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Unraveling the Molecular Mechanisms That Lead to Supernumerary Teeth in Mice and Men: Current Concepts and Novel Approaches

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Abstract
Supernumerary teeth are defined as those that are present in excess of the normal complement of human dentition and represent a unique developmental anomaly of patterning and morphogenesis. Despite the wealth of information generated from studies on normal tooth development, the genetic etiology and molecular mechanisms that lead to congenital deviations in tooth number are poorly understood. For developmental biologists, the phenomenon of supernumerary teeth raises interesting questions about the development and fate of the dental lamina. For cell and molecular biologists, the anomaly of supernumerary teeth inspires several questions about the actions and interactions of transcription factors and growth factors that coordinate morphogenesis, cell survival and programmed cell death. For human geneticists, the condition as it presents itself in either syndromic or non-syndromic forms offers an opportunity to discover mutations in known or novel genes. For clinicians faced with treating the dental complications that arise from the presence of supernumerary teeth, knowledge about the basic mechanisms involved is essential. The purpose of this manuscript is to review current knowledge about how supernumerary teeth form, the molecular insights gained through studies on mice that are deficient in certain tooth signaling molecules and the questions that require further research in the field.

Abbreviations used in this paper

BMP	bone morphogenic protein
CCD	cleidocranial dysplasia
Eda	ectodysplasin gene
FGF3–10	fibroblast growth factors 3–10
FGFR1, 2	fibroblast growth factor receptors 1, 2
M1	first molar
MSX1	homeo box, msh-like 1
PAX6, PAX9	paired box genes 6, 9
RUNX2	runt-related gene 2
SHH	sonic hedgehog
Spry2, 4	sprouty genes 2, 4
Wnt	an oncogene

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Introduction

In contrast to the reduced monophyodont rodent dentition in which only incisors and molars are present, the diphyodont human dentition exhibits all four tooth classes at maturity. The deciduous complement of 20 teeth consists in each quadrant two incisors, one canine and two molars that are shed during childhood while the permanent or secondary dentition consists of 32 teeth, namely, two incisors, one canine, two premolars and three molars in each quadrant. Molecular and genetic studies performed in the past two decades have shown that the patterning of dentition is a highly complex process that provides a valuable developmental model for studying genes that control three-dimensional patterning and morphogenesis. Taken together, these advances have improved our understanding that the precise control of the number, position, size and shape of teeth within the maxilla and mandible requires signaling between odontogenic (tooth-specific) epithelium and mesenchyme. Such interactions involve diffusible morphogens and nuclear transcription factors that operate within parallel signaling pathways to give rise to an exquisitely patterned dentition. That such a complex process frequently goes awry is not surprising, and patterning defects in human dentition occur often and are characterized by alterations in the number, size and shape of teeth. The importance of the genetic control of the patterning of human dentition is underscored by the findings that mutations in genes that encode the transcription factors *PAX9* and *MSX1* lead to non-syndromic tooth agenesis or the congenital absence of teeth, among the most commonly inherited disorders in humans [hypodontia (OMIM 106600)]. Another condition affecting the patterning of dentition is the presence of supernumerary teeth, a relatively rare condition that is characterized by the presence of more than the normal complement of 20 deciduous and 32 permanent teeth. In contrast to advances made in our understanding of the genetic etiology of tooth agenesis and its underlying molecular mechanisms, not as much has been reported about the genetic and molecular defects that lead to supernumerary tooth development. The objectives of this review are to survey classical literature concerning the etiology, frequency and classification of supernumerary teeth, to highlight molecular insights gained from the use of genetically engineered mouse models, and to propose new research opportunities in human molecular genetics.

The Dental Lamina

Knowledge about the origin, development and fate of the dental lamina is critical to our understanding of how supernumerary teeth arise. In humans, the primitive oral cavity or stomatodeum becomes apparent around day 25 of development and is demarcated laterally by the first pair of branchial (pharyngeal) arches [Krause and Jordan, 1965; Nanci, 2003]. The latter consist of an external layer of ectoderm, an intermediate layer of lateral plate mesoderm and an internal layer of endoderm. Microscopically, the stomatodeum is lined by a two- to three-cell layer of epithelial cells that overlies a layer of embryonic connective tissue derived from cranial neural crest ectomesenchyme. Around day 37 of embryonic development, the presumptive maxilla and mandible become visible as each is covered by a continuous layer of thickened epithelium, called the primary epithelial band. Shortly after, this band of tissue divides into two distinct structures, the vestibular lamina and the dental lamina. While both structures are composed of highly proliferative cells that rapidly grow into surrounding ectomesenchyme, cells of the vestibular lamina enlarge and undergo programmed cell death to form a cleft (the space between the cheek and the arch). In contrast, the dental lamina remains proliferative, extending outgrowths into the ectomesenchyme but only at future sites of odontogenesis. The epithelial outgrowth or tooth bud then progresses from the cap to the bell stages of morphogenesis but remains attached to the dental lamina through a stalk-like extension called the lateral lamina. During the bell stage and prior to the differentiation of odontoblasts and ameloblasts, the dental lamina and the lateral lamina that connects the tooth organ to the overlying oral epithelium fragment into small clusters of cells. These cells normally undergo programmed cell death but sometimes persist to form structures called epithelial pearls. The formation of eruption cysts from these epithelial remnants is known to interfere with the normal eruption pathway of the underlying tooth organ [Moskow and Bloom, 1983; Pindborg, 1970]. Thus far, studies on the fate of the dental lamina have been limited to histologic and ultrastructural analyses that suggest that the sequence of events leading to its degeneration is initiated by underlying mesenchyme [Khaejornbut et al., 1991].

The primary dental lamina that gives rise to the deciduous dentition is also responsible for the formation of the succedaneous (permanent) incisors, canines and premolars. These develop as lingual extensions of the dental lamina and are clearly visible in a coronal section through a developing human jaw. The three permanent molars that

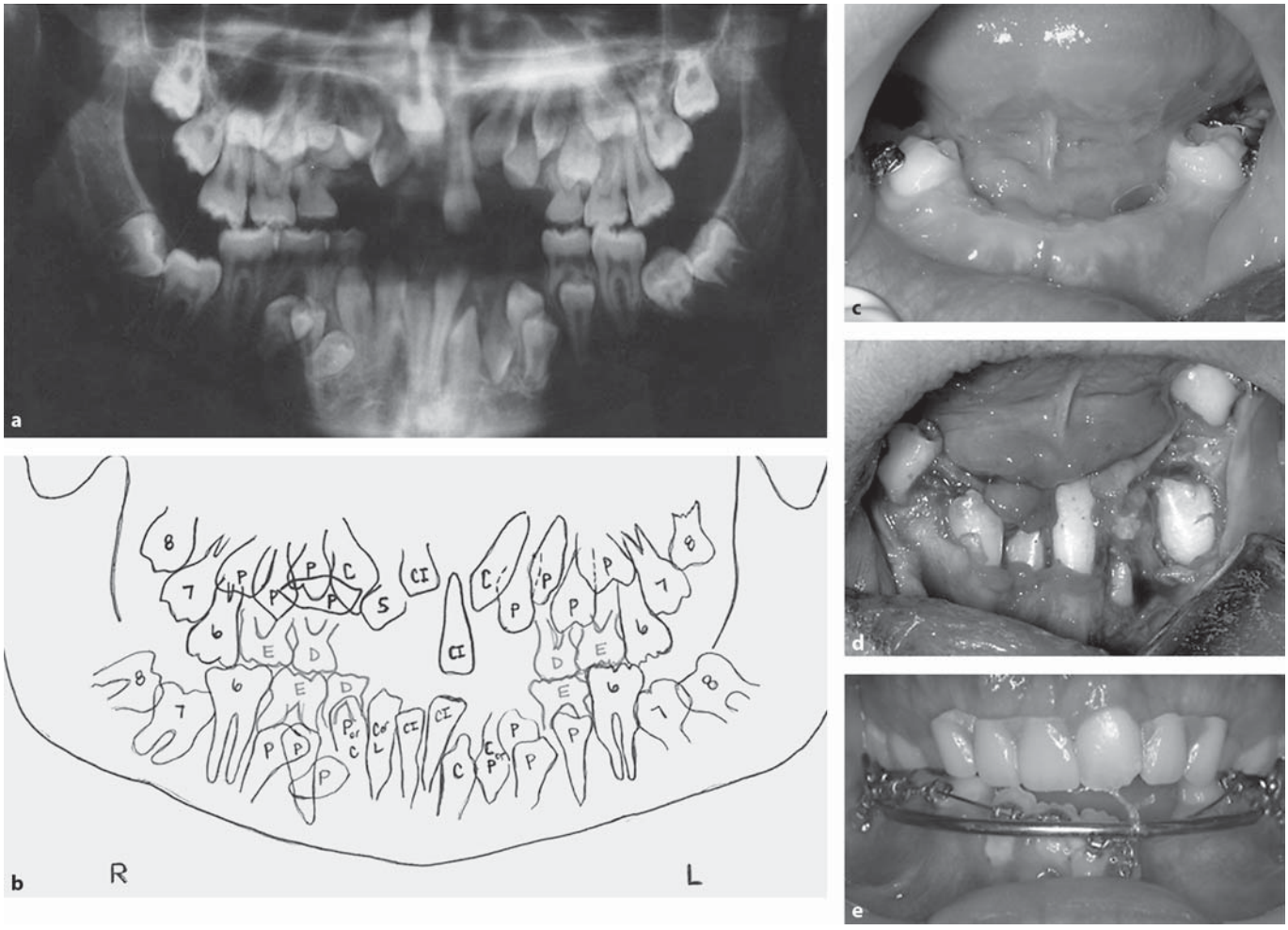

Fig. 1. Panel describing the dental complications and the sequence of treatment for a 17-year-old male affected by CCD. **a**, **b** Panoramic radiograph and tracing. CI = Central incisor; L = lateral incisor; C = canine; P = premolar; 6 = permanent first molar; 7 = permanent second molar; 8 = permanent third molar; D = deciduous first molar; E = deciduous second molar. The presence of multiple supernumerary teeth creates several problems. Note that the only permanent teeth which have erupted are the maxillary left central incisor and all first molars. Multiple retained deciduous teeth are also present. **c** Preoperative intraoral frontal photograph showing that the majority of permanent teeth are unerupted. **d** Surgical exposure and extraction of all supernumerary teeth and deciduous teeth with the exception of the right deciduous second molar, which was not extracted for orthodontic anchorage. **e** Intraoral view of mandibular arch, showing lip bumper appliance. Orthodontic traction, through bonded attachments with chains, will be applied to promote eruption of the remaining teeth. A series of orthodontic interventions are planned to restore normal occlusion.

do not have deciduous predecessors develop from an outgrowth of the primary dental lamina backward into the jaw ectomesenchyme. There is relatively little known about the genetic factors that control the fate of the dental lamina. Future studies on the patterns of differential gene expression within this specialized epithelium and its surrounding mesenchyme are needed to increase our understanding of the molecular mechanisms that lead to aberrations like missing teeth or the formation of supernumerary teeth.

Supernumerary Teeth in Humans

Supernumerary teeth exist in excess of the normal complement of deciduous and permanent teeth and are easily diagnosed by clinical examination and/or radiographs. Most common among the supernumeraries are mesiodens, or teeth that appear on the palatal side between the maxillary incisors. Accessory canines and premolars are typically located within the arch form while

supernumerary molars develop buccal to the permanent molars or distal to the third molars. Supernumerary teeth can present unilaterally, bilaterally, singly or multiply, and in the maxilla and/or mandible. Non-syndromic cases of supernumerary teeth are reported to occur with varying frequencies in different ethnic populations while a few cases of inherited or familial forms have been reported [Burzynski and Escobar, 1983; Yusof, 1990]. Supernumerary teeth can also be associated with other organ anomalies [D'Souza et al., 2006]. Syndromes that involve supernumeraries include X-linked conditions like focal dermal hypoplasia syndrome and orofaciodigital syndromes types I and III, autosomal recessive disorders like steroid dehydrogenase deficiency and Rothmund-Thomson syndrome, and autosomal dominant conditions that include syndromic cleft lip and palate, cleidocranial dysplasia (CCD), Gardner syndrome and Nance-Horan syndrome [for review, see Zhu et al., 1996].

Although the presence of supernumerary teeth can greatly compromise esthetics, the condition is not life-threatening. Several predictable dental complications can arise that range from mild to severe, depending on the number and location of supernumeraries. These include but are not limited to: dentigerous cysts; malocclusion due to impaction and pressure on adjacent teeth; resorption of bone; pericoronitis and impingement on nerves leading to paresthesia and/or pain [Bodin et al., 1978; Primosch, 1981; Burzynski and Escobar, 1983]. Figure 1 describes the sequence of treatment involved in correcting malocclusion associated with CCD.

The overall patterns of presentation and incidence of supernumerary teeth support the various theories that are proposed to explain how the condition arises. One theory hypothesizes that these teeth are derived from remnants of the dental lamina that fail to degenerate and become reactivated to form accessory tooth organs. Another theory proposes that the dental lamina, while intact, continues to proliferate due to a failure of programmed cell death, which may be brought on by defects in signaling between epithelium and mesenchyme. Finally, the possibility that supernumerary teeth arise from the division of a single tooth bud is supported by a few case reports. One describes differences in the mesiodistal width of central incisors depending on unilateral or bilateral occurrence of mesiodens. Another report describes gemination of a deciduous incisor on the same side of a mesiodens [Stellzig et al., 1997]. From a developmental and molecular viewpoint, each theory is plausible and can explain the origin of different types of supernumerary teeth.

Mouse Models of Supernumerary Teeth

When using animal models to study changes in tooth number, it is important to keep in mind that tooth number varies dramatically among species, presumably as a functional adaptation in response to environmental pressures [Line, 2003]. Both humans and rodents have reduced mammalian dentition in comparison to the ancestral eutherian formula, in which up to three incisors, one canine, four premolars, and three molars can occur in each dental quadrant. This primitive formula can be seen in some extant mammals, such as certain species of mole. Compared with humans, the adult mouse dentition is severely reduced. Each dental quadrant contains three molars and one incisor, separated by an edentulous region called a diastema (fig. 2), and no premolars or canines.

The absence of teeth in the adult mouse diastema does not reflect a lack of tooth development during embryogenesis. Transient epithelial primordia originate in the mouse diastema and reach the bud stage before regressing [Peterkova et al., 2002]. These primordia are presumably evolutionary remnants of the developmental program for tooth formation in species that have teeth between the incisors and molars. Although the diastema buds initially appear quite robust, they do not progress to the cap stage. In the mandibular antemolar region, two primordia are detected anterior to the presumptive first molar (M1). The more anterior of these structures is thought to regress completely, whilst the more posterior of these, adjacent to M1, may be partially absorbed into the developing M1. The mouse maxilla has not been examined in as much detail as the mandible, but the maxillary diastema is believed to contain as many as seven buds [Peterkova et al., 2000]. The reason for the different number of buds in the maxilla and mandible is not well understood. The elimination of the diastema tooth buds involves apoptosis [Tureckova et al., 1996; Peterkova et al., 2000; Peterkova et al., 2003], but it is not known if cell death is a primary event in failure of the diastema tooth bud to develop, or if it is secondary to failure of the bud to progress to the cap stage.

Supernumerary diastema teeth have been found in a few mutant mouse strains, including mice null for the fibroblast growth factor (FGF) antagonists *Spry2* or *Spry4* [Klein et al., 2006] (fig. 2), Polaris hypomorphs [Zhang et al., 2003], mice that overexpress ectodysplasin (*Eda*) or its receptor [Mustonen et al., 2003; Pispa et al., 2004], *Pax6* mutants [Kaufman et al., 1995], and mice that are

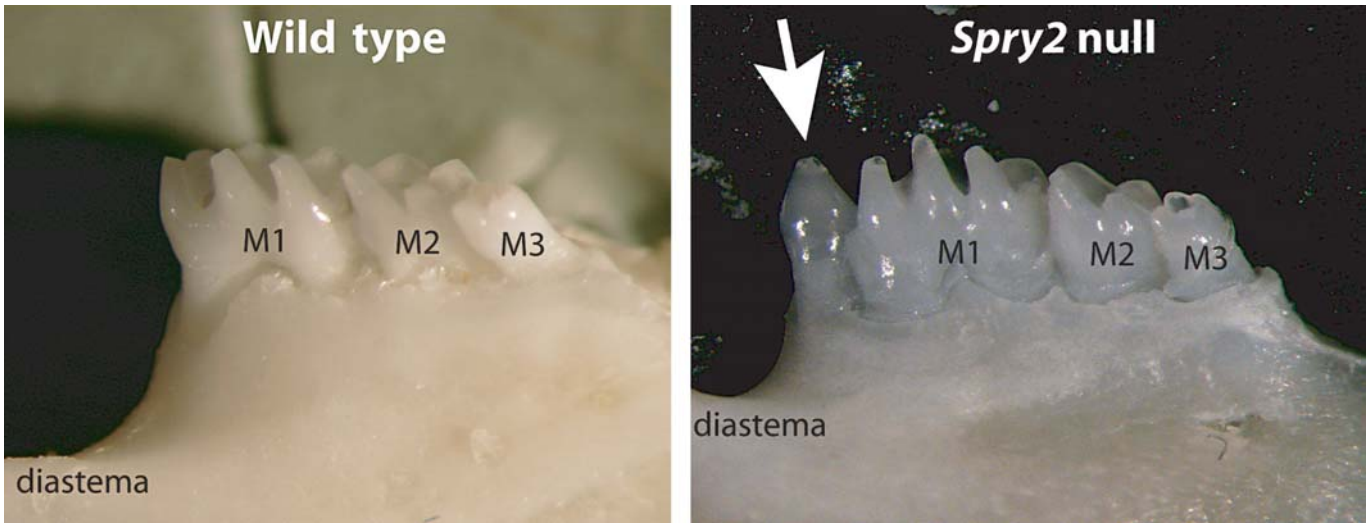

Fig. 2. Views of the mouse diastema and the presence of diastema teeth in Spry2 null. Side views of the molar and diastema region of wild-type (left) and *Spry2*-null (right) mice. M1, M2, M3 = 1st, 2nd, and 3rd molars. White arrow points to a diastema tooth.

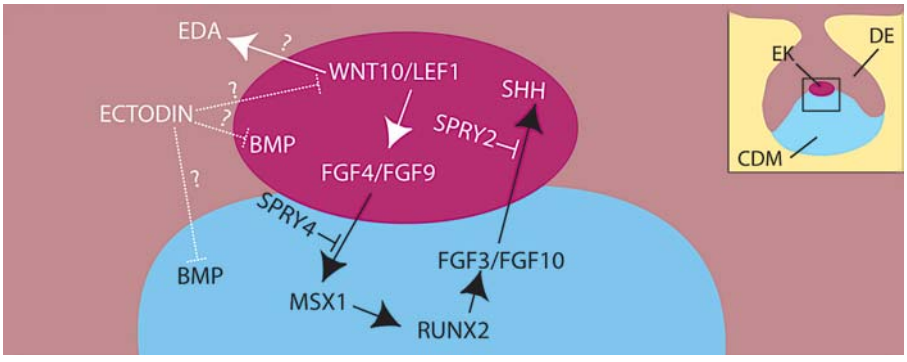

Fig. 3. Perturbation of signaling pathways can lead to supernumerary teeth by affecting epithelial-mesenchymal interactions. This model summarizes some of the critical signaling interactions between the enamel knot (EK) in the dental epithelium (DE) and the condensing dental mesenchyme (CDM) in a cap-stage molar tooth germ. It is based on data from both gene expression studies and manipulations of tooth germs in vitro [Kettunen et al., 2000; Kratochwil et al., 2002] and from genetic studies on knockout mice [Kassai et al., 2005; Klein et al., 2006]. Arrows indicate a stimulatory effect. The symbol indicates an inhibitory effect of one molecule on the expression of another when solid, and inhibition of signaling by a ligand when dashed. Wnt signaling induces epithelial FGFs, which in turn induce mesenchymal FGFs via MSX1 and RUNX2. Mesenchymal FGFs induce SHH in the epithelium. FGF signaling to the epithelium and mesenchyme is blocked by SPRY2 and SPRY4, respectively. Loss of function of *Spry2* or *Spry4* leads to supernumerary tooth development by upregulating FGF signaling. Wnt signaling may also induce ectodysplasin (EDA), a molecule that can lead to supernumerary teeth when overexpressed. Ectodin/WISE is a putative inhibitor of both BMP and Wnt signaling, and loss of ectodin/WISE function leads to supernumerary teeth. The precise signaling pathways modulated by ectodin/WISE function have yet to be elucidated.

null for ectodin/WISE, a bone morphogenic protein (BMP) and/or Wnt inhibitor [Kassai et al., 2005]. Interestingly, mutations in the gene encoding Polaris affect sonic hedgehog (SHH) signaling [Murcia et al., 2000; Huangfu et al., 2003; Liu et al., 2005], and mutations in the *Eda* pathway can be partially rescued by increasing FGF signaling in organ culture [Pispa et al., 1999]. Thus, modulation of pathways that are necessary for molar and incisor development, such as those initiated by SHH, FGFs, and BMPs, can lead to the development of diastema

teeth in mice. *Pax6* mutants have also been reported to have supernumerary incisors [Quinn et al., 1997].

Another major difference between mouse and human dentition is that mice have only a single set of teeth, whereas in humans the first set of teeth (primary or deciduous teeth) is replaced by a permanent set during childhood. The generation of progressive cycles of teeth appears to be the primitive (ancestral) condition for vertebrates. For example, lower vertebrates like fish replace their teeth throughout life. Most mammals, including humans, have only one cycle of tooth replacement, in which a deciduous (primary) dentition is supplanted by a permanent (secondary) dentition. The secondary dentition develops from lingual buds off of the deciduous lamina. Mice, in contrast, have only one dentition. The mouse therefore provides a simplified model for tooth formation in humans.

Molecular Insights

The development of diastema teeth could potentially be due to events either at the placode or at the bud stage. An expansion of the dental lamina at an early stage could lead to the development of more teeth. Later events, such as survival of the diastema bud, can also lead to diastema teeth [Klein et al., 2006]. Because recent work has focused on signaling events at the bud-cap transition leading to survival of the diastema bud, we have focused on this below.

Whether the diastema bud normally has a functional enamel knot is still a matter of controversy. It appears, though, that in mutants with diastema teeth, the presence of a functional enamel knot is essential for maintenance of the diastema bud. The pathways that are important in this process are diagrammed in figure 3, and they include members of the FGF, EDA, BMP and Wnt signaling families [reviewed in Tucker and Sharpe, 2004]. As an example of how changes in the function of one of these pathways can lead to development of diastema teeth, we focus below on the role of the FGF signaling pathway in this process.

The FGF signaling pathway appears to mediate epithelial-mesenchymal interactions at several stages of tooth morphogenesis in mammals and other vertebrates [Thesleff and Sharpe, 1997; Jernvall and Thesleff, 2000; Mandler and Neubüser, 2001; Jackman et al., 2004]. In the developing mouse molar, at least five different FGF ligands (FGF3, FGF4, FGF8, FGF9, and FGF10) and two receptors (FGFR1 and FGFR2) are expressed in complex, overlapping patterns in the epithelium and/or mesenchyme [Niswander and Martin, 1992; Neubüser et al., 1997; Kettunen et al., 1998; Kettunen and Thesleff, 1998; Kettunen et al., 2000]. Molar development is thought to be initiated by signaling via FGF8 [Trumpp et al., 1999], but *Fgf8* does not appear to have a function later in tooth development, because *Fgf8* expression is not detected at later stages [Kettunen and Thesleff, 1998].

Subsequent development of the tooth is thought to depend on signaling via other FGFs (fig. 3). This hypothesis has been based primarily on gain of function studies in organ culture and gene expression analyses. Epithelial cell proliferation and morphogenesis throughout the cap and bell stages is stimulated by FGF3 and FGF10 produced in the dental mesenchyme from the bud stage onwards [Jernvall et al., 1994; Kettunen and Thesleff, 1998; Kettunen et al., 2000]. These FGFs signal to the epithelium by activating the 'b' isoforms of FGFR1 and FGFR2, which are expressed exclusively in the epithelium [Kettunen et al., 1998]. Conversely, FGF4 and FGF9 produced in the epithelium are presumed to be key mediators of enamel knot activity in coordinating tooth morphogenesis. These signals bind to and activate the 'c' isoforms of FGFR1 and FGFR2 expressed in the mesenchyme [Kettunen et al., 1998]. Their major function is to maintain *Fgf3* and *Fgf10* expression in the dental mesenchyme, which – as discussed above – is thought to be critical for sustaining tooth morphogenesis. Some of the downstream targets of this FGF signaling cascade have been identified (fig. 3). Of particular interest, two genes known to be involved in dental anomalies in humans – *Msx1* and *Runx2* – are thought to be transcriptional targets of FGF signaling [Bei and Maas, 1998; Åberg et al., 2004]. In addition to being a mesenchymal target of FGF signaling, *Runx2*, the gene responsible for CCD, has been proposed to directly induce mesenchymal *Fgf3* expression [Åberg et al., 2004]. Thus, *Runx2* appears to be a critical link in the epithelial-mesenchymal FGF signaling loop. In patients with CCD, which is caused by haploinsufficiency for RUNX2 [Mundlos et al., 1997], supernumerary teeth arise from the permanent teeth, representing a third dentition [Jensen and Kreiborg, 1990]. The underlying molecular mechanism is proposed to result from an incomplete resorption of the dental lamina of the secondary dentition [Lukinmaa et al., 1995]. In *Runx2* homozygous null mutant mice, molar development ceases at the bud stage, a time during which there is normally strong expression of *Runx2* in the dental mesenchyme [D'Souza et al., 1999]. However, in *Runx2* heterozygote mice there appears to be the beginning of successional tooth development: lingual epithelial buds

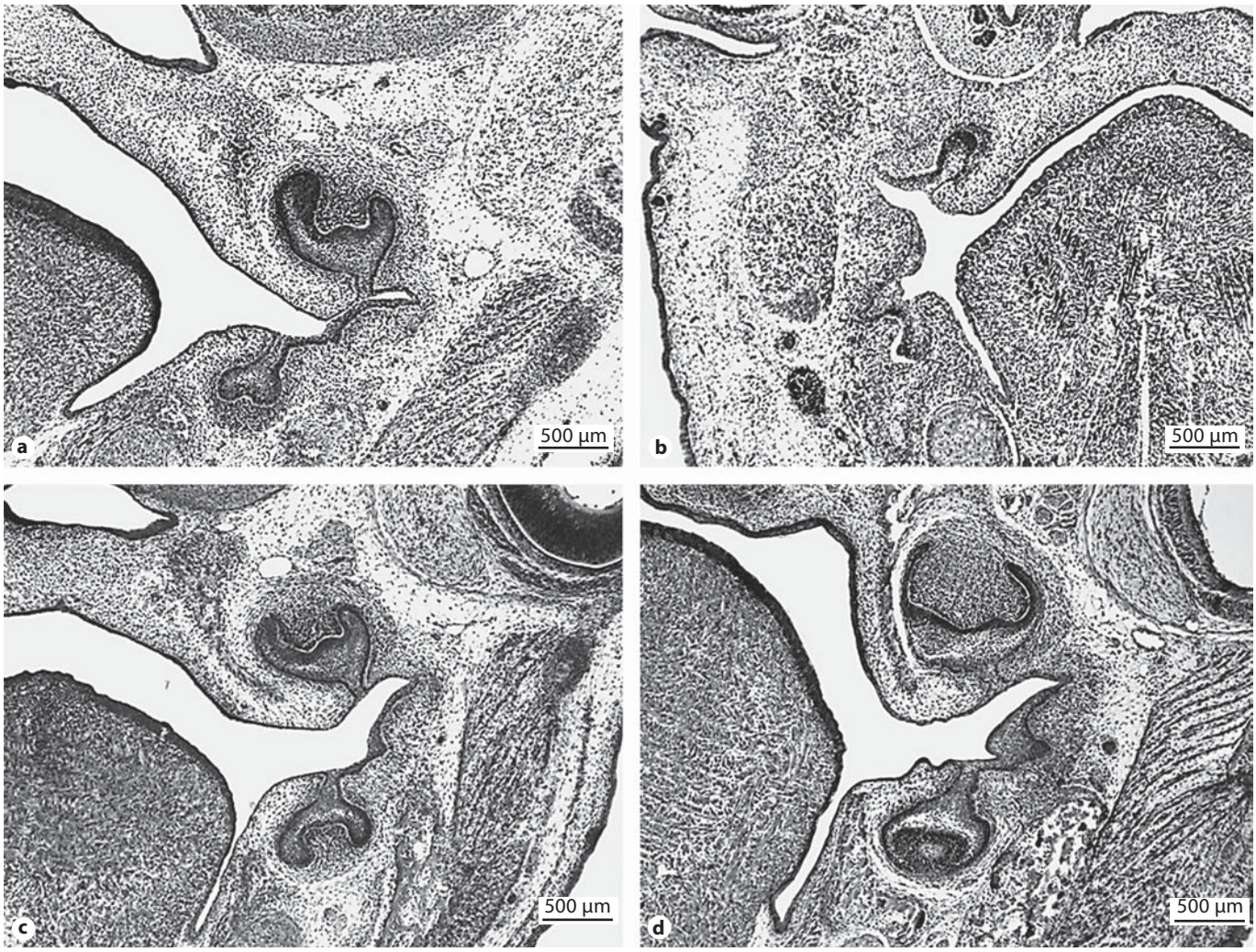

Fig. 4. Runx2 mutant tooth phenotype and rescue of arrest in Runx2/Twist-1 double heterozygote mutant mice. **a** E 16.5 WT. **b** E 16.5 Runx2 (–/–). **c** E 16.5 Twist-1 (+/–). **d** E 16.5 Runx2 (+/–)/Twist-1 (+/–). E = Embryonic day; WT = wild-type.

with active *Shh* signaling are present [Wang et al., 2005]. Thus, in both mice and humans, an important role of the Runx2 protein appears to be prevention of excess budding of successional laminae.

Interestingly, both FGF receptor levels and Runx2 activity are modulated by Twist-1 [Bialek et al., 2004; Guenou et al., 2005], a transcription factor involved in Saethre-Chotzen syndrome, and patients with this condition have been reported to have dental anomalies [Goho, 1998]. The relief of a functional antagonism between Runx2 and Twist-1 proteins leads to the onset of osteoblast differentiation [Bialek et al., 2004], suggesting a potential mechanism for the formation of supernumerary teeth in human CCD. Presently, experiments are underway to evaluate whether the formation of accessory tooth buds in Runx2 homozygous null mice are due to a relative overabundance of Twist-1. The latter may result in a prolonged survival of the dental lamina. Tooth development progresses normally in Runx2/Twist-1 double heterozygote mutant mice, suggesting that the in vivo genetic interaction between the two molecules is critical for tooth morphogenesis (fig. 4). Interactions between Runx2 and Twist-1 proteins may thus modulate a variety of events in both development and homeostasis.

For each of the major intercellular signaling pathways in development, antagonists have been identified. Signal-

ing via FGFRs is inhibited by a molecule called Sprouty (*spry*), which was first identified in a screen for mutations that affect tracheal branching in *Drosophila melanogaster* [Hacohen et al., 1998]. Because Sprouty is a negative feedback regulator of FGF signaling, the FGF pathway affects the expression of its own antagonist and thereby limits the range over which FGF signaling is active. When subsequent experiments in *Drosophila* showed that *spry* also regulates epidermal growth factor receptor signaling and other receptor tyrosine kinase pathways, the notion arose that *spry* is a general inhibitor of receptor tyrosine kinase signaling pathways [Casci et al., 1999; Kramer et al., 1999; Reich et al., 1999].

Four mouse genes have sequence similarity to *Drosophila spry*, and all have human orthologs [de Maximy et al., 1999; Minowada et al., 1999]. Three of the four mouse Sprouty genes, *Spry1*, *Spry2*, and *Spry4*, are expressed at various stages of embryonic development, whereas *Spry3* expression has been detected only in the adult [Minowada et al., 1999]. As Sprouty gene expression is induced by FGF signaling, it is observed in association with FGF signaling centers throughout the embryo in numerous developing organs, including the brain, lungs, digestive tract, kidneys and limb buds [Minowada et al., 1999; Zhang et al., 2001]. Sprouty family members act intracellularly to negatively regulate FGF and other receptor tyrosine kinase signaling through diverse biochemical mechanisms, primarily via effects on the mitogen-activated protein kinase pathway [Dikic and Giordano, 2003; Guy et al., 2003; Kim and Bar-Sagi, 2004].

In the tooth buds that form in the wild-type embryonic diastema, the genetic program that normally controls progression from the bud to the cap stage is not active. One mechanism by which diastema bud development is normally suppressed is via inhibition of FGF gene expression, including *Fgf4* in the enamel knot and *Fgf3* in the dental mesenchyme. Sprouty genes are required to prevent diastema tooth development even though there is little or no FGF gene expression in wild-type diastema buds. It is likely that the normal function of *Spry2* is to prevent the relatively low level of signaling via FGF10 produced in diastema bud mesenchyme from inducing/maintaining *Shh* expression. Likewise, the normal function of *Spry4* in the mesenchyme is to prevent any epithelial FGF signals, including FGF4 and FGF9 produced in the adjacent M1 tooth germ, from inducing/maintaining *Fgf3* expression. As a result of the combined activities of *Spry2* and *Spry4*, the diastema bud regresses and there are no teeth in the adult diastema.

Future Challenges and Directions

As is evident from the discussion above, the molecular events that lead to the prolonged survival or proliferation of the dental lamina, and possibly to abnormal division of the tooth bud, are complex and warrant further analysis. Elucidating the precise etiology of supernumerary tooth development will require the use of multifaceted approaches that involve both mouse and human genetic studies. The availability of individuals and families with non-syndromic forms of supernumerary teeth offer a valuable resource to identify genes and underlying mutations that give rise to this condition. Furthermore, information gained from understanding the precise pathogenesis of supernumerary tooth development can be translated to regenerative strategies aimed at bioengineering replacement tooth forms.

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