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Tooth Morphogenesis *in vivo*, *in vitro*, and *in silico*

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One of the aims of developmental biology is to understand how a single egg cell gives rise to the complex spatial distributions of cell types and extracellular components of the adult phenotype. This review discusses the main genetic and epigenetic interactions known to play a role in tooth development and how they can be integrated into coherent models. Along the same lines, several hypotheses about aspects of tooth development that are currently not well understood are evaluated. This is done from their morphological consequences from the model and how these fit known morphological variation and change during tooth development. Thus the aim of this review is two-fold. On one hand the model and its comparison with experimental evidence will be used to outline our current understanding about tooth morphogenesis. On the other hand these same comparisons will be used to introduce a computational model that makes accurate predictions on three-dimensional morphology and patterns of gene expression by implementing cell signaling, proliferation and mechanical interactions between cells. In comparison with many other models of development this model includes reaction–diffusion-like dynamics confined to a diffusion chamber (the devel-

Current Topics in Developmental Biology, Vol. 81
0070-2153/08 $35.00

DOI: 10.1016/S0070-2153(07)81012-X

oping tooth) that changes in shape in three-dimensions over time. These changes are due to mechanical interactions between cells triggered by the proliferation enhancing effect of the reactants (growth factors). In general, tooth morphogenesis can be understood from the indirect cross-regulation between extracellular signals, the local regulation of proliferation and differentiation rates by these signals and the effect of intermediate developing morphology on the diffusion, dilution, and spatial distribution of these signals. Overall, this review should be interesting to either readers interested in the mechanistic bases of tooth morphogenesis, without necessarily being interested in modeling *per se*, and readers interested in development modeling in general.

I. Introduction

One of the aims of developmental biology is to understand how a single egg cell gives rise to the complex spatial distributions of cell types and extracellular components of the adult phenotype. Ideally this problem can be separated into the problems of cell differentiation, or how cells become who they are, and the problem of how cells become arranged in specific spatial patterns, that is, how cells end up where they are. This review considers this second problem for the case of teeth, that is: how a tooth develops its morphology. This review discusses the main genetic and epigenetic interactions known to play a role in tooth development and how they can be integrated into coherent models. Computational models provide a method to explore the logical implications of hypotheses about developmental dynamics. It will be discussed whether existing models in tooth development provide precise predictions about how three-dimensional morphologies and patterns of gene expression change during development. By precise it is meant that computational models can give quantitative descriptions of morphology by specifying the three-dimensional positions of cells, which cells they have as neighbors and what are their levels of expression of several genes. More importantly computational models relate these morphological changes to a specific set of genetic and epigenetic interactions and to their specific organization of those. Then the morphological consequences of a genetic or epigenetic manipulation can be explored by making the same manipulations on model "genetic" and "epigenetic" interactions and seeing the *in silico* morphologies appearing from the model. This provides an objective way to see if the results of experimental manipulations are consistent or not with a specific hypothesis about morphogenesis. The studies and models here reviewed provide developmental explanations for morphological changes that are gradual and complex. The models presented do not focus, as many studies in development do, on gross and discrete morphological alterations of morphology so as to identify the genes involved in development. Instead the focus, and the model predictions, are on complex multivariate gradual changes and on how small genetic variation regulates these morphological changes and developmental dynamics. Along the same lines several hypotheses about aspects of tooth development

that are currently not well understood are evaluated. This is done from their morphological consequences in the model and how these fit known morphological variation and change during tooth development. This review aims, thus, to provide mechanistic insights about overall tooth developmental dynamics and about specific aspects of it. In that sense tooth computational models are not an end result but a way to summarize understanding of the development of an organ and an analytical tool to allow precise comparison of hypothesis with results. Thus the aim of this review is two-fold. On one hand the model and its comparison with experimental evidence will be used to explain current understanding about tooth morphogenesis. On the other hand these same comparisons will be used to introduce a computational model that makes accurate predictions on three-dimensional morphology and patterns of gene expression by implementing cell signaling, proliferation and mechanical interactions between cells. The model includes a reaction–diffusion-like part and a biomechanical part that changes the three-dimensional shape in which diffusion and reaction is taking place. These changes are due to mechanical interactions between cells triggered by the proliferation enhancing effect of the reactants (growth factors). Overall, this review should be interesting to either readers interested in the mechanistic bases of tooth morphogenesis, without necessarily being interested in modeling *per se*, and readers interested in development modeling in general.

II. The Use of Mammalian Tooth for Developmental and Evolutionary Biology

There are several characteristics of teeth that are very convenient for studies in development and evolution. The tooth organ develops without too many influences from other parts of the body. In fact, teeth can develop *in vitro* to give rise to nearly normal morphologies. *In vitro* teeth, however, do not fully mineralize. In addition they exhibit more morphological variability than *in vivo* teeth. Tooth development is finished by the time teeth erupt. After that point tooth morphology changes only because of mechanical wear or accident. Tooth morphology is thus relatively independent of environmental effects. In that sense tooth morphology has been shown to exhibit high heritability (Matsumoto *et al.*, 1990), although more accurate measurement of morphology indicates that such heritabilities may not be as high as previously thought (Townsend *et al.*, 2003). Overall, teeth, in contrast to many other mammalian organs, can be studied in relative isolation.

Teeth are probably the mammalian organs that are most often preserved in the fossil record. In fact, many extinct mammalian species are known only from tooth remains. The fossil record, thus, provides a relatively good description of the morphological transitions that occurred in tooth evolution (Butler, 1983, 1995). There is also substantial knowledge about the patterns of variation in morphology within populations of several species (Colyer, 1936; Wolsan, 1989; Szuma, 2002). Overall, the tooth literature provides a solid knowledge of the morphological diversity that can arise from

changes in tooth development and, thus, the tooth is a good system for studies in development and morphological evolution.

The developmental systems that are currently best understood are often characterized by a relative decoupling between cell signaling and cell movement. Thus early anteroposterior and dorsoventral patterning in *Drosophila* blastoderm, wing imaginal disc patterning in *Drosophila*, hair and feather primordia spacing in vertebrate skin, can all be basically understood from the signaling between cells that do not move or move little. In most cases these patterning events occur, or can be understood, in a two-dimensional field. These kinds of systems do not necessarily represent the whole of animal development. In general cells signal to each other most of the time and animal development involves numerous events of active or passive cell movements. Thus, it is often the case that cells are signaling to each other while their relative locations are changing. In that situation the spatial distribution of the cells receiving a signal changes depending on how the relative distances, shapes and orientations of those cells and the cells sending the signal change over time. In those development systems, called morphodynamic (Salazar-Ciudad *et al.*, 2003), it may be more complex and more difficult to understand how different cell types get arranged in specific spatial locations. Then common models of pattern formation and the insights acquired from more static developmental systems cannot directly be applied to morphodynamic systems (Salazar-Ciudad *et al.*, 2003). Teeth provide a relatively simple system in which cells change their relative locations while signaling. They are also eminently three-dimensional morphologies. In that sense teeth provide an advantageous entry point for the study of more complex and representative developmental dynamics and morphologies.

III. Morphological Changes During Tooth Development

This section describes the main morphological changes occurring during tooth development. This description is merely phenomenological. The genetic and epigenetic bases of such changes are covered in later sections. This section, and most of the article, is focused on the mouse first molar because it is the best described tooth but most of the discussion is valid for many other teeth. In that sense the description, in this section, of the morphological changes during tooth development should be seen as the description of the problem that the model and the research review in this article tries to understand.

Teeth start from an invagination of the oral epithelium. In this initial bud stage, the invagination deepens in the underlying mesenchyme and extends in an anterior and posterior direction from its starting point. By embryonic day E13 it is deep where it started and shallow in its anterior and posterior borders (Viriot *et al.*, 1997). The driving force for such invagination is unknown but at that stage most

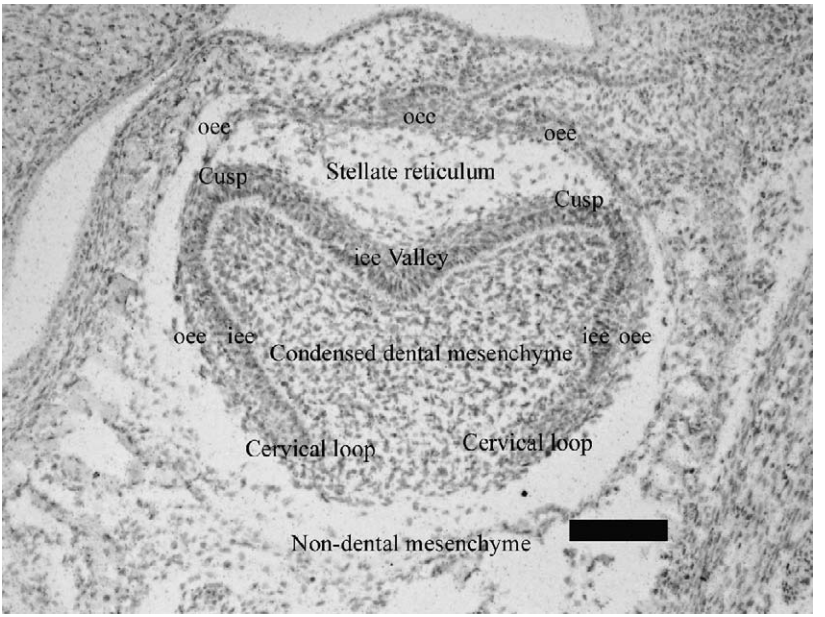

Figure 1 Frontal section of a first mouse molar at embryonic day 17 (E17) showing the basic anatomy of a developing tooth. Scale bar indicates 100 μm. Buccal is to the left and lingual to the right. Abbreviations: iee—inner enamel epithelium; oee—outer enamel epithelium.

dental epithelial cells are proliferating (Lesot *et al.*, 1996; Jernvall *et al.*, 1994; Coin *et al.*, 1999). The apical side of the invagination (the side in contact with the forming oral cavity) is very narrow and has cells that, over time, become vacuolized and form a pressurized structure called the stellate reticulum. The maturation of this structure also seems to proceed into an anterior and posterior direction and seems to be more advanced in parts that invaginate earlier, but no systematic study of that has been performed. The mesenchyme underlying the deepest parts of the invagination (see Figs. 1 and 2) starts to condense at this point. Soon after that, in the cap stage, the part of the epithelium just above the condensating mesenchyme stops growing and its cells become more packed (Jernvall *et al.*, 1994). This structure is called the enamel knot or, here, simply the knot. Next, the epithelium grows more extensively near the knot, but not in the knot itself, leading to the formation of two epithelial loops (called the cervical loops; one on the buccal side and one on the lingual) that invaginate deeper in the underlying mesenchyme. These loops also form in an anterior and posterior direction (see Fig. 2) and proceed at a distance from the knot. At E15, the beginning of the so-called bell stage, another knot forms buccally from the first one. Over time, the two knots end up in the tip of two bumps in the epithelium while the epithelium in between continues to proliferate and deepens into the underlying mesenchyme (Fig. 2). As a result, a valley forms in the epithelium between these bumps. By E16 a new

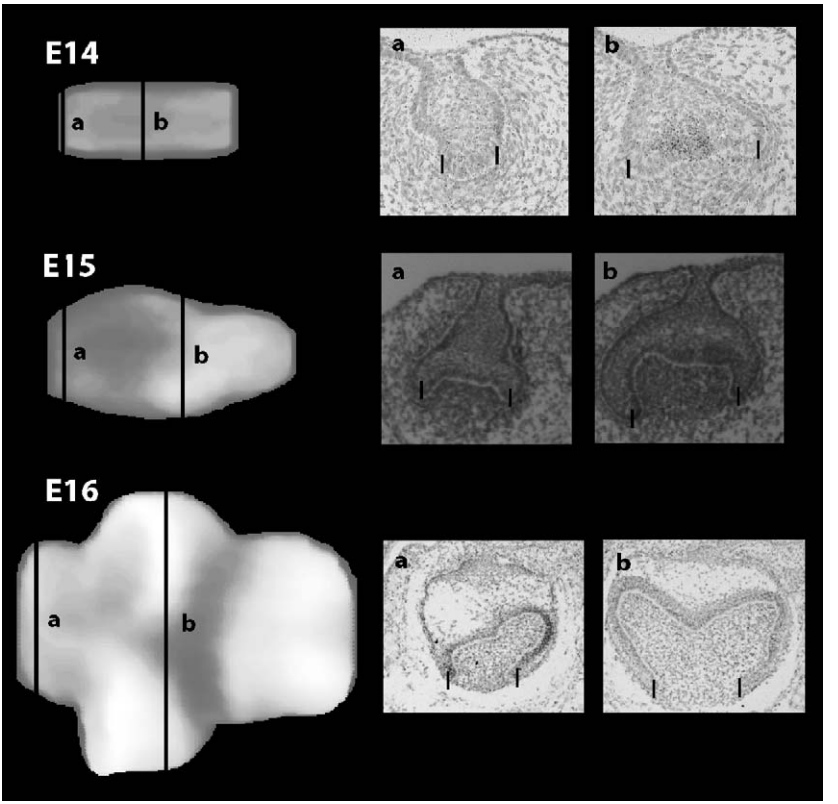

Figure 2 In the left side a three-dimensional reconstruction of the inner enamel epithelium surface for embryonic stages E14, E15, and late E16. For the three-dimensional reconstructions anterior is to the left and posterior to the right. The images in the right are frontal sections for each stage. The levels at which these sections were performed are indicated on the three-dimensional reconstructions. In each section two vertical black lines indicate the boundary between inner and outer enamel epithelium (the three-dimensional reconstructions include only the inner enamel epithelium).

knot forms, posteriorly from the first one, and, soon afterwards, a fourth one forms lingually from it. These also end up in the tips of some bumps in the epithelium while the epithelium around them continues to grow. Progressively, the epithelium that is not enclosed by the cervical loops (called the outer enamel epithelium in contrast with the inner enamel epithelium) stops proliferating and its cells flatten. Meanwhile the cervical loops continue to grow and extend in the anterior and posterior extremes of the tooth. They also change from growing laterally and downwards to grow mainly downwards. Progressively growth becomes confined to the tips of the cervical loops while the epithelium near the knots starts to become columnar and eventually starts mineralization. This process of differentiation proceeds as a wave from the knots to

the cervical loops. Ultimately the inner enamel epithelium outlines the surface of the formed tooth. The cells that formed the knot end up at the tips of the tooth cusps (see Fig. 2) while the cervical loops form the lateral walls of the tooth. The cervical loops continue to develop to give rise, in mouse but not in all other rodents, to the tooth root, by a process that is beyond the scope of this review. The mechanisms by which teeth erupt is also outside the scope of this review.

IV. Gene Networks in Tooth Development

There is over 300 genes known to be expressed in teeth (Thesleff *et al.*, 2001; see http://bite-it.helsinki.fi/ for a database on those patterns). It is not the purpose of this review to consider all, or even a significant proportion, of them. There are already some excellent reviews on this (Jernvall and Thesleff, 2000; Thesleff *et al.*, 2001). Here I only consider genes that, when mutated, produce a specific change in tooth morphology (other than the nonformation of teeth). Until recently there were relatively few mutants with a clear specific effect on tooth morphology (Pispa *et al.*, 1999; Biben *et al.*, 2002; Wang *et al.*, 2004a, 2004b; Kangas *et al.*, 2004; Kassai *et al.*, 2005; Järvinen *et al.*, 2006). Many other genes are involved in the terminal differentiation and mineralization of teeth and are not considered in here. In general terms, most patterns of gene expression can be categorized in terms of few types. Many genes are expressed only in the main tooth tissues (or in combinations of them) such as in the epithelia (inner, outer or both), or in the mesenchyme or in the stellate reticulum. Many other genes seem to be expressed in concentric patterns around the knots (either in the epithelium or mesenchyme or both), or everywhere but in the knots. Some few genes are expressed in the anterior and/or posterior borders of the tooth primordia. So far no single gene has been shown to be expressed in only one knot (except for the first knot). This relative simplicity suggests that many insights about how gene interactions regulate the production of morphology can be acquired without a detailed description of all the genes involved.

Gene regulation ultimately acts on development by affecting a limited number of cell behaviors (proliferation, apoptosis, matrix and signal secretion, differentiation, adhesion and consequent changes in cell shape and motility). Some understanding of morphogenesis is achievable without having to consider the details of intracellular signaling but by simply considering in which ways those behaviors change when cells receive specific growth factors. In this review the account of developmental mechanisms is based on considering how cells respond to specific growth factors by secreting other growth factors, proliferating, differentiating, expressing some receptors and changing their adhesive properties. What happens inside cells is then largely simplified to facilitate the understanding of the collective behavior of enamel epithelium and dental mesenchyme during morphogenesis. As I will try to show, the rise of the whole tooth morphology can be understood from these relative simple developmental rules and some few epigenetic constraints.

V. The Formation of the Cusps

At E12, when a starting invagination is morphologically visible, intense expression of several signals appears in a subset of invaginating cells. This transient, early epithelial signaling center expresses signals in all four major morphogen families (BMPs, Shh, Wnt, and FGFs) as well as other genes associated with signaling such as p21, Msx2, and Lef1 (Dassule and McMahon, 1998; Keränen *et al.*, 1998). Many of these signals remain expressed during the bud stage, especially at the tip of the invagination, and in the first and subsequent knots (Vaahtokari *et al.*, 1996). It is not clear how the knots form but since the first knot appears in a location where all these signals are expressed intensively my hypothesis in current and previous models has been that epithelial cells differentiate into knot cells after receiving a threshold concentration of some signal. Which signal that may be is not totally clear but recent studies (Kassai *et al.*, 2005) show that Bmp4 added to *in vitro* tooth cultures accelerates the formation of knots and tooth differentiation. That seems to be especially the case in mutants lacking ectodin, a BMP sequesterer, which has been suggested to buffer the levels of diffusing Bmp4. Moreover, Bmp4 has been shown to induce p21 (Jernvall *et al.*, 1998), a gene known to arrest the cell cycle at the G1/S transition (Steinman *et al.*, 2001). p21 is one of the earliest markers of knot formation. Activin, another TGFß superfamily member, has also been shown to induce p21 and is expressed in the mesenchyme just under the knots (Wang *et al.*, 2004a). Fgf-2, -4, -8, and -9 have been shown to induce, *in vitro*, the proliferation of dental epithelium and mesenchyme (Jernvall *et al.*, 1994; Kettunen *et al.*, 1998) while Fgf-10 acts similarly only in the dental epithelium (Kettunen, 2000). These FGFs are expressed in the knots and several FGF receptors are expressed throughout the dental primordia (except, significantly in the knot itself). Shh is also expressed in the knot and has been suggested to promote proliferation in the dental epithelium (Cobourne *et al.*, 2001; Gritli-Linde *et al.*, 2002). Bmp2 and Bmp4 are also expressed in the knots but, as mentioned, they promote differentiation of the surrounding tissues. The spreading of differentiation from the knots downwards is correlated with a gradual spreading of the expression of many knot genes from the knots downwards to the cervical loops.

The proliferation that knots promote around them combined with their lack of proliferation has been proposed (Butler, 1956; Butler and Ramadan, 1962; Osman and Ruch, 1976; Ruch, 1990; Jernvall *et al.*, 1994; Jernvall and Thesleff, 2000) to produce the folding of the epithelium around the knots and the subsequent formation of cusps. The relative sharpness or bluntness of cusps has been proposed to arise from either the relative rates of growth of the epithelium (by itself or in proportion to mesenchymal growth) near the knots (Jernvall and Thesleff, 2000; Salazar-Ciudad and Jernvall, 2002) and/or the relative efficiency of Bmp2 and 4 in producing the differentiation of cells near the knot (Salazar-Ciudad and Jernvall, 2002). Although these hypotheses can explain the formation of single cusps (as in canine teeth) it does not explain how different cusps, and the spacing between them, arise nor how whole tooth morphology arises.

VI. Spacing Between Cusps

In mammals, cusps in a tooth appear at a distance from each other. Grossly the shape of a tooth can be described by the relative positions and heights of cusps and the border of the tooth. If, as suggested, knots appear after exposure of dental epithelium to Bmp4 there has to be some mechanism to restrict the expansion of these knots and allow the formation of different discrete knots (instead of a single large one). Knots have been suggested to secrete some signal that would inhibit neighboring cells from becoming knots (Jernvall and Thesleff, 2000). Since this hypothesis was proposed, our understanding on the signaling taking place during tooth development has greatly advanced. It seems that this later inhibition may involve several signals. Activin, that as mentioned is expressed in the mesenchyme under the knots, has been shown to promote the expression of follistatin (Heikinheimo *et al.*, 1998; Wang *et al.*, 2004a). Follistatin is a diffusible gene product that sequesters both activin and Bmp2 and Bmp4 (Nakamura *et al.*, 1990; Iemura *et al.*, 1998) and thus acts as its inhibitor. However, follistatin knock out mice seems to be able to form several individual knots and cusps (Wang *et al.*, 2004a). Thus follistatin cannot be the signal mediating lateral inhibition, at least not alone. Activin also promotes Edar expression, which in turn seems to promote Shh expression in the knots (Laurikkala *et al.*, 2001; Pummila *et al.*, 2007). It is possible that the proliferative effect of Shh or FGFs on dental epithelium may prevent it from differentiating and thus from forming a knot.

More generally it has been suggested that the spacing between knots in the tooth would arise by a reaction–diffusion or reaction–diffusion-like mechanism (Jernvall and Thesleff, 2000). In that respect, Bmp4 has been shown to, indirectly through Msx1, activate its own expression (Vainio *et al.*, 1993; Bei and Maas, 1998). Since it also seems to promote knot formation Bmp4 is a perfect candidate to be an activator in a reaction–diffusion mechanism, although activin could also be involved. Although the exact molecular mechanisms may not yet be clear, it is likely that Shh, FGFs and follistatin may be, or may mediate, the inhibitory molecule in this reaction–diffusion-like mechanism. In contrast with reaction–diffusion models, the inhibitor is not expressed by all cells that receive activator but only in knot cells. This is drawn from the previous experimental evidence and produces, as I will show in the model, dynamics that are slightly different from those of pure reaction–diffusion mechanisms.

Reaction–diffusion mechanisms produce symmetric regular patterns in which a number of repeated elements, either stripes or spots, are homogeneously spaced through a (cellular) field. However, when looking at the morphological disparity of mammalian molars it is clear that molars tend to be nonsymmetric and not all cusps are of the same size and height. In addition, not all cusps are equally spaced nor, always, regularly arranged in the tooth. In fact, the disparity of molar shape can be described as variation in these aspects of cusps. Therefore, a simple reaction–diffusion-like mechanism alone cannot account for the development of the morphologies of molars. As briefly explained in the introduction, growth and its interaction with signaling is the additional factor that needs to be considered. This growth signaling interdependence

happens, as I will explain in the next section, at several levels. The components of the reaction–diffusion-like mechanism, at least BMP and Shh, affect proliferation in the epithelium and mesenchyme. This affects local growth that in turn affects the amount and shape of the space in which these molecules can diffuse. This regulates the distances at which Bmp4 concentration reaches the threshold to form new knots and indirectly regulates the heights and positions at which new knots form. Thus these morphodynamic mechanisms cannot be understood as the mere addition of growth to reaction–diffusion mechanisms, but, as I will show, represent a distinct class of dynamics that allows a repertoire of morphologies larger than that of reaction diffusion or growth alone (Salazar-Ciudad *et al.*, 2003). Even with growth, reaction–diffusion mechanisms cannot produce teeth that are asymmetric in the anteroposterior or buccolingual axis. This is achieved, as I will detail, because of asymmetries in Bmp4 and other molecular expression in the mesenchyme surrounding teeth.

The next sections describe these morphodynamic dynamics in detail. This is done by introducing the models and how they can be used to explore the implications of several alternative hypotheses about the interaction of signaling with specific aspects of growth. Thus, much relevant experimental evidence is introduced along with the models to make explicit the biological assumptions upon which the models are built. Two computational models of tooth morphogenesis will be reviewed here (Salazar-Ciudad and Jernvall, 2002, 2007). The models differ in the accuracy of their predictions and the detail and realism of the cellular behaviors and gene networks involved in tooth formation.

VII. Morphodynamic Model 1

The model includes four cell behaviors: cells can secrete signaling molecules; cells can receive signaling molecules (and change their behaviors in consequence); cells can also divide and differentiate. The model includes a network of gene products that regulates these behaviors and interact between them. Model 1 includes an activator which, as Bmp4 does indirectly, activates itself and, after a concentration threshold, knot formation. The inhibitor is produced from the knot at a rate proportional to the activator concentration in the knot and it inhibits activator production by epithelial cells.

The model starts with four epithelial cells distributed in a regular rectangular grid. Three layers of mesenchymal cells lie under these epithelial cells. All epithelial cells secrete activator at an intrinsic rate (k_3) and also in response to the local activator concentration. Over time, in areas where the local activator concentration exceeds a set threshold, the epithelial cells differentiate irreversibly into nondividing knot cells. These knot cells also secrete inhibitor at a rate equal to the local activator concentration. This inhibitor counteracts activator secretion and, in addition, enhances growth of the mesenchyme. The mesenchyme is mainly a three-dimensional space where diffusion and growth take place.

Diffusion takes place inside the three-dimensional space (subdivided into a three-dimensional grid of boxes) of the growing tooth. The system has zero-flux boundary conditions in the epithelium (diffusion is not allowed in their apical side) and open boundary conditions in the mesenchyme (molecules exit the system through the borders). The mesenchyme is surrounded by the epithelium (where diffusion is allowed), except in the ventral border where lies the nondental mesenchyme (where the activator and inhibitor can diffuse out of the system). The rate of activator secretion in nonknot epithelial cells is:

$$\frac{\partial A}{\partial t} = \frac{k_1[A]}{k_2[I]+1} + k_3 + D_A\nabla^2[A], \tag{1}$$

where $D_A\nabla^2[A]$ is the diffusion term and D_A is the diffusion coefficient of the activator. The k_1 and k_2 constants can be related to biochemical aspects as the affinity of each molecule for its receptor or to the signal amplification produced by its chain of signal transduction. The rate of inhibitor secretion by knot cells is:

$$\frac{\partial I}{\partial t} = [A] + D_I\nabla^2[I], \tag{2}$$

where $D_I\nabla^2[I]$ is the diffusion term and D_I is the diffusion coefficient of the inhibitor.

In model 1 all cells are positioned in a three-dimensional grid made of regular cubes. In that sense the initial tooth primordium is made of 4 columns of cells (with epithelial cells in the top of each column). Epithelial growth is implemented by making epithelia increase its depth into the mesenchyme. This is made by displacing epithelial cells downwards as they growth. Epithelial growth rate is $R_e - [A]$ and at least zero. When a single epithelial cell shifts ventrally one cell length into the mesenchyme, it displaces ventrally all the underlying cells in that column (including the stellate reticulum space apical to the top epithelial cell). Since this growth rate depends of the concentration of the activator it can differ between different parts of the epithelium. As a result of these processes, part of the epithelium folds into the mesenchyme leaving the knots isolated in the tips of the forming cusps. At the same time mesenchymal growth produces localized lateral expansion affecting cusp sharpness. Any cell which, due to the relative displacement of columns, gets in contact with the stellate reticulum is considered, in model 1, an epithelial cell.

Mesenchymal growth occurs mainly in the direction offering least resistance (away from the space apical to the epithelium). This growth is proportional to the concentration of inhibitor in each mesenchymal cell. Thus the inhibitor in the model has two effects (although *in vivo* these effects may be mediated by distinct molecules coming from the knots). This propensity of movement towards less occupied space was calculated as the sum of the concentration of inhibitor in all the mesenchymal cells of a column multiplied by a constant (R_m) that reflects the sensitivity of cells to the inhibitor's growth effect. Specifically, the lateral pressure of cells in a column i is distributed into four nearest neighboring columns (the anterior, posterior, buccal and lingual columns) by the following rules: (i) pressure distribution can only occur to

columns shorter than column i. (ii) The resistance $(1/Sj)$ of each neighboring column shorter than column i is the total number of cells that all the columns in one direction have (for example, all the posterior columns next to the column i). This reads:

$$S_j = 1 \Big/ \left(\sum_{k=0}^{k=n(i,j)} m(k) \right), \tag{3}$$

where j can be any of the four directions possible, p, posterior, a, anterior, b, buccal, l, lingual. These correspond to the four axes of a tooth. $n(i, j)$ is the number of columns between column i and the border of the tooth in the direction j, and $m(k)$ is the number of cells in column k. Note that $n(i, j)$ and $m(k)$ depend on tooth shape at each time point and are not external functions or fixed parameters of the model but, at each iteration, the result of previous dynamics. (iii) The pressure of column i is distributed to its neighbors in inverse proportion to their resistance. This is defined as

$$R_j(i) = D_j R_m \sum_{k=0}^{k=m(i)} [I]_{ik}, \tag{4}$$

where

$$D_j = S_j/(S_p + S_a + S_b + S_l) \quad \text{for } j[p, a, b, l].$$

$R_j(i)$ is the resulting lateral pressure exerted by a column i in direction j. $[I]_{ik}$ is the concentration of the inhibitor in cell k in column i and R_m is the rate constant of mesenchymal growth. S_j is the inverse of the resistance and $(S_p + S_a + S_b + S_l)$ is the overall inverse of the resistance in all directions.

The lateral expansion is mimicked by adding new cells to a column when it receives lateral pressure in a given direction that exceeds a unit corresponding to a cell size in a given direction. For a column that is not in the border of the tooth, the lateral force received from neighboring columns causes the whole column to displace upwards. Note that mesenchymal pressure only moves from tall to short columns and thus this upward movement is equivalent to the growth of a column (or dome) to the sides. When this displacement upwards exceeds one unit a new cell is added at the bottom of the column. Mesenchymal pressure from a column in the border of the tooth to the border leads to the addition of a new column in that direction (when accumulated pressure in that direction exceeds unity). A new column starts as a single epithelial cell. This way the tooth can grow in width (and this is due to the effect of signals emanating from the knots). Mesenchymal cells are also added at the bottom of the tooth at the level of the deepest column. This does not represent a real biological phenomenon, it simply indicates that more space is considered for the model calculation (essentially these additional mesenchymal cells were already there but were not considered for the calculations). Lateral growth can be biased in the anterior, posterior, buccal and/or lingual direction by increasing the lateral force on cells in the perimeters of

the tooth in any of these four directions. There is a bias in the posterior (B_p), anterior (B_a), buccal (B_b) and lingual (B_l) direction. For cells in the border equation (4) becomes:

$$R_j(i) = D_j R_m \sum_{k=0}^{k=m(i)} [I]_{ik} + B_j \quad \text{for } j[p, a, b, l]. \tag{5}$$

These biases are B_p, B_a, B_b, and B_l. These biases are necessary because without them all teeth would be symmetrical. The teeth of mouse and other mammals are known to grow asymmetrically in these directions.

VIII. Model 1 and Tooth Dynamics

In spite of the overall coarseness by which the model implements cell mechanical interactions and growth, the model is able to reproduce, to a large extent, the three-dimensional changes in morphology and patterns of gene expression observed during development in mouse and vole first molars (see Fig. 3). The model reproduces these changes with a relative timing very comparable to that of real mouse and vole tooth development. These molars were chosen for testing the model because they are the ones for which changes in morphology and patterns in gene expression are best described (Jernvall *et al.*, 2000). In addition, the model is also able to reproduce the molar morphology of many other species (Salazar-Ciudad and Jernvall, 2002).

The model also has some obvious limitations that have motivated the construction of a second model. These have to do, mainly, with the way growth and mechanical interactions between cells are implemented. These are also the aspects of tooth development that are less well understood. Model 1 does not explain how epithelial cells grow down but it simply takes it for granted. Although it is possible that on average increased epithelial grow leads to sharper cusps, dividing epithelial cells tend to bud-off in the plane of the epithelium. Thus, epithelial growth does not necessarily move cells downward but moves cells in the plane of the epithelium (which changes locally and over time). Although, on average, the implementation of mesenchymal growth pressure may give rise to lateral growth towards the borders, it is unlikely that this pressure is the result of summing up the growth along a column of cells. Instead, it may be more realistic to assume that every mesenchymal cell grows and divides in any possible direction. In the model the cells can only move as cohesive columns in a fixed rectangular grid. Lateral growth in a column is mimicked by vertical growth in neighboring columns. This provides a rather discrete description of space, cell position and cell displacements. Morphodynamic model 2 (Salazar-Ciudad and Jernvall, 2007) provides a more spatially continuous implementation of these aspects as well as a better low level implementation of epithelial and mesenchymal growth and cell division.

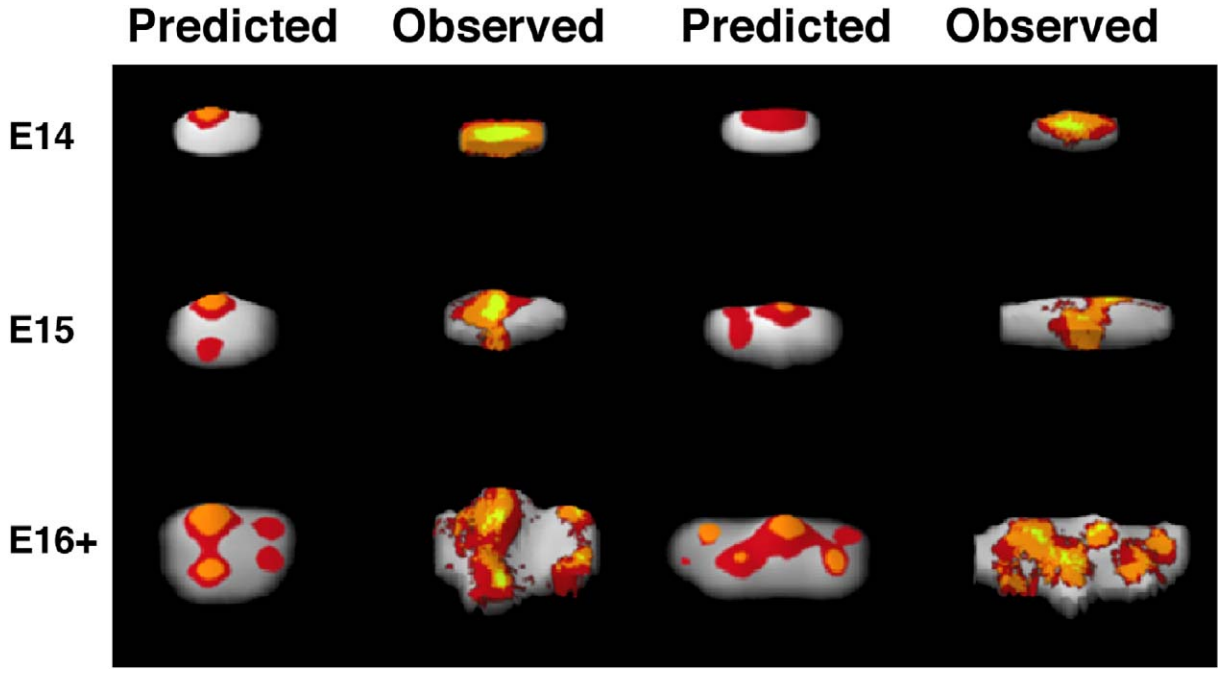

Figure 3 Model 1 predicts gene expression patterns in mouse (left) and vole (right). The activator and inhibitor concentration peaks predict the observed nested gene coexpression patterns among genes of different signaling families. On predicted shapes, the coexpression patterns of activator and inhibitor (in orange) inside the activator domain (in red) resemble the observed coexpression patterns where Fgf4, Shh, Lef1, and p21 (in yellow) mark the cores of the enamel knots, surrounded by areas lacking Fgf4 (in orange) and Fgf41Lef1 expressions (in red). Anterior side is toward the left and buccal side is toward the top; ages are in embryonic days. See color insert.

IX. Morphodynamic Model 2

Gene networks in model 2 are implemented in a similar way to model 1. Three more genes are included, *BMP2*, ectodin, and *FGF-4*. Ectodin is an extracellular sequesterer of several BMPs and in the model it acts by decreasing the concentration of free diffusible Bmp2 and 4. Fgf4 is also included. As in mouse teeth (Jernvall *et al.*, 1994), it is secreted from the knots and it promotes proliferation of the underlying mesenchyme. Also as in mouse teeth (Åberg *et al.*, 1997), Bmp2 is secreted from the knots and enhances differentiation. In the model, Bmp4 is the activator and Shh is again assumed to be the inhibitor. These genetic differences, however, have a rather mild effect on model dynamics. The more significant changes have been made at the level of growth dynamics, cell biomechanics and proliferation. In contrast to model 1, model 2 does not constrain cell position and displacement to a rectangular discrete grid. Instead, the cells in the epithelium form a grid that deforms and grows due to cell growth and division.

The model only considers tooth development from the later moments of the bud stage. The model's initial conditions consist of an initially flat epithelium. This epithelium represents the tip of the invagination before the first knot forms (see Fig. 2). The model starts with 19 hexagonal epithelial cells arranged in a hexagon (see Fig. 4B) with 20 layers of mesenchymal cells under them. Each cell has six neighbors and is situated at an arbitrary distance of 1 from them. Cells in the borders have only 3 or 4 neighbors.

Each cell is a three-dimensional volume that includes the cell itself and its immediate extracellular space. Molecular diffusion between two cells is proportional to the area of contact between those cells and their surroundings (finite volume method). This method is used because it allows accurate calculations even when cells change their shapes. The contour conditions for diffusion are the same than in model 1. The initial condition includes the expression of Bmp4 in the borders of the tooth at the level of the epithelium. The concentration of Bmp4 is kept constant and asymmetric in the borders all the time: Border epithelial cells that extend buccally from the midline have concentration set to a model parameter, *bib*, while cell extending lingually are set to *bil*. This asymmetry in Bmp4 expression in the borders of the tooth germ exists in mouse first molar through development (Åberg *et al.*, 1997).

Epithelial cells grow by pushing their neighbors in the plane of the epithelia. Each epithelial cell pushes each of its neighboring epithelial cells away from it. In other words, this pushing is, for each pair of cells, in the direction of a unit vector pointing from the center of one cell to the center of the other. For each cell the direction of push made by all neighboring cells is summed up and normalized to determine the direction of growth. The amount of growth by a cell is, in a similar way to model 1, proportional to R_e multiplied by 1 minus the amount of differentiation of that cell (cells differentiate at a rate proportional to Bmp2 concentration). When the distance between two original neighbor cells is larger than two, a new daughter cell appears at the midpoint in between them. The gene product concentrations of that daughter cell

are the average of the two "mother" cells. This way the grid made by the positions of the epithelial cells grows by intercalation of new cells and the average size of cells remains relatively constant (as in many animal epithelia). New cells have as neighbors the two "mother" cells and a subset of their mother's neighbors (chosen in such a way that the lines uniting cells with their neighbors do not cross each other). As in model 1 knot cells do not grow nor divide.

With the above-mentioned rules the model's teeth would simply grow as flat epithelia. As explained above, once the first knot forms two epithelial folds form buccally and lingually from the knot. Sections at successive times show that the cervical loops tend to bend towards the midline as time progresses. In some molars that bending is so large that the buccal and lingual cervical loops get very close to each other at their tips (Fig. 1). Tooth biomechanics are currently not understood sufficiently well to explain this phenomenon. In fact it is not known why cervical loops grow downwards and why they bend medially. Several hypotheses can be advances to explain this. Here I present the one that is implemented in the current version of the model. Later sections describe how the other hypotheses have been tried in the model and how and why they fail to produce dynamics consistent with those found in mouse molars. My preferred hypothesis is that the cervical loops and their growth dynamics are a result of the epithelium having a strong binding affinity for the dental mesenchyme and the dental mesenchyme having a strong binding affinity for itself. This could happen either because dental and nondental mesenchyme express different adhesion molecules or simply because dental mesenchyme is more condensed and thus has more adhesion molecules per relative volume. That way the morphology and the medial bending of the cervical loops would be a result of the epithelium trying to engulf the dental mesenchyme (Steinberg, 1970). In that sense the cervical loops would expand in the interface between the dental mesenchyme and the normal mesenchyme of the jaw. The condensed dental mesenchyme extends as a spheroidal mass under the inner enamel epithelium. Morphologically it seems that the cervical loops do not surround mesenchyme that is not condensed. In that sense it is also possible that the contact with this dental mesenchyme is involved in making the inner epithelium different from the outer one. At the same time it is possible that signals emanating from the epithelium or from the knots are responsible for the condensation of the mesenchyme. This could happen early on in the bud or cap stage or continuously throughout tooth development. This differential affinity hypothesis is implemented in the model and produces growth dynamics consistent with those observed in the cervical loops of mouse molars.

Instead of simulating the whole dynamics of spreading and condensation of the dental mesenchyme the current version of the model takes a much simpler approach. The borders of the tooth in the model, analogous to the cervical loops, have a tendency to grow following the interface between the dental and jaw mesenchyme. The more condensed mesenchyme there is, the less steep is that interface and the more the epithelium grows laterally. In the model I assume that Fgf-4 enhances mesenchymal proliferation [as has been shown by bead experiments (Jernvall *et al.*, 1994)]. In practice this is implemented by altering the direction of displacement by adding an

arbitrary downward vector and multiplying the x and y components of displacement by a factor proportional to mesenchymal growth (produced by Fgf-4). As in any other epithelial cell, however, the total amount of displacement is equal to $R_e(1 - d_i)$.

Mesenchymal growth is implemented as a pressure exerted in each epithelial cell in a direction normal to the apical surface of the epithelium. This force is proportional to the concentration of Fgf-4, to a model parameter specifying how strongly Fgf-4 promotes mesenchymal proliferation and to 1 minus the differentiation of the epithelial cell. This implementation assumes that proliferation in the mesenchyme produces local increases in its volume that exert a pressure in all directions. Pressures exerted downwards to the mesenchyme simply displace nondental mesenchyme and have no effect on the form of the epithelium. Then, only the pressures that are perpendicular to the epithelium surface need to be considered.

In addition to growth, cells interact by pushing each other if their centers become too close. This is meant to add physical realism to the model and can be visualized as the cells being united by an elastic spring. This simply simulates the inevitable physical resistance of cells to external pressures. A traction force that tends to decrease the distance between neighboring cells when they get very far away from each other is also implemented to provide physical realism.

Overall, all of these displacement equations are summed up in each iteration. It is assumed that the stellate reticulum exerts a pressure on the epithelia. Then, all displacements in its direction are discarded (this rarely happens however). In addition, knots are not allowed to move down because they are supposed to be attached to the stellate reticulum and they do not seem to move down in any species examined (Butler, 1956). Cells with odd shapes can produce, as in the case of finite element analysis, inaccuracy in the calculations, either on morphology or/and diffusion. The rules of division of large cells in model 2 ensure that there is a limited variation in cell size and shape and thus ensure the accuracy of calculations.

A special situation applies to the cells in the anterior and posterior borders. At these borders the cervical loops form very late while the oral epithelium is still extending anterioposteriorly. This seems to happen at different rates in the anterior and posterior border and this asymmetry seems to be responsible for the anteroposterior asymmetry of the molars. Thus, this asymmetry needs to be implemented in the model. However, the genetic or/and epigenetic interactions that give rise to this asymmetry are not known. The only thing that is known is that Bmp3 and follistatin, molecules that sequester Bmp2, Bmp4 and activin, are expressed in the anterior border of the first mouse molar at E14 (Åberg *et al.*, 1997; Wang *et al.*, 2004a). It is not clear how, or whether, that may affect the anteroposterior asymmetry. Due to this lack of specific understanding these biases are simply implemented as a factor, model parameters *bia* and *bip*, multiplying the displacement along the anteroposterior axis (this is the x coordinate) in the anterior, *bia*, and posterior, *bip*, borders of the tooth. Anterior border cells are those whose y coordinate is smaller (in absolute value) than a model parameter, AP, and whose x coordinate is larger than zero. Posterior cells are those whose y coordinate is smaller (in absolute value) than

AP and whose x coordinate is smaller than zero. In addition, the z coordinate (ventral direction) in both borders is multiplied by a factor, model parameter *bo*. This is used to phenomenologically describe the delayed formation of the cervical loops in the anterior and posterior borders and is assumed to be due to the existence of some incipient condensed dental mesenchyme through the anteroposterior axis along the invagination.

X. What Do Model Dynamics Reveal About Developmental Dynamics

Fig. 4 shows the changes in mouse *in silico* morphology over early tooth development from the initial conditions described. In spite of the simplicity with which the model deals with cell behaviors and gene interactions, the model provides a reasonable match with the three-dimensional changes in molar morphology and patterns of gene expression occurring during development. Moreover, these *in silico* morphological changes occur with a timing that is very close to the timing observed in mouse first molars. The model outcomes have not yet been compared with later stages because in mouse molars cusps exhibit, after E15, an anteroposterior bending that cannot be explained from present hypotheses.

Although the model can produce a tooth shape that reasonably resembles that of the adult mouse, the model does not currently include any aspects of the mineralization process. Thus, although the morphology of the epithelium gives a clear outline of the final adult morphology, the deposition of enamel may slightly modify that outline. This is specially the case in species which, unlike mouse, have substantially thick enamel. In general, more enamel deposition (Avishai *et al.*, 2004) makes cusps to appear relatively blunter.

Three points are worth considering in evaluating the validity of both models:

1. The models, implementations and parameters come from some understanding and hypotheses about the genetic and epigenetic interactions involved in tooth development. Thus the models are not statistical models that fit some parameters to a given target. Different morphologies can be produced by changing the parameters of the model but the model cannot be fit to any arbitrary morphology nor do the parameters keep their biological validity for all values. Both models are also able to produce tooth morphologies, and its change during development, of other species, for example: voles, seals and early tribosphenic mammals. Overall these are first (developmental) principles models where morphological changes are predicted from hypotheses and assumptions. More generally, however, these models provide a tool with which these hypotheses can be tested by comparing their morphological consequences in the model with real morphological variation arising from artificial manipulation of tooth development or arising in evolution. Thus the hypotheses of the models can be changed or revised according to how well they ex-

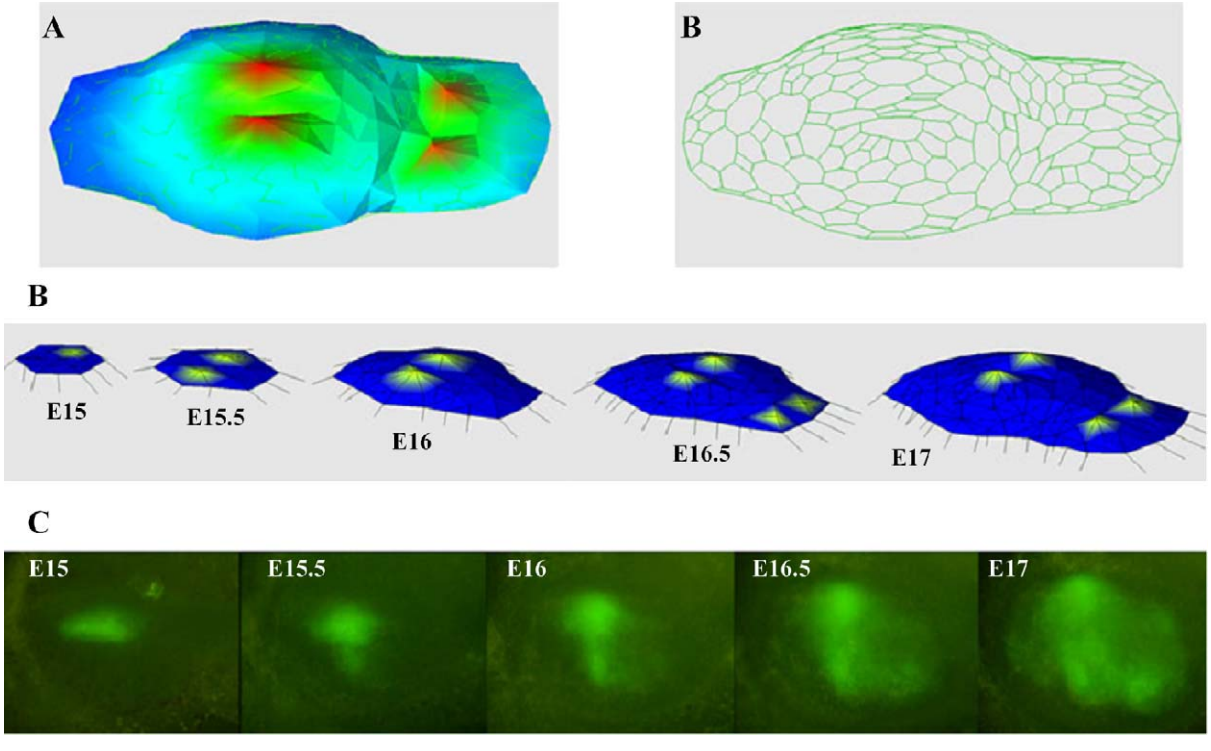

Figure 4 The figure shows in (A) an outcome of the model 2 for a middle stage in tooth development. In color it is plotted the concentration of Fgf-4 in the inner enamel epithelium (red is the highest concentration and blue is the lowest). In (B) the contours of inner enamel epithelium cells are plotted. In (C) several time slides of the outcomes of model 2 are plotted. In (D) it is shown the *in vitro* first molar development from embryonic day 15–17. The green color indicates the presence of fusion protein between GFP and Shh (in a heterozygous mouse) and thus indicates the spatial distribution that Shh may exhibit. See color insert.

plain new morphological variation. This has, in fact, been done to develop model 2 from model 1 (Salazar-Ciudad and Jernvall, 2002).

2. The morphological outcome of the models arises from the dynamics of the model. In other words, all the parameters and hypotheses of the models (except for the anteroposterior asymmetry) are at the low level of genetic and epigenetic interactions but explain high level phenomena such as the overall morphology of the tooth. This is performed without arguing for the existence of complex spatial prepatterns or genetic interpretations. In other words, nowhere in the models it is prespecified where knots are going to arise.
3. That the hypotheses of the models are consistent with mouse first molar morphology and development does not necessarily mean that the hypotheses of the models are correct. That would require the design and realization of further experiments on the bases of the models. In particular, other hypotheses could also explain the development of mouse first molar. In the next section model dynamics are discussed in detail and compared with tooth development to explain how specific hypotheses of model 2 produce specific outcomes and how alternative hypotheses can be discarded (or not).

A. The Formation of the Cusps

In model 2 Bmp2 signaling from the knot slows down proliferation rate in surrounding tissue by enhancing cell differentiation. This way cells around the knot are left behind by the rest of the epithelium that is dividing more intensively. This gives rise to a morphologically distinct cusp with slowly dividing cells near the top and the knot at the top. However, in the model, if Bmp2 secretion is totally inhibited cusps still form as long as there are nonproliferating knots. Without knots, proliferation in the epithelium, and the pushing between its cells, results, in the model, in the progressive flow of the epithelium towards the edges of the developing tooth (where the cervical loops are engulfing the dental mesenchyme). This leads to the formation of teeth that are nearly flat. This situation is also found in tooth tissue cultures in which a "tooth" without knots also forms (Iwatsuki *et al.*, 2006). The presence of a knot leads the epithelium to flow away from the knot in all directions. This mechanical effect is due to the nonproliferation of knots. This meant that the knot height does not change and that the knot is left behind as a morphologically distinct cusp. If two knots form close to each other, then the flow of epithelial cells from them leads to the formation of a valley in between. This is very similar to what happens in mouse molars and other teeth where valleys form between cusps. All these morphological changes occur without a prespecification, in the model, of where or how those cusps would form. The model simply specifies how individual cells push each other and how they respond to growth factors. In other words, the cusps simply emerge as a dynamic output of model 2. In that sense the model suggests a simple mechanistic basis for the folding of the epithelia.

Although the model suggests that Bmp2 (and its differentiation enhancing effect) may not be strictly required for cusp formation, the absence of Bmp2 leads to cusps that are much sharper than in mouse first molar. Bmp2 increases cusps bluntness by producing a gradual, rather than sharp, extension of the area around the knots where proliferation is decreased. A similar effect is produced by mesenchymal growth. These hypotheses about the effect of differentiation and mesenchymal growth on cusps sharpness could be further tested by specifically inhibiting Bmp2 (and other BMPs with possible redundant functions as Bmp4) expression in the knots. Similar experiments could be performed for Shh. These manipulations could be rather complex because these genes are extensively used through development. Conditional knockouts or/and overexpression transgenes would then be required. Ideally, manipulations that produce gradual changes in the expression of these genes from the knots would be much more informative.

In model 2 the formation of valleys between cusps also produces, indirectly, the movement of cusps away from one another (for the mouse mainly away from the midline buccally and lingually). For the stages considered in Fig. 4 this pushing is relatively small but increases at later stages. A similar displacement has been observed for mouse molars (Jernvall *et al.*, 2000). In the model this seems to occur by the simple traction of the knots by the epithelium flowing towards the cervical loop and by the pushing of the epithelium flowing towards the valley side (while the invagination of this valley is taking place). Then, again, the model suggests a simple explanation for complex morphogenetic phenomena on the bases of simple developmentally grounded cell to cell interactions.

The hypothesis that nondiving cells are responsible for the bending of the epithelium and the formation of cusps is technically more difficult to test because it is a biomechanical explanation that is not so readily approachable from the molecular techniques most commonly used in developmental biology. Ideally, this hypothesis could be tested by inhibiting proliferation in specific locations in the epithelia. However, the enamel epithelium is surrounded, even *in vitro*, by the vacuolated stellate reticulum and it is thus difficult to manipulate mechanically. Local administration of cell division inhibiting substances is, then, also difficult. This problem could be overcome by the design of transgenes in which a cell division inhibiting construct could be expressed in a specific area of the epithelium. However, as described, gene expression in the epithelium is either around the knots, in the whole epithelium or away from the knots. Thus it is difficult to target gene expression to arbitrary parts of the inner enamel epithelium for the stages considered in the model. A viable alternative would be a construct that is expressed in the enamel epithelium in random spots after some heat-shock or other signal is provided (as achieved by Zong *et al.*, 2005, for the cerebellum of mouse). That way proliferation could be inhibited in areas of the inner enamel epithelium where there are no knots. If the inhibition of proliferation is responsible for the formation of cusps these transgenes should produce a cusp under each nonproliferative zone in the enamel epithelium. The overall morphology and the positions of valleys and cusps should then be predictable from the model (for a given

random distribution of nonproliferative zones). If that is not the case, then it is likely that something else (like some mechanical attachment of nonproliferative zones to the stellate reticulum or some signaling from the knot) is also required to explain cusp formation.

As mentioned above, bead experiments (Jernvall *et al.*, 1994) indicate that Fgf-4 secreted from the knots may induce proliferation in the enamel epithelium. However, if this proliferation-enhancing effect is concentration dependent, then the higher rates of proliferation should be encountered at medium distances from the knot. In other words, at short distances from a knot the differentiating effect of Bmp2 dominates while at large distances Fgf-4 concentration would be too low to induce strong proliferation. This spatial distribution of proliferation intensity (from the knots to the cervical loops) does not produce, when implemented in the models, cusp morphologies consistent with those found in most mammalian teeth. Nor does information about the distribution of proliferation rates (Jernvall *et al.*, 1994) support the existence of maximal proliferative rates at medium distances from the knots. This suggests that either the proliferative effect of Fgf-4 is not concentration dependent (cell proliferation would occur at a fixed rate after a Fgf-4 concentration threshold is reached or Fgf-4 concentration in the enamel epithelium would rapidly saturate its receptors in epithelial cells) or, as assumed in here, the proliferative effect of Fgf-4 is much stronger in the mesenchyme. If the model is changed to allow Fgf-4 to activate epithelium proliferation in a nonconcentration-dependent manner then the range of morphologies found is very similar to the ones found in the current version of the model (although the actual parameter combinations in which specific morphologies arise changes).

B. The Positioning of Knots

In mouse molars, as well as in many other rodent molars, several cusps have, roughly, the same height. Taking into account that knots form at a distance from each other and that the knots rapidly become higher than the surrounding proliferating epithelium it would seem difficult to explain how that happens. This may seem inconsistent with what is expected from a reaction diffusion model. In reaction–diffusion models the distances between peaks of activator concentration tend to be constant (over a field of cells) and depend on the several kinetic parameters (such as the diffusion rates and the inhibition of activator production). However, in model 2, the initial asymmetric expression of Bmp4 in the borders of the developing tooth results in the second knot forming very close to the first one. The second knots forms close to the side where Bmp4 is expressed less strongly (lingual side). This happens early, before too much growth occurs. In mouse first molar (see Fig. 4D) the second knot also forms on that side and before too much growth occurs. This predicts that in species where the second knot forms more anteriorly or posteriorly than the first knot, the Bmp4 bucolingual asymmetry would be smaller or/and the early bud stages in which the second knot

forms would be narrower than in mouse. Both situations seem to apply to the vole (*Microtus rossameridionalis*), another species of rodent (Keränen *et al.*, 1998).

In the mouse first molar the third and fourth knots form at the same level in the anterior–posterior axis. This pattern seems to require some specific positioning of the Bmp-4 sources. As mentioned, Bmp4 is expressed in the borders of the tooth throughout tooth development. This means that new knots tend to form towards the borders of the tooth (and away from existing knots). Without this external source of Bmp-4 the third knot forms, in model 2, where there is more space (and away from existing knots). That is, the third knot forms in the midline (in the buccolingual axis) in the posterior part of the tooth. The fourth knot forms then much posteriorly and the resulting tooth morphology does not resemble that of the mouse first molar. The Bmp4 expressed in the borders results in the third knots forming close to the buccal border (Bmp4 border expression is larger: $bib > bil$) and a fourth knot can form close to the lingual border (and that same anterior–posterior level) as occurs in the mouse first molar.

In mouse first molar the distance from the first knot at which the second knot forms is much smaller than the distance from the first knot at which the third and fourth knots form. This seems to be due, as will be discussed in following sections, to the morphodynamic interrelationship between signaling and growth.

C. Cervical Loop Growth

Other than the hypothesis implemented in the current version of the model there could be other hypotheses explaining the bending and growth pattern of cervical loops. It could be that the cervical loops would bend due to differential growth rates between inner and outer enamel epithelium. It could also be that the dental mesenchyme exerts some mechanical pressure to the epithelium that is larger near the knots and produces, then, a tooth that is wider near the knots and narrower near the cervical loops. As a side effect of that the cervical loops would seem to bend towards the midline. With the model I have explored these possibilities. Differential growth between inner and outer epithelium is unlikely to be responsible for the morphological changes occurring in the cervical loops. Proliferation in the outer enamel epithelium seems to stop by E15. In addition, when the model included extensive outer enamel epithelium proliferation the tooth primordium developed into an inflated spherical balloon-like shape that did not resemble tooth primordia morphology at early or late stages. Mesenchymal growth is, as described, implemented in model 2. The vacuolization of the stellate reticulum seems to be stronger near old knots and away from the tips of the cervical loops. Increased vacuolization may imply higher hydrostatic pressure. This suggests that mesenchymal growth would more efficiently push the epithelium near the cervical loops (where the stellate reticulum is less developed). When that is implemented in the model the loops do not tend to bend inwards medially but outwards laterally. It

is possible that the mesenchyme exerts some pressure on the epithelium but the previous arguments and simulations suggest that it may not be able to explain, by itself, the bending growth trajectory of the cervical loops. Thus, at present, the differential adhesion hypothesis implemented in the model is the only one that seems consistent with the cervical loop growth pattern observed mouse molars. This hypothesis could be further tested by experimentally manipulating the amount or adhesiveness of the dental mesenchyme. For example, specific inhibition of proliferation in the dental mesenchyme (locally) or experimental subtraction of part of it should lead to steeper cervical loops. These latter experiments are under way in our laboratory.

D. Morphodynamics

The fact that signaling and cell movement is happening at the same time has some important consequences for the dynamics of the model. In reaction–diffusion models the distance between knots (or activator peaks) depends on several kinetic parameters such as the diffusion rates and inhibitor and activator productions rates. The inclusion of growth and its interdependence with signaling means that knots can appear at a larger range of distances. Moreover, this interdependence affects where new knots form. First, new knots will appear where there is (epithelial) space for them. That depends on the growth biases (*bil* and *bib*) but also on the overall proliferation rates and relative rates of growth of the epithelium and mesenchyme. Since molecules are leaving the tooth from its borders the shape of those new areas, and not only their size, affects whether, and when, Bmp4 concentration can reach the threshold value to produce a knot. Thus, for example, slender epithelial contours with a high ratio between border perimeter and surface tend to produce more outflow of inhibitor and more inflow of activator from the tooth (and then knot formation).

In addition, the volume enclosed by tooth morphology also affects the concentration and dilution of growth factors. Thus, sharp cusps produce, because of smaller volume relative to blunter cusps, a smaller dilution of inhibitor and thus new knots form at larger distances from the knot in the tip of the cusp (although that also depends on the relative rates of diffusion of the inhibitor and activators). Blunter cusps produce the contrary effect and in general the local morphology of the tooth at each time point affects the patterns of diffusion of all the growth factors and thus subsequent morphological changes. Since growth factors also affect local growth in the epithelium and mesenchyme there is a strong interdependence between the effects of model parameters affecting signaling and model parameters affecting growth. Extensive model simulation has shown that this interdependence between growth and signaling allows for a larger spectrum of tooth morphologies compared with a situation where both things would act independently (Salazar-Ciudad and Jernvall, 2004). At the same time, however, this means that some small changes in one parameter can affect overall tooth morphology and that other parameter changes in model parameters may have a modest effect in morphology. Overall, morphodynamic mechanisms

produce a complex relationship between genetic and morphological variation. This produces developmental dynamics, both in the model and in the organism, that are more difficult to understand in comparison with a nonmorphodynamic, morphostatic, situation (Salazar-Ciudad and Jernvall, 2004). In that sense mathematical modeling provides a useful tool to identify and study these complex dynamics.

E. Developmental Dynamics and Tooth Diversity

This review has explained how current experimental evidence in tooth development can be integrated into a predictive coherent model. The current and previous models have been shown to be able to produce the forms of mouse first molars and also many other teeth (Salazar-Ciudad and Jernvall, 2002, 2004, 2006). In that sense the model should be able to generate some hypotheses about which developmental changes are responsible for the generation of the morphological diversity of mammalian teeth. For example, as few as two changes in model parameters can transform a mouse first molar into a vole first molar (Salazar-Ciudad and Jernvall, 2002). In general by simply changing model parameters it is possible to produce a large diversity of mammalian-looking teeth. My hypothesis would then be that most mammalian tooth morphologies would be possible by tinkering with those parameters. Since most of these parameters have clear molecular bases the model can offer some hints about what genetic changes may be involved in the morphological differences between species or within populations. Of course, to the extent that the model is a necessary simplification of reality it may underestimate the number of parameters. In addition, our incomplete understanding of tooth developmental dynamics may conflate several parameters and changes in parameters with small changes in gene network topology. In that respect the present model should be considered as an identification of the basic possible developmental logic of tooth morphogenesis.

Studies in the previous version of the model have shown that the model is also able to predict the morphology of a mutant (Järvinen *et al.*, 2006). That mutant is a genetic construct in which β-catenin is under the regulation Keratin-14 promoter and thus canonical Wnt signaling is constitutively activated in the whole dental epithelium. Wnt is not included in the model but its effect has been suggested to be mediated through an increase in activator production. In that way the role of a gene not yet directly included in the model can be explored through its effect in some of the existing model parameters and its effect on the whole dynamics of tooth development.

Model 2 has also been shown to be able to produce the morphology of ringed seal (*Phoca hispida*) premolars (seals tend to be homodont), its change during development and the main patterns of morphological variation found in their populations (Jernvall and Salazar-Ciudad, 2007).

Most of the model parameters (all except *bip* and *bia*) are based on genetic or cellular features of tooth development and probably have a genetic basis. Variation in some of these parameters, like the diffusion rates, may arise from genetic variation in

a single gene (or few genes). These could be genes such as the growth factor themselves (although chemical modification of these proteins may also affect their function and diffusion rates and then involves variation in other genes). Functional constraints on protein structure (like, for example, to be able to bind to its receptor) may result in numerical variation in these parameters being only possible in some ranges. Other parameters, like the efficiency of Fgf-4 in promoting dental mesenchyme proliferation R_m, could change due to variation in several genes (for example, in all the genes involved in the signal transduction of Fgf-4 in dental mesenchymal cells). Parameter changes, however, do not affect the topology of the gene network considered in the model (except for the trivial case that two genes stop interacting because their interaction strength is close to zero) nor which cell behaviors are regulated by growth factors, and how. These are aspects of development that can also change over the course of evolution (although they may require more or larger changes at the genetic level) and thus some tooth morphological transitions may be explainable by these kinds of changes. This view contrasts with claims that the diversity of teeth in a heterodont individual (like, for example, mouse) comes from the differential expression of transcriptional factors that precisely control the development of each tooth type (McCollum and Sharpe, 2001). This view is not very explicit about the mechanisms by which that may happen but it seems to favor a complex genetic hierarchic regulation ("control") of several unspecified morphogenetic events by each of these transcriptional factors. However, it may be easier to change, by random genetic variation, the strength by which a gene interacts with some others than the identity of the genes with which a gene interacts (Salazar-Ciudad, 2006). This suggests that morphological evolution may often proceed by changes in developmental parameter values (or to genetics changes leading to small topological changes) rather than by complex changes in network topologies (although that may of course also occur). In contrast, it would be simpler to suggest that the transcriptional factors regulating each tooth type act by simply regulating some gene products so as to change one or several of the developmental parameters (as in the model). That could involve, for example, subtle regulatory changes such as quantitative increases in the expression of a receptor, signal, signal transducer, or/and adhesive molecule.

XI. Tooth Model in Comparison to Other Models of Organ Development

With few exceptions (Goodwin and Trainor, 1985; Dillon *et al.*, 2003), mathematical and computational models of morphogenesis and pattern formation in animal development do not explicitly consider the interdependence between signaling and cell movements. There are, however, some other models that consider how signaling affects growth (Miura *et al.*, 2006) and also some models in which signaling and growth happen at the same time but signaling does not affect growth (Meinhardt, 1982, 2007; Harris *et al.*, 2005). There are also some few reaction–diffusion

models on teeth. These consider, however, the relative positioning of teeth in the jaw in Alligator (Kulesa *et al.*, 1996a, 1996b; Murray and Kulesa, 1996) and not teeth morphology as in this review. Very few mathematical models of development give as an outcome complex three-dimensional morphologies and patterns of gene expression as the current and previous versions of the tooth morphodynamic model (Salazar-Ciudad and Jernvall, 2002). Some of the models that consider cell movements and signaling use a different simulation approach than the one presented in here. In Glazier's model (Glazier and Graner, 1993) cells are composed of elements that move in a prespecified rectangular grid. These elements move to neighbor grid positions depending on the energy change they would produce with the change. This mainly relates to the adhesive context on these grid positions. This approach has been successful in simulating several developmental systems (Chaturvedi *et al.*, 2005; Merks *et al.*, 2006) and is probably applicable to many more. Since each cell needs to consist of several of these elements, with increasing numbers of elements, cell behavior is more accurate and more computation is required per cell compared to my model. This model inevitably introduces a discretization of space while model 2 does not. More importantly model 2 may be more able to deal with mechanical changes occurring in tightly linked cells and only slightly deformable groups of cells (like in the invagination of epithelia or the traction of one tissue by another, as in this model). In contrast, my approach cannot deal, as these other approaches do, with phenomena in which cells dramatically change their neighborhood (like in adhesive cell sorting). In that sense this model provides a different approach to the simulation of organs development.

XII. Concluding Remarks

In summary this review has described a number of hypotheses about how tooth morphology develops from genetic and epigenetic interactions. By implementing these hypotheses into a mathematical developmental model the capacity of these hypotheses to produce the developmental and morphological transformation observed in mouse first molar have been evaluated. The studies and models here reviewed provide developmental accounts for morphological changes that are gradual and complex. In other words, these studies do not focus on gross and discrete morphological alterations of morphology to identify the genes involved in development. Instead, the focus, and the model predictions, is on complex multivariate gradual changes and on how small genetic variation regulates these morphological changes. The models have been used as a framework to compare different hypotheses in developmental biology. In this way I have reviewed our current understanding of tooth morphogenesis and outlined possible future lines of investigation in which this understanding can be improved. At the same time I have provided an example of how close collaboration between theoretical and experimental developmental biologists can give rise to operative models than

can be used as reference for discussion and evaluation of hypotheses, experiments and computational implementations of organ development.

Acknowledgments

I thank Bruno Julia, Janne Hakanen, Alistair Evans, and Jukka Jernvall for comments and Juselius foundation (Helsinki, Finland) for funding.

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