestigial incisor dI^1

At 18 dpf, the first upper incisor is already mineralized with dentin surrounded by dental soft tissues very close to the oral epithelium. This dI^1 is a vestigial tooth; it will not give rise to a functional incisor (Fig.2, A). At 24 dpf, the dI^1 has passed through the oral epithelium and is now trapped in the oral mucosa (blue triangle in Fig.1, Fig.S1). At 28 dpf, the dI^1 is shed.

dI^2

At 18 dpf, the dI^2 is at the cap stage, linked mesialy to the other dI^2 and in rotation. As in mouse (Mucchielli & Mitsiadis, 2000), the dI^2 rotates antero-posteriorly and become parallel to the long axis of the jaws, contrary to molars that stay perpendicular to the jaw axis (Fig.1). At 20 dpf, the dental lamina that links the teeth together is completely disorganized and the two dI^2 are no longer linked. The dI^2 has already secreted a thin layer of dentin and the 3D reconstructions show that the vestibular and lingual cervical loops are well formed. These

structures are supposed to contain the stem cells that will allow the continuous growth of the tooth during the animal's entire life. At 22 dpf, the teeth are completely separated. At 24 dpf, the dI^2 occlusal surface is very close to the oral epithelium and highly mineralized. The dI^2 presents enamel in its entire mineralized surface. We observed a big cervical loop at the vestibular side and a very small one at the lingual part and this incisor has a fold on its vestibular surface on the mesial side. At 26 dpf, the dI^2 is close to erupt. The secretion of enamel at the vestibular and lingual part of the tooth continues. At 28 dpf, the dI^2 is erupting. At birth, the unworn dI^2 presents enamel in the entire erupted occlusal surface. In direction to the growth zone, we detect enamel in the vestibular and the lingual part, and then the enamel production in the lingual part stops and enamel is secreted only in the vestibular part of the tooth. At 4 dpn, the dI^2 are worn in their occlusal surface, showing the dentin. In the juvenile, around 1 month post-natal, we observed that the dI^2 present enamel only in the vestibular surface, so the entire part with enamel in the lingual and vestibular position is already worn (Fig.2, A). In adult, the dI2 is ever-growing. We observed that the dI^2 and the I^3 do not have the same shape at the occlusal surface, the dI^2 has a sharper beveled edge form compared to the I^3, linked to the gnaw ability of the dI^2.

dI^3

At 18 dpf, we observed the dI^3 in a transitional stage between bud and cap at the distal position of the dI^2. At the vestibular part of the dI^3, we observed an epithelium structure distally oriented that is probably a dental lamina rest (Fig.1, pink triangle). At 20 dpf, the dI^3, still connected to the dI^2, is now in a cap stage and rotates antero-posteriorly. At 22 dpf, dI^3 is still linked to the oral epithelium but separated to the dI^2. We observed a lingual budding of the replacement dental lamina in the dI^3 (Fig.1, black triangle). At 24 dpf, the dI^3 is already well mineralized and now perfectly orientated compared to the working position. The dI^3

presents small cervical loops at the vestibular and lingual part of the tooth. We detect the replacement dental lamina of the dI^3 attached to the antero-lingual part of the tooth, already parallel to the oral epithelium. At 26 dpf, the cervical loops of the dI^3 are reduced; we observed the start of the root formation. The replacement dental lamina has grown but is still linked to the dI^3. At 28 dpf, the dI^3 has erupted to the oral epithelium but still do not exit of the oral mucosa. The dI^3 is the only incisor that develops roots; the pulpal cavity seems to close. The I^3 is in a bud stage and no longer linked to the dI^3. At birth, the dI^3 present enamel in the entire occlusal surface. The I^3 continues its development in a cap stage. At 4 dpn, the permanent I^3 begins to mineralize, the roots of the dI^3 are still in mineralization. The dI^3 is worn in the occlusal surface, showing the dentin. In the juvenile, the roots of the dI^3 have finished mineralizing and we observed root resorption. The I^3 is now well mineralized and will soon replace the dI^3 (Fig.2, A). Interestingly, the I^3 is entirely covered by enamel in juveniles on the while the dI^2 have enamel only in the vestibular part of the tooth. In adult, the I^3 is covered by enamel in the vestibular and lingual part of the tooth (Fig.2A, Fig.S2).

Specificity of the rabbit incisors: dentin holes in the newborn incisors

The dI^2 incisor mineralizes at 24 dpf, a thin and continuous layer of dentin is secreted by the odontoblasts. Enamel is first secreted in all the surface of the dI^2, then restricted to the vestibular and lingual sides to finally be secreted only in the vestibular part of the tooth (Fig.S2). In samples from 24 dpf to 28 dpf, the dentin layer of the dI^2 is continuous; no uneven formation of dentin is detected. However, we observed dentin defects in newborn rabbits. In rabbits from 0 to 4 dpn, we detect the presence of holes in the dentin of the dI^2, opening the pulp cavity at specific locations, the lower incisors are not affected (Fig.3, A-D). Older samples, from juveniles to adults, do not have holes in their incisors, indicating a mechanism of tooth repair.

To study the appearance and repair of the holes, we identified 59 holes in 10 dI^2 incisors from six individuals from 0 to 4 dpn. We precisely localized each hole in frontal section of the incisor by calculating an angle in function of the incisor centroid and the mesio-distal and linguo-vestibular planes (Fig.3, A). We observed that the majority of the holes are at the distal side of the tooth (73%), and some in the mesial part (27%). No hole is observed in the vestibular or lingual part of the incisor. By comparing the repartition of the holes in right and left incisors of a same individual, we conclude that holes are independently localized in the two incisors on the proximo-occlusal axis; we did not observe any symmetry (Fig.3, B). Moreover, the number of holes is variable from one tooth to another, even in the same individual. Interestingly, on the 3 and 4 dpn rabbits, we observed a reparation system by the presence of dentin and cells inside the hole (Fig.3, D). This reparation seems very efficient as the holes appear at birth and are repaired two days later. We decided to use *Osteocalcin in situ* staining as a marker for the dentin formation process (Fig.3, E). We observed that the cells in the holes are odontoblasts still expressing *Osteocalcin*, so still in a secretion stage. These secreting odontoblasts, coming from the pre-existing odontoblast layer, could explain the presence of new dentin in the holes. *Keratin 14* staining indicates the epithelial cells; we wanted to check if the holes could deform the layer of epithelial cells. We detect in the incisor a perfect layer of ameloblasts that is not disrupted by the presence of the hole that are formed at the mesial or distal edges of the enamel layer (Fig.3, F).

Therefore, these holes are present only in newborns at the mesial or distal limits of the enamel layer, are repaired by the pre-existing odontoblasts and no longer observed during adulthood. Holes seem thus to occur in a very short period of development around or just after the birth of the animal.

276 *Chronology of cheek teeth development and replacement*

277 We observed the establishment of the cheek teeth on the upper (Fig.4) and lower
278 (Fig.5) jaws. We focus here our description on the mechanisms of tooth replacement. The
279 steps of the tooth replacement are illustrated in the figure indicating all the tooth tissues
280 (Fig.6). All our 3D-reconstructions of the epithelial parts of the cheek teeth are available in
281 Morphomuseum (Bertonnier-Brouty et al., 2019).

282

283 At 12 dpf, the oral epithelium is regular in thickness; we detect no morphological trace
284 of tooth development initiation. Two days later, the oral epithelium has thickened in the upper
285 and lower jaws, forming the dental lamina (Figs.4, 5). The dental lamina thickens
286 continuously over a large part of each mouth quadrant and the mesenchyme seems condensed
287 around it, forming the dental placode. In contrast to humans (Juuri & Balic, 2017), the rabbits
288 do not have a unique continuous dental lamina around the jaw arch but distinct dental laminas
289 for the right cheek teeth, the incisors and the left cheek teeth. At 16 dpf, we observed the
290 budding of the dental lamina with a high condensation of mesenchyme around it. At this
291 stage, it is still impossible to name the teeth, the epithelium being continuous in thickness and
292 shape over the entire length of the bud, we never detect individual tooth buds. In the upper
293 jaw, the budding tooth row is rounded, following the vestibular commissure, and measures
294 1.3mm long from the mesial to distal part of the bud (Fig.4). In the lower jaw, the budding
295 tooth row is straighter and smaller with only 0.8 mm long (Fig.5).

296

297 From 18 dpf on, we can identify the teeth and so we will now described the
298 development of each tooth individually. Rabbit cheek teeth have a thin and long dental stalk
299 compared to the mouse or the fruit bat (Dosedělová et al., 2015; Popa et al., 2016). We
300 observed that the development of the dP_4 is the fastest, followed by the dP_3, dP^3 and dP^4 that

are synchronous and then by the dP^2 so we described tooth development following these categories. For the molars, the M1 is the first to develop and then the development timing follows the mesio-distal organization plan. The lower molar begins their development before the upper molar.

dP^2-P^2

At 18 dpf, the dP^2 is at a transition stage between bud and cap (Fig.4). The tooth is connected to the oral epithelium and to the followed tooth by the interdental lamina. At the mesial part of the dP^2, the interdental lamina is present in almost all the diastema length with an oral epithelium link only in the mesial and distal extremities. At 20 dpf, the dP^2 is in a cap stage. The interdental lamina at the mesial part has disappeared but is still present in the distal part of the dP^2. The dental lamina stalk begin to detach from the oral epithelium. At 22 dpf, the dP^2 is in a bell stage. Initiation of the replacement begins with an epithelial bud from the dental lamina in the lingual part of the dP^2, called the replacement dental lamina (also named successional lamina). At 24 dpf, the dP^2 is completely detached from the oral epithelium. The replacement dental lamina, still connected to the dP^2, plunges into the mesenchyme by keeping a lingual orientation (Fig.4). The interdental lamina is now very thin but still connects the replacement dental lamina of the dP^2 to the replacement dental lamina of the dP^3. At 26 dpf, the dP^2 begins its mineralization. The dental replacement lamina of the dP^2 is still growing in a lingual direction. At 28 dpf, the dP^2 have the crown completely mineralized and just begins to mineralize at the root level. The P^2 is in a cap stage and is localized in a disto-lingual plane compared to the dP^2. At birth, the dP^2 is completely mineralized from the occlusal surface to the roots and the P^2 is still in a cap stage. Four days later, the P^2 is now at the bell stage and begins to move from its lingual position to apical to the dP^2. Then, around 1 month old, the rooted dP^2 is pushed by the P^2 (Fig.2, B). The dP^2 has a completely worn

crown surface and the roots begin to reduce, the P^2 is ready to erupt. P^2 is hypselodont (with an unlimited growth) whereas the dP^2 was hypsodont. In adult, the P^2 display a complex shape with numerous folds filled with cementum at the mesial part of the tooth.

dP^3-P^3 / dP_3-P_3 / dP^4-P^4

At 18 dpf, the dP_3, dP^3 and dP^4 are at the cap stage. The teeth are linked to the oral epithelium and connected to each other with the interdental lamina (Fig.4). At 20 dpf, the dP_3, dP^3 and dP^4 are in a bell stage (Figs.4, 5). Initiation of the replacement begins in the lingual part of the teeth; we detected the replacement dental laminas. The tooth replacement begins in the dP^4 and we observed a shift in the initiation of replacement with a gradient of development from the distal premolar towards the most mesial premolar. The interdental lamina continues to link all the teeth together but the dental lamina begins to be detached from the oral epithelium. At 22 dpf, the dP^3, dP_3 and dP^4 are at the bell stage. All the premolars display a replacement dental lamina in the lingual side, and in the dP^4, we can see that this dental lamina begins to dive in the mesio-lingual direction. Each upper and lower tooth is still connected to the others but dental lamina is completely detached from the oral epithelium. At 24 dpf, the premolars have finished their morphogenesis and now mineralize by secreting dentin and enamel. The replacement dental laminas are highly invaginated into the mesenchyme and are still attached to some elements of the interdental laminas. The replacement dental laminas from the dP^3 and dP^4 grow in a mesial orientation whereas the replacement dental lamina of the dP_3 grows towards the base of the deciduous tooth while maintaining its lingual orientation. The interdental lamina is resorbed around the dP_3, detached of the dP^3, broken between the dP^3 and the dP^4, and very thin between the other upper teeth. At 26 dpf, the deciduous premolars are now highly mineralized in their occlusal surface. The replacement dental laminas are budding to give the replacement teeth. The P_3 is

developing at the lingual basis of the dP_3, the P^3 in a mesio-distal plane of the dP^3 and the P^4 in a mesio-lingual plane of the dP^4. At 28 dpf, the dP_3, dP^3 and dP^4 are mineralized to the roots. The permanent premolars are all in the cap stage, localized at the basis of the deciduous teeth in different positions: The P_3 is localized at the basis of the dP_3 in the lingual side, the P^3 is in a mesio-vestibular plane compared to the dP^3 and the P^4 is in a mesio-lingual plane compared to the dP^4. At birth, the deciduous premolars are well mineralized with dentin, enamel and cementum. The P_3, P^3 and P^4 are in a bell stage. The P_3 begins to migrate from its lingual position to under the dP_3 whereas P^3 and P^4 are still in their developing position. At 2 dpn, the P^4 begins to mineralize followed two days later by the P_3 and P^3. These permanent premolars are now positioned under their deciduous teeth (Fig.2, B, C, Fig.S1). Then, around 1 month old, the rooted deciduous teeth dP_3, dP^3 and dP^4 are pushed by the permanent premolars (Fig.2, B). The deciduous premolars have a completely worn crown surface and the roots begin to reduce, the permanent teeth are ready to erupt. All the permanent cheek teeth are hypselodont. In adult, the P^3 and P^4 have a molarized bilophodont shape with a lingual fold filled with cementum, whereas the P_3 has a complex shape with more folds at the mesial part of the tooth (Fig.2, B, C).

dP_4-P_4

At 18 dpf, the dP^4 is at the cap stage. The dP_4 is connected to the oral epithelium and to the other teeth of the row by the interdental lamina (Fig.5). 2 days later, the dP_4 is at the bell stage and the initiation of the replacement has begun at the lingual part of the tooth, where we detect the replacement dental lamina. At 22 dpf, the dP_4 is the first cheek tooth to mineralize. The tooth is detached from the oral epithelium, but the replacement dental lamina is still connected to the deciduous tooth. At 24 dpf, the replacement dental lamina is detached from the dP_4 that is detached to the other teeth. The replacement dental lamina plunges into

376 the mesenchyme keeping the lingual orientation. At 26 dpf, the P_4 is budding from the
377 replacement dental lamina at the lingual basis of the dP_4 crown. At 28 dpf, the dP_4 is
378 completely mineralized and the P_4 is at the cap stage, localized at the basis of the dP_4 in the
379 lingual side. At birth, the P_4 is the first permanent cheek tooth to mineralize and begins to
380 migrate from its lingual position to below the fully mineralized dP_4. Two days later the
381 permanent tooth is well-localized bellow the dP_4. At 4 dpn, the dP_4 is the first cheek tooth to
382 erupt (Fig.2, C). Then, around 1 month old, the dP_4 has a completely worn crown surface and
383 the roots begin to reduce, the P_4 is ready to erupt. P_4 is hypselodont with a continuous growth.
384 In adult, the P_4 has a molarized bilophodont shape (Fig.2, C).
385
386 M_1-M^1
387 At 18 dpf, the M_1 is in a bud to cap stage transition and the M^1 in a bud stage (Figs.4,
388 5). At 20 dpf, the M_1 and M^1 are in a cap stage. At 22 dpf, the M_1 is at the early bell stage and
389 the M^1 is in the cap stage. We observed an epithelial bud at the lingual part of the M_1 and M^1,
390 indicating a transient rudimentary successional lamina. At 24 dpf, The M^1 and M_1 are in a bell
391 stage and the lingual buds are no more visible. Molars are connected distally to the M^2 and M_2
392 but only the M^1 is still connected to a premolar mesialy. At 26 dpf, the M_1 begins its
393 mineralization and the M^1 finishes its morphogenesis. At 28 dpf, the M_1 and M^1 have the
394 crown completely mineralized, with dentin, enamel and cementum. Then, in juveniles and
395 adults, we observed that the molars are hypselodont with a bilophodont shape (Fig.2, B, C).
396
397 M_2-M^2
398 At 22 dpf, we observed the budding of the continual lamina at the distal part of the
399 tooth row that will give the M^2 and M_2 by serial addition of molars (Figs.4, 5). At 24 dpf, the
400 M_2 and M^2 are in a cap stage, and these teeth are still attached mesialy to the interdental

401 dental lamina. At 26 dpf, the M_2 and M^2 are in the cap stage and are the only teeth connected
402 with the remains of dental and interdental lamina. At 28 dpf, the M_2 is now detached from the
403 dental lamina and is in a bell stage. The M^2, in cap stage, is still linked to the dental lamina.
404 At birth, the M_2 begins to mineralize its occlusal surface whereas the M^2 is in bell stage. At 2
405 dpn, the M^2 and M_2 are mineralizing with dentin, enamel and cementum (Fig.2, B, C). Then,
406 in juveniles and adults, we observed that the molars are hypselodont with a bilophodont
407 shape.
408
409 M_3-M^3
410 At 28 dpf, we observed a completely separated dental lamina that is budding in the
411 distal part of the lower tooth row that will gives the M_3 (Fig.5). In the upper row, we also
412 detect the budding of the dental lamina in the distal part of the M^2 that will give the M^3
413 (Fig.4). At 2 dpn, the M_3 is at the cap stage, followed two days later by the M^3. Then, in a
414 juvenile around 1 month old, we observed that the M^3 is well mineralized but not erupted
415 (Fig.2, B). In adult, the M_3 and M^3 are highly reduced compared to other molars. Among 188
416 observed adult *Oryctolagus cuniculus* specimens, two lacked the upper third molars with no
417 sign of loss, indicating that this tooth can sometimes not be fully developed.
418
419 To summarize, the development of the maxillary teeth is late compared to the
420 mandible counterparts and all the teeth of a same row are not synchronized. In the upper and
421 lower rows, the dP4 is the first forming tooth, that will be followed by the dP3, and then, only
422 in the maxillary, by the dP^2 (Fig.7). The development of replacement teeth follows the same
423 order. Regarding the molars, the M1 is the first to develop, followed by the M2 and finally the
424 M3.
425

Discussion

We show here the morphology (Fig.6) and the chronology of rabbit teeth development(Fig.7). Using 3D reconstructions, we were able to clearly identify the different steps of tooth development and replacement. Our study contains more developmental stages and we separated the results for each tooth, allowing us to obtain a complete and more precise chronology than the ones available in the literature (Hirschfeld et al., 1973; Michaeli et al., 1980; Navarro et al., 1975; Ooë, 1980). 3D-reconstructions from the placode stage lead to the conclusion that the canine and first premolars dP^1 and dP_{1-2} are never detected in rabbit embryos. These results suggest a complete loss of these teeth in rabbits, contrary to vestigial tooth rudiments in mice (homologous to the dP_3 and dP_4), detected in early tooth development (Sadier et al., 2019; Viriot, Peterková, Peterka, & Lesot, 2002). However, some observations still need to be clarified, as the epithelium link between the vestigial incisor dI^1 and the dI^2. This relationship was already problematic for Ooë (1980), who discussed the localization of the dI^2 compared to the dI^1. In mammals, replacement teeth begin their development at the lingual part the deciduous predecessor (Järvinen et al., 2009). Here the dI^1 and dI^2 incisors are in a mesio-distal plan and not in a vestibulo-lingual plan, so it seems more similar to serial development with an interdental lamina between deciduous incisors. However, the rabbit dI^2 has the specificity ofbeing the only tooth to be deciduous and ever growing. For other teeth, the ever-growing ability appears only in permanent teeth. Hirschfeld et al. (1973) concluded that due to its ever-growing capacity the so-called dI^2 is the permanent tooth coming from the dI^1. More data on the precise dynamics of the tooth development between 16 and 18 dpf are needed to fully understand the nature of the so-called dI^2. Reconstructions of the concerned structures during the transition from bud to separated teeth in 3D should help to conclude on the links between these incisors.

One particularity of incisor development in rabbits is the appearance of holes that occur only in newborns. The cause of these holes is unknown. These tooth defects have been observed in all our samples, indicating a common phenomenon. We did not find in literature any description of systematic presence of tooth defects at one specific age in rabbits or other species. In this study, we worked on common rabbit breeding regularly crossed with wild rabbits to ensure genetic mixing, limiting the possibility that these dental abnormalities are specific to one genetic selection in a closed breeding. Some hypotheses can be considered to explain the hole formation, as a possible mechanical compression due to the development of adjacent teeth or bone mineralization or the result of molecular signals. It could be interesting to identify which mechanical forces at birth could be responsible for tooth fractures without morphologically affecting the other tissues. These causes could be purely mechanical (development of the bones or the other upper incisors) or molecularly facilitated (involvement of signaling pathways involved in root resorption for example). The observed reparation system, which systematically takes place in the first days of an animal's life, indicates an ability of quick tooth repair, probably due to the high secretion capacity of the odontoblasts in the ever-growing incisor. This speed in tooth repair is incompatible with the classical reparative dentin that require differentiation and migration of new odontoblasts from a pulpal progenitor cell (Moses, Butler, & Qin, 2006). So here, another type of tertiary dentin could close the hole, the reactionary dentin that is formed from a pre-existing odontoblast.

The 3D-reconstructions of the rabbit premolars indicate that the permanent upper premolar begins its morphogenesis in a mesio-vestibular plane compared to its deciduous tooth, so in the opposite side in contrast to the other teeth. Popa et al. (2016) presented in the fruit bat different modalities of tooth replacement throughout the jaw. They showed, using 3D-reconstructions, that the canine, the second and the third premolar do not have the same

476 modalities of replacement due to the relative position of the deciduous and permanent teeth.
477 In rabbit, the initiation of the tooth replacement always begins by a growth of the dental
478 lamina at the lingual position of the tooth that will give the replacement dental lamina as in
479 the ferret or fruit bat (Jussila et al., 2014, Popa et al., 2016). Then, the separation between the
480 first generation of teeth, the oral epithelium and the replacement dental lamina can vary. First,
481 the teeth lose their connection with the oral epithelium. Variations are then observed; the
482 replacement dental lamina can first be separated from the tooth or from the interdental lamina.
483 Concerning the tooth position of the second generation compared to the first it also depends
484 on the teeth as in the fruit bat. For the lower premolars and the upper incisor, the permanent
485 teeth develop directly on the lingual side of the deciduous teeth. For the upper cheek teeth, the
486 position of the second-generation development varies for each premolar. The tooth
487 replacement begins at the lingual part of the deciduous tooth, but then the replacement dental
488 lamina plunges and develops in different orientation depending on the tooth. The P^2 is
489 localized in a disto-lingual plane compared to the dP^2, the P^3 is in a mesio-vestibular plane
490 compared to the dP^3 and the P^4 is in a mesio-lingual plane compared to the dP^4. Indeed, the P^2
491 and P^3 develop on the same frontal plan, with one lingualy and the other vestibulary. Then, all
492 the permanent teeth migrate under the associated deciduous teeth. Compared to the fruit bat or
493 the ferret (Järvinen et al., 2009; Popa et al., 2016), the rabbit dental replacement lamina seems
494 to plunge much deeper into the mesenchyme before it begins to initiate its permanent tooth
495 morphogenesis, as observed in the minipig (Wang et al. 2014).
496
497 During rabbit molar development, we observed buddings at the lingual part of the first
498 molars that quickly disappear. Same tooth shape has been observed in the mouse; a
499 rudimentary successional dental lamina (RSDL) is visible in the lingual part of the M_1 tooth
500 germ (Dosedělová et al., 2015). The RSDL in mouse as in the rabbit does not have

odontogenic potential (Popa, Buchtova, & Tucker, 2019). However, by stabilizing the Wnt/ β-catenin signaling in the RSDL, it is possible to obtain a new tooth (Popa et al., 2019). The molars can be considered as deciduous teeth that are never replaced (Luckett, 1993); the study of this budding observed in the rabbit with the molecular information obtained in mutant mice could identify why these teeth are not replaced.

In this study, we have shown that the rabbit seem to be a good model in dental research. Rabbits are already used in odontological studies but we showed here that they are also useful for studying tooth morphogenesis. Studies of tooth development and replacement in rabbits could allow understanding the mechanisms of mammalian tooth replacement. We showed that common molecular experiments can be done in rabbit tissues as *in situ* hybridization and rabbits can also be a relevant model to use more advanced techniques as CRISPR/Cas9 technology (Yan et al., 2014). Compared to other species used to study tooth replacement (the ferret, the fruit bat, the shrew and the minipig), rabbit embryos are easy to get, with large litters of embryos and a pretty short gestation time (31 days). The main disadvantage to the study of the rabbit compared to the other models already proposed is an incomplete dentition with the lack of the canines. However, rabbits have also ever-growing permanent teeth, allowing study of other issues such as dental stem cells maintaining in addition to mammalian dental replacement.

Conclusion

We provided here the description of the complete histo-morphological chronology of the tooth development and replacement in rabbit. It can be considered as a new dataset that allows a better understanding of the dental replacement among mammals. The 3D-reconstructions gave useful information about the geometric organization of the dental

526 replacement and the precise knowledge of the dental development and replacement
527 chronology in this species is a starting point that will allow further studies of gene functions at
528 specific time windows.
529
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539
540 **Author contributions**
541 L.B.B: contributed to design, to acquisition, analysis, and interpretation, drafted manuscript.
542 L.V: contributed to conception, to analysis and interpretation, critically revised manuscript.
543 T.J: contributed to interpretation, critically revised manuscript. C.C: contributed to conception
544 and design, to analysis and interpretation, critically revised manuscript.
545
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Figure legends

Fig.1. 3D-reconstructions of the epithelial part of the upper incisors during development. Development of the dI^1, dI^2, dI^3 and I^3 from 14 to 28 days post-fertilization (dpf). Indicated in blue, the vestigial incisor; in pink, the interdental lamina; in black (dotted line), the dental tissues; in black (arrow), the replacement tissues. M, mesial; L, lingual; O, occlusal.

Fig.2. 3D-reconstructions of the mineralized part of the rabbit teeth. (A) Mineralized tissues of the upper incisors from embryo to adult. (B) Mineralized tissues of the upper cheek teeth from newborn to adult. (C) Mineralized tissues of the lower cheek teeth in newborn and adult. (D) Tooth nomenclature: "I", "P" and "M" for incisor, premolar and molar; the deciduous teeth are indicated with a "d"; the tooth number is in exponent for the upper teeth and in an index for the lower teeth. In blue the vestigial incisor, in red all the deciduous teeth that will be replaced.

Fig.3. Hole repartition in the upper incisor. (A) Distribution of the holes in 10 upper incisors from 0 to 4 days post-natal (dpn). (B) Distribution of the holes in the two dI^2 from a same individual. Each arrow indicates a fold. (C) Histological sections of a hole in a 0 and (D) 4 dpn individual. (E) *Osteocalcin* staining and (F) *Keratin 14* staining in a 0 dpn incisor.

Fig.4. 3D-reconstructions of the epithelial tissues and histology in rabbit upper cheek teeth from 14 dpf to 28 dpf. 3D-reconstruction of the epithelial tissues of the upper cheek teeth and frontal sections associated for each tooth. Indicated with the black arrow, the permanent premolars. M, mesial; L, lingual; O, occlusal; D, distal; V, vestibular.

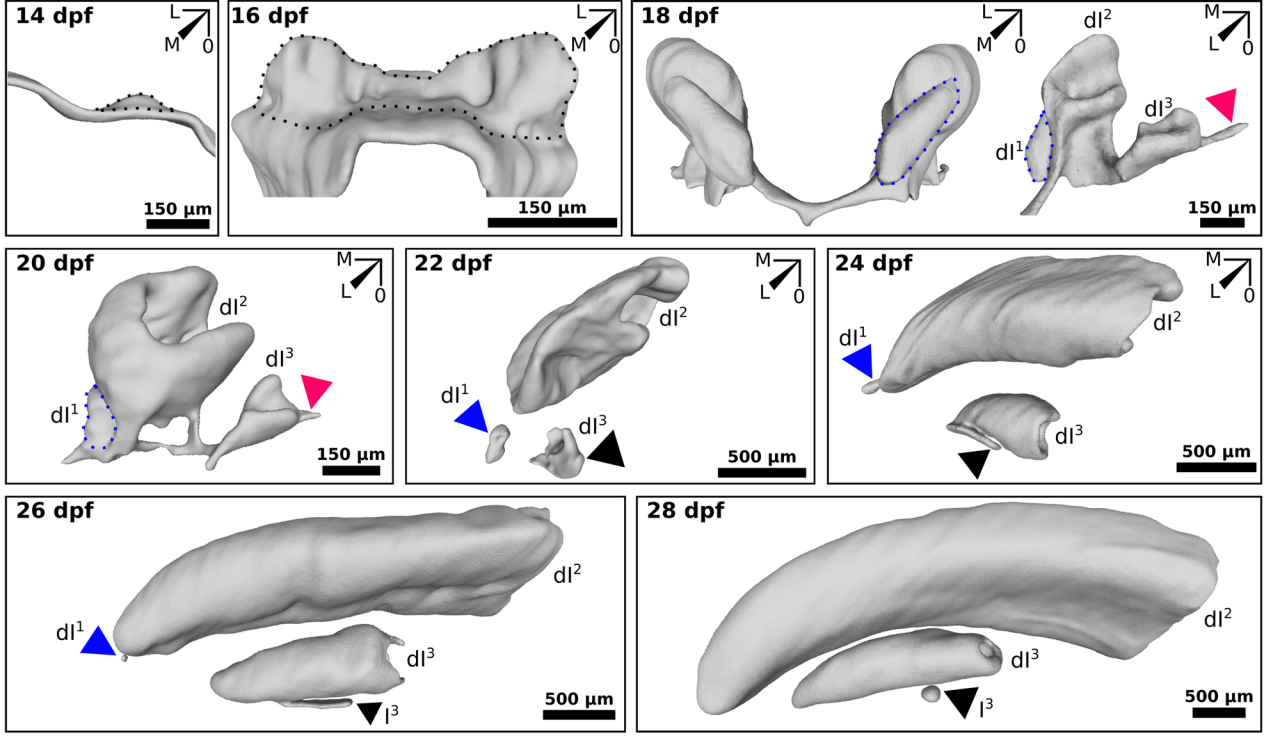
Fig.5. 3D-reconstructions of the epithelial tissues and histology in rabbit lower cheek teeth from 14 dpf to 28 dpf. 3D-reconstruction of the epithelial tissues of the lower cheek teeth and frontal sections associated for each tooth. M, mesial; L, lingual; O, occlusal; D, distal; V, vestibular.

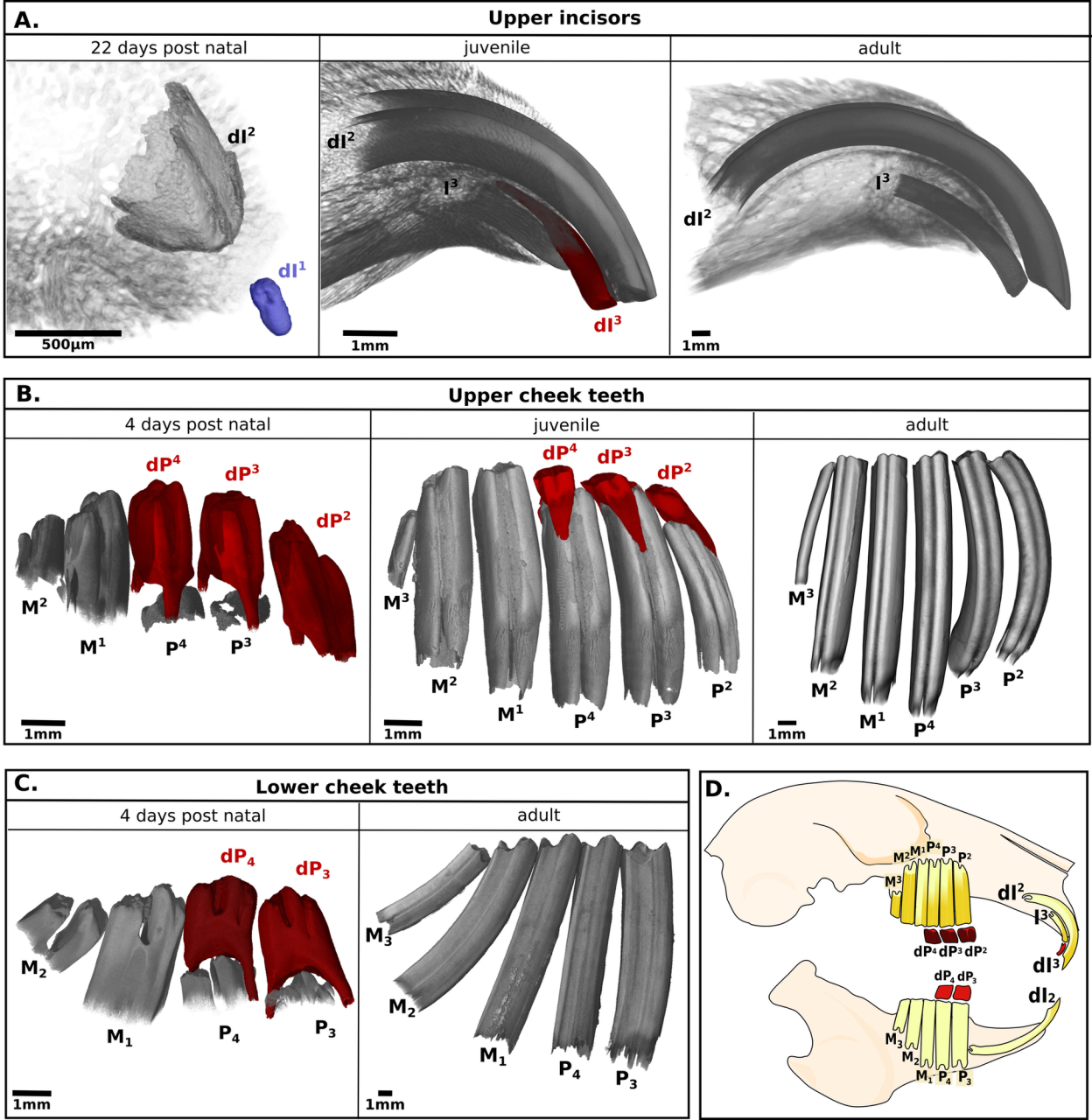
Fig.6. Summary of the dental replacement progression in rabbit. The black triangle shows the initiation of the replacement dental lamina, the black star the separation of the replacement dental lamina and the deciduous tooth and the white triangle the morphogenesis initiation of the permanent tooth. Scale bar: 200µm

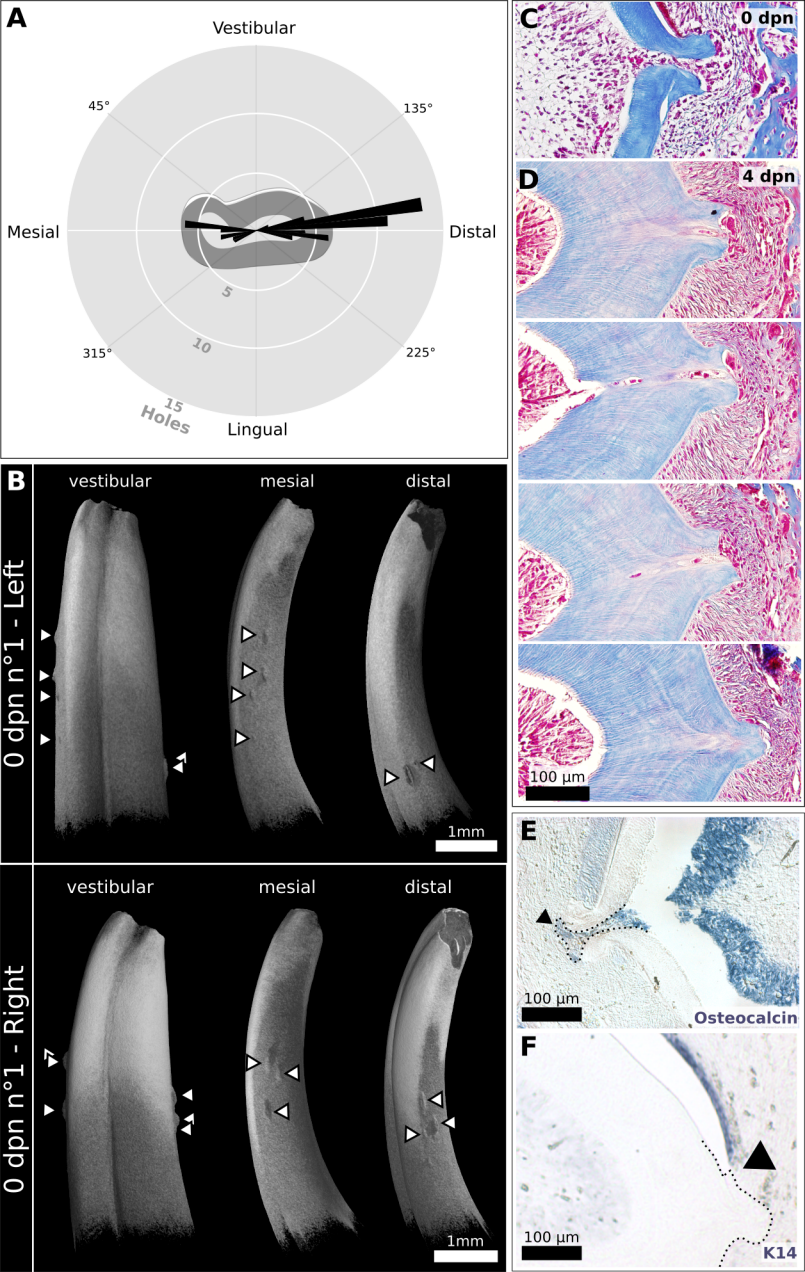
Fig.7. Chronology of dental development and replacement for each rabbit tooth from 12 dpf to 4 dpn.

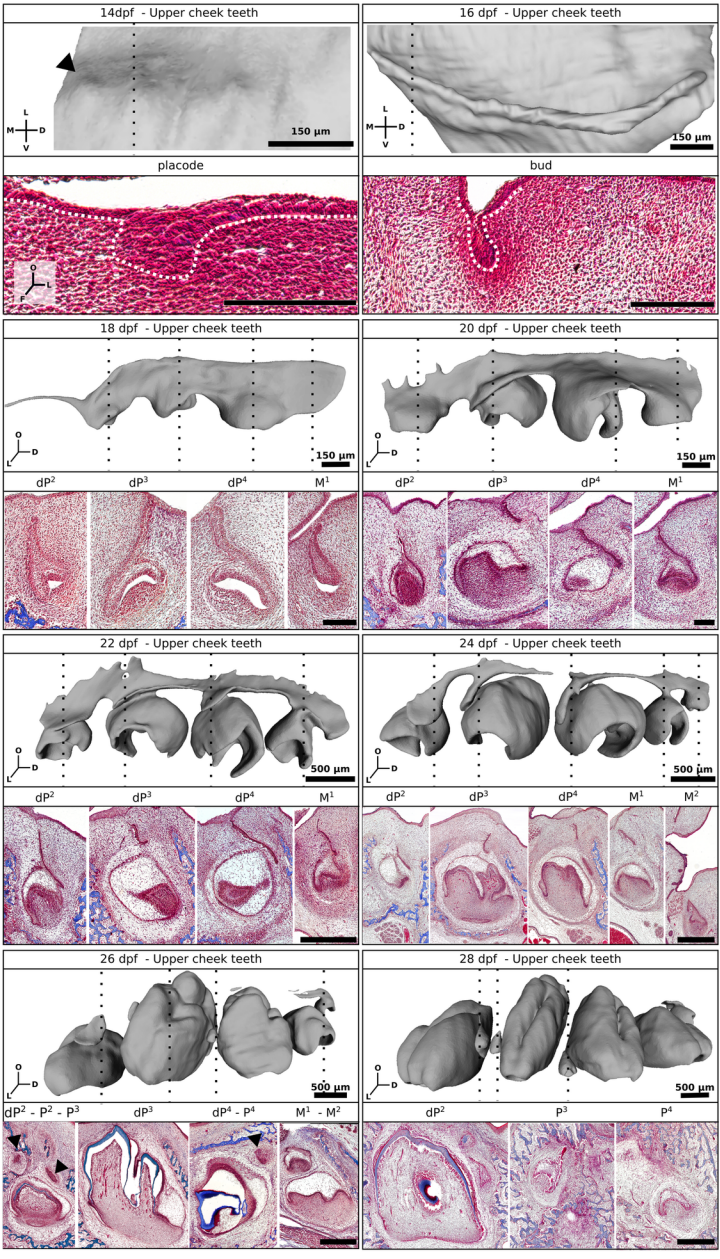
Fig.S1. Histological sections of the rabbit upper incisors and lower cheek teeth. (A) Frontal and sagittal sections of the upper incisors from 18 dpf to 4 dpn. Indicated with black arrows, the replacement dental lamina. (B) Frontal sections of the lower third premolar from 0 dpn to 4 dpn. L, lingual; O, occlusal. Scale bar: 200µm.

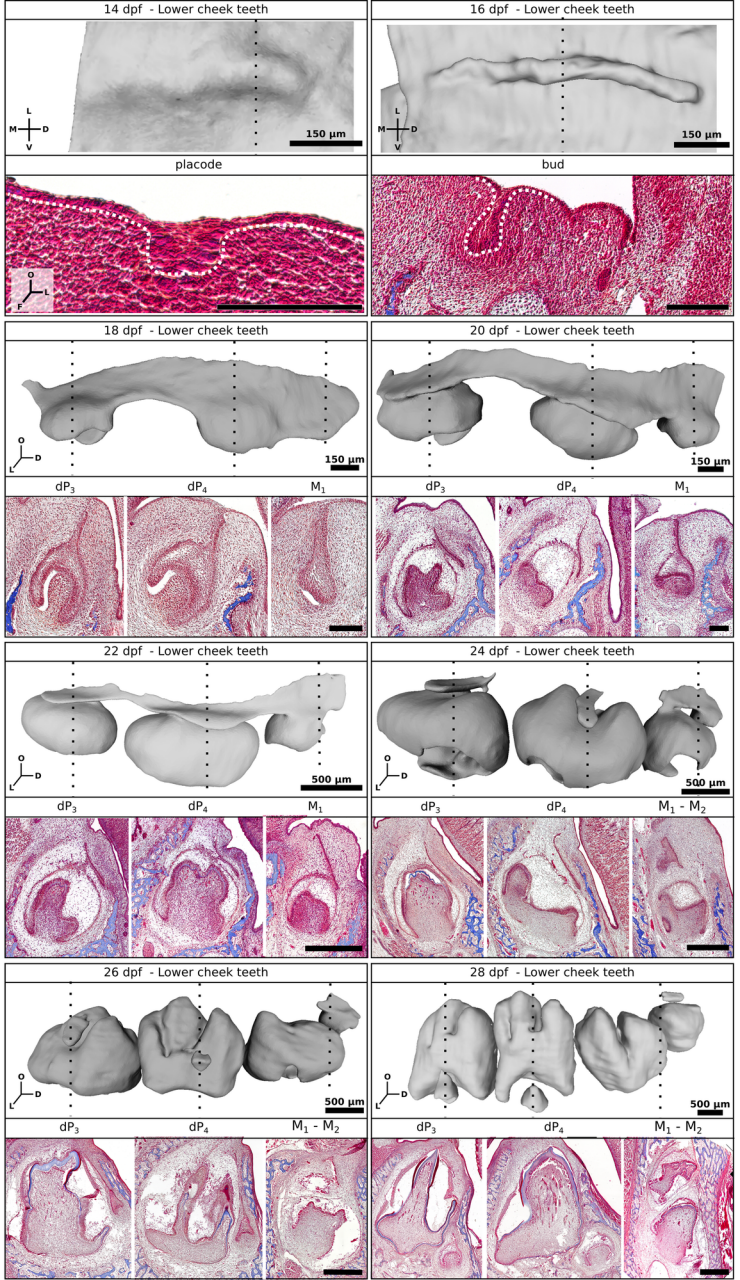
Fig.S2. Views of the 3D reconstructions of the upper incisors mineralized tissues and virtual sections of the incisors. In sections, the dentin appears in grey and the enamel in white. Indicated in blue, enamel layer in the dI^2; in yellow, enamel layer in the dI^3; in red, enamel layer in the I^3. The white dotted line indicates the frontal section plane illustrated in the white rectangle. V, vestibular; M, mesial. Scale bar: 1mm.

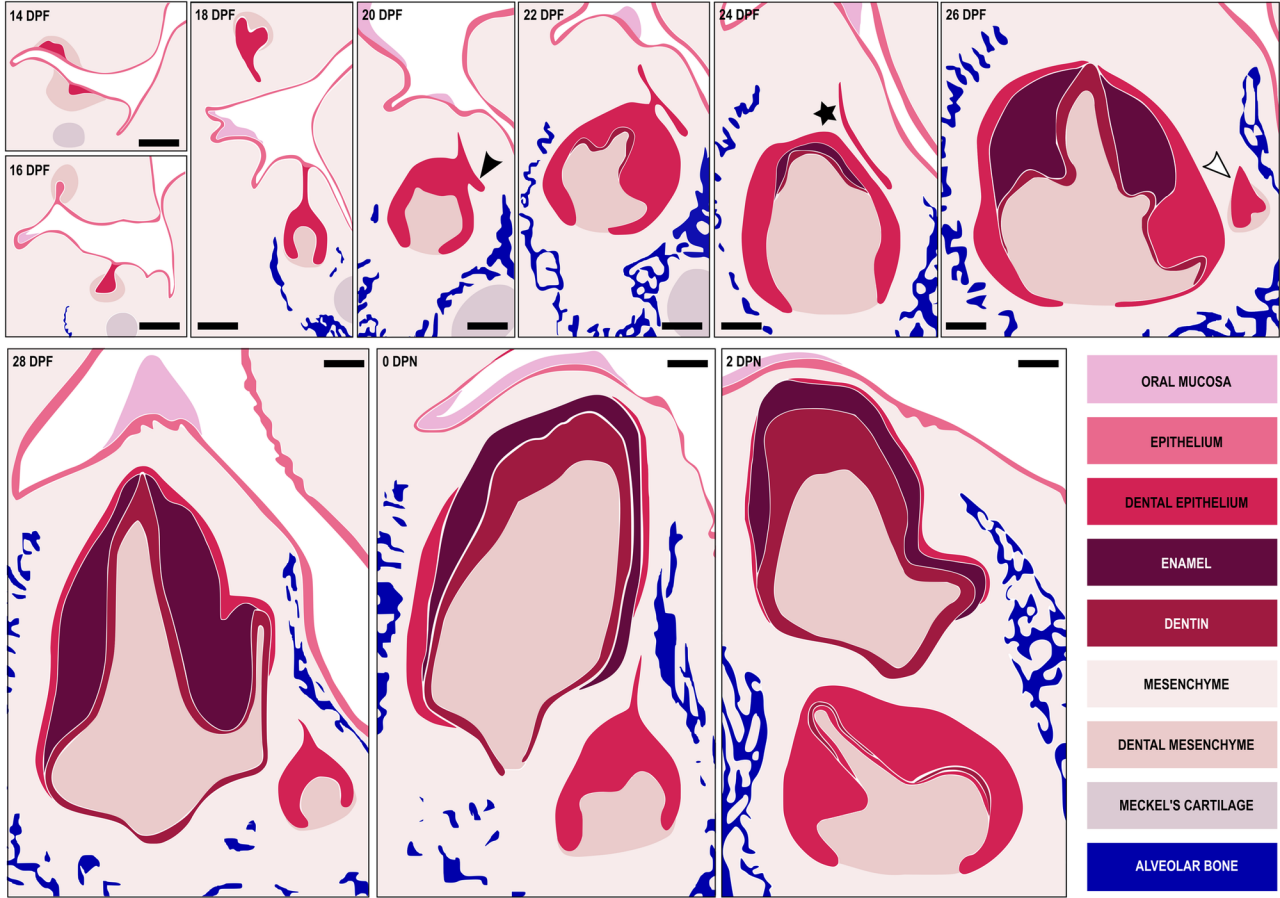

14 dpf
L
M
0
150 µm
16 dpf
L
M
0
150 µm
18 dpf
L
M
0
dl2
dl3
dl1
M
L
0
150 µm
20 dpf
M
L
0
dl2
dl3
dl1
150 µm
22 dpf
M
L
0
dl2
dl1
dl3
500 µm
24 dpf
M
L
0
dl1
dl2
dl3
500 µm
26 dpf
dl2
dl1
dl3
l3
500 µm
28 dpf
dl2
dl3
l3
500 µm

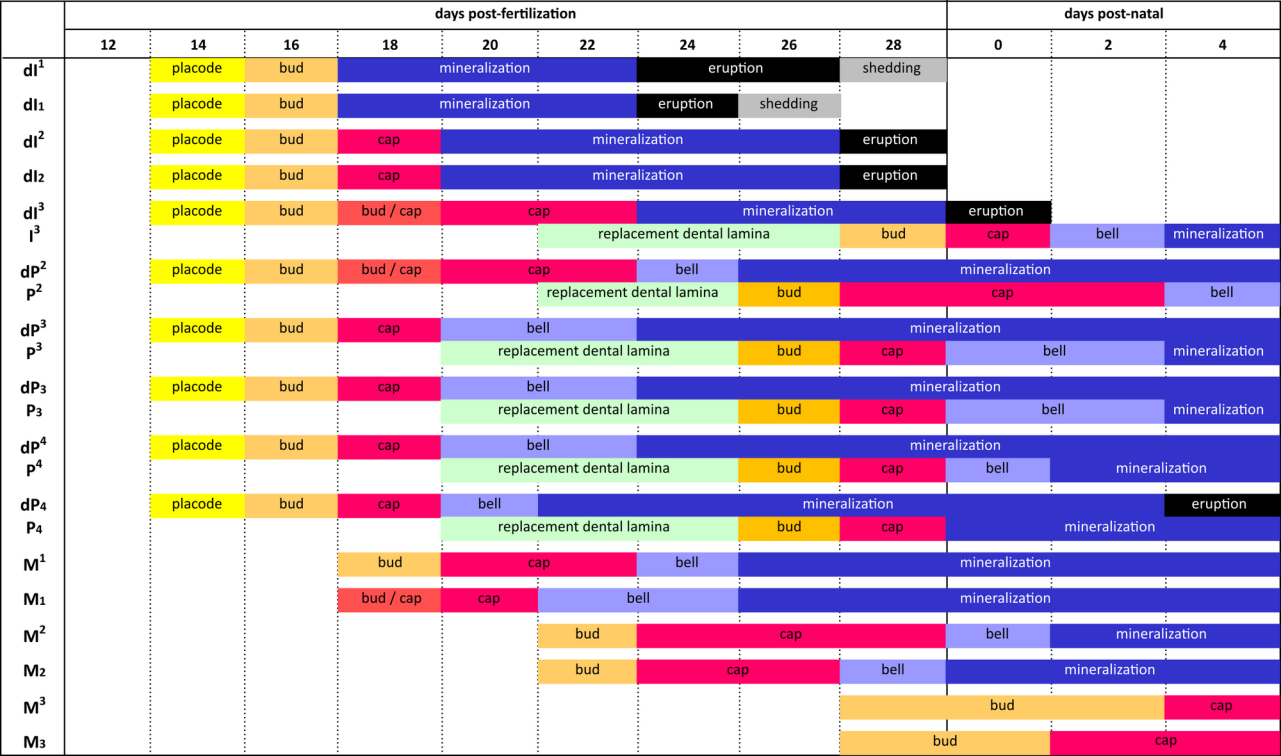

A.
Upper incisors
22 days post natal
juvenile
adult
dI2
dI1
500µm
dI2
I3
dI3
1mm
dI2
I3
1mm
B.
Upper cheek teeth
4 days post natal
juvenile
adult
dP4
dP3
dP2
M2
M1
P4
P3
1mm
dP4
dP3
dP2
M3
M2
M1
P4
P3
P2
1mm
M3
M2
M1
P4
P3
P2
1mm
C.
Lower cheek teeth
4 days post natal
adult
dP4
dP3
M2
M1
P4
P3
1mm
M3
M2
M1
P4
P3
1mm
D.
M2 M1 P4 P3 P2
M3
dI2
I3
dP4 dP3 dP2
dI3
dP4 dP3
dI2
M3
M2
M1 P4 P3


A
Vestibular
45°
135°
Mesial
Distal
315°
225°
5
10
15
Holes
Lingual
B
vestibular
mesial
distal
0 dpn n°1 - Left
1mm
vestibular
mesial
distal
0 dpn n°1 - Right
1mm
C
0 dpn
D
4 dpn
100 µm
E
100 µm
Osteocalcin
F
100 µm
K14


14dpf - Upper cheek teeth
16 dpf - Upper cheek teeth
150 µm
placode
bud
18 dpf - Upper cheek teeth
20 dpf - Upper cheek teeth
dP2
dP3
dP4
M1
22 dpf - Upper cheek teeth
24 dpf - Upper cheek teeth
M2
500 µm
26 dpf - Upper cheek teeth
28 dpf - Upper cheek teeth
dP2 - P2 - P3
dP4 - P4
M1 - M2
P3
P4


14 dpf - Lower cheek teeth
16 dpf - Lower cheek teeth
150 µm
placode
bud
18 dpf - Lower cheek teeth
20 dpf - Lower cheek teeth
dP3
dP4
M1
22 dpf - Lower cheek teeth
24 dpf - Lower cheek teeth
500 µm
M1 - M2
26 dpf - Lower cheek teeth
28 dpf - Lower cheek teeth


14 DPF
16 DPF
18 DPF
20 DPF
22 DPF
24 DPF
26 DPF
28 DPF
0 DPN
2 DPN
ORAL MUCOSA
EPITHELIUM
DENTAL EPITHELIUM
ENAMEL
DENTIN
MESENCHYME
DENTAL MESENCHYME
MECKEL'S CARTILAGE
ALVEOLAR BONE


days post-fertilization
days post-natal
12
14
16
18
20
22
24
26
28
0
2
4
dI1
placode
bud
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replacement dental lamina
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