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# Manipulation expérimentale de la dent et de l'os à travers une fenêtre osseuse dans la mandibule de rat.

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Ce mémoire intitulé:

**Manipulation expérimentale de la dent et de l'os à travers une fenêtre osseuse dans la mandibule de rat.**

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# Sommaire

Le but de notre travail est de mieux comprendre les phénomènes cellulaires et matriciels impliqués dans la formation des tissus calcifiés. Afin d'intervenir sélectivement sur l'organe de l'émail, un agent expérimental toxique à l'échelle systémique a été administré à travers une fenêtre osseuse dans la mandibule de rat. Des techniques de cytochimie avec des lectines et des anticorps contre les protéines de l'émail et l'albumine ont été utilisées pour caractériser la matrice extra-cellulaire. Un nouvel anticorps dirigé contre l'amélogénine principale a été préparé. Cet anticorps, appelé M179y, nous permet de détecter très spécifiquement les formes sécrétrices de l'amélogénine. Des informations sur le rôle et la fonction de la lame basale qui sépare les améloblastes de l'émail en cours de maturation ont été ainsi obtenues. Nos travaux démontrent qu'une rupture de l'intégrité de la lame basale, générée expérimentalement par blocage de la N-glycosylation, nuit grandement à son rôle de filtre des protéines exogènes à l'émail comme le montre la présence d'albumine dans la matrice amélaire. Notre modèle chirurgical s'est avéré également très utile pour étudier l'influence de l'administration d'un extrait de facteurs de croissance plaquettaires sur l'os en périphérie de la zone de trépanation trans-corticale. Les résultats prometteurs dans ce domaine nous encouragent à approfondir l'observation au niveau ultra-structural et à examiner le rôle inducteur de ces facteurs sur la morphogenèse et le développement dentaires. En conclusion, cette approche très ciblée pourrait être employée pour évaluer l'efficacité et la performance de vecteurs viraux de transduction et, pour des études de "knock-in" et "knock-out" de gènes, visant à clarifier le rôle des différentes protéines matricielles dans la morphogenèse et la physiologie des tissus calcifiés.

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# LISTE DES SIGLES ET DES ABREVIATIONS

| | |
|---|---|
| AMBN | améloblastine (ameloblastin) |
| AMEL | amélogénine (amelogenin) |
| AMELy | anticorps anti-amélogénine |
| ARN | acide ribonucléique |
| BL | basal lamina |
| BM | basement membrane |
| BSP | sialoprotéine osseuse (bone sialoprotein) |
| °C | degré Celsius |
| EDTA | acide éthylène diamine tétra-acétique (ethylene diamine tetraacetic acid) |
| EO | organe de l'émail (enamel organ) |
| EPs | enamel proteins (protéines de l'émail) |
| HPA | agglutinine de *Helix Pomatia* (*Helix Pomatia* agglutinin) |
| hr | heure (hour) |
| kDa | kiloDalton |
| kV | kiloVolt |
| LB | lame basale |
| M | mole |
| M179y | anticorps de poule anti-amélogénine de souris (chicken anti-mouse amelogenin antibody) |
| µg | microgramme |

| | |
|---|---|
| µl | microlitre |
| µm | micromètre |
| mg | milligramme |
| ml | millilitre |
| mm | millimètre |
| mvB | corps multivésiculaire (multivesicular body) |
| N | noyau (nucleus) |
| NaOH | hydroxyde de sodium (sodium hydroxide) |
| nm | nanomètre |
| OPN | ostéopontine (osteopontin) |
| PBS | tampon de sel de phosphate (phosphate buffered saline) |
| PBS-Oval | tampon de sel de phosphate avec ovalbumine |
| rER | réticulum endoplasmique rugueux (rough endoplasmic reticulum) |
| RNA | ribonucleic acid |
| Sg | granule de sécrétion (secretion granule) |
| TEM | transmission electron microscopy (microscopie électronique à transmission) |
| WGA | agglutinine du germe de blé *Triticum vulgaris* (wheat germ agglutinin) |

**"Quelli che s'innamoran di pratica sanza scienza,**
**son come 'l nocchiere ch'entra in navilio sanza timone o bussola,**
**che mai ha certezza dove si vada"**

(*Leonardo da Vinci, Pensieri, 10*: Dell'orror di quelli che usano la pratica senza scienza)

# Introduction

## Les tissus calcifiés dentaires

L'organe dentaire comporte trois tissus calcifiés: l'émail, la dentine et le cément. L'os alvéolaire environnant supporte la dent par l'intermédiaire du ligament parodontal. La formation de ces tissus résulte d'un processus complexe impliquant diverses activités cellulaires et des interactions entre les composants organiques et inorganiques de la matrice extra-cellulaire. Une comparaison systématique entre les évènements cellulaires et extra-cellulaires qui ont lieu pendant la formation des tissus calcifiés fait ressortir des similitudes dans leur formation (Nanci et Smith, 2000).

Les cellules responsables du développement des divers tissus calcifiés sont polarisées, favorisant une sécrétion vectorielle des constituants organiques de la matrice minéralisée. Ceux-ci s'accumulent en une pré-matrice non minéralisée qui devient calcifiée progressivement. Les tissus calcifiés à base de collagène ont un rôle de support et de protection des tissus mous et, dans le cas de l'os, de réservoir de calcium. La matrice organique de l'émail, tout comme la partie acellulaire et afibrillaire du cément, et contrairement aux autres tissus calcifiés, ne contient pas de collagène. Elle est composée essentiellement de protéines non-collagéniques (Fincham *et al.*, 1999) et on y trouve des traces de lipides, dont des phospholipides (Goldberg et Boskey, 1996). Cependant, l'émail peut davantage être considéré comme couche protectrice de la couronne dentaire, très résistante à l'abrasion et aux contraintes occlusales. L'émail ne peut se régénérer en cas d'altération ou d'agression et, afin de compenser cette limitation inhérente, il est formé d'une organisation tissulaire complexe et d'un taux de minéralisation très élevé qui en fait le tissu le plus dur de l'organisme puisqu'il contient,

dans sa forme mature, plus de 95% de minéraux, peu d'eau et seulement quelques traces de matrice organique.

**Amélogenèse**

La particularité de l'émail est en partie liée à son origine épithéliale et à la nature des améloblastes, cellules responsables de la création et du maintien d'un environnement extra-cellulaire propice à la formation de l'émail et qui expriment différentes protéines non-collagéniques contrôlant l'apposition de la phase minérale. Cependant, contrairement à la dentine et à l'os, l'émail ne possède pas de couche distincte de matrice non-calcifiée (ostéoïde, prédentine), qui s'interpose entre la cellule et le tissu minéralisé (Nanci et Smith, 2000). Trois stades peuvent être schématiquement considérés au cours de l'évolution des améloblastes: pré-sécréteur, sécréteur et post-sécréteur (Sasaki *et al.*, 1990; Warshawsky et Smith, 1974).

L'améloblaste pré-sécréteur se différencie et développe les organites impliqués dans la synthèse et la sécrétion des protéines de l'émail qui débute d'ailleurs déjà à ce stade (Nanci *et al.*, 1989a; Nanci *et al.*, 1998). Puis, au moment de la sécrétion (ou de formation), l'améloblaste développe à sa surface apicale une extension cellulaire, appelée prolongement de Tomes, qui serait en partie responsable de l'organisation tridimensionnelle de l'émail. Durant cette phase de formation, les diverses protéines matricielles sont activement sécrétées de façon "constitutive" (Nanci et Smith, 1992), c'est-à-dire que les améloblastes n'accumulent pas leurs granules de sécrétion, comme les glandes salivaires ou le pancréas, et la libération de leur contenu se fait de façon

continue; dès lors, la couche entière de l'émail se forme (Boyde, 1989; Frank et Nalbandian, 1967; Nanci *et al.*, 1989a; Nanci et Smith, 1992; Sasaki *et al.*, 1990).

Les améloblastes, en cours de maturation, subissent d'importantes réorganisations morphologiques: ils deviennent plus courts et larges et perdent le prolongement de Tomes (Sasaki *et al.*, 1990; Smith et Nanci, 1995; Warshawsky et Smith, 1974). La morphologie de leur surface varie cycliquement d'un état plissé (80% de la vie d'un améloblaste) à un état lisse ("modulation"), afin de créer un environnement propice à l'apposition du minéral (Smith, 1998). Au cours de cette alternance, le complexe de jonction apicale passe d'un état étanche, à un état perméable, permettant le "flushing" du milieu dans lequel baignent les cristaux. Par ce mécanisme, la phase de modulation permettrait également à certains composants du liquide interstitiel d'entrer dans l'émail pour contribuer à la neutralisation du milieu (Nanci et Smith, 1992). Une fois que l'émail est complètement mature, les améloblastes cessent leur "modulation" et régressent; leurs organites cellulaires diminuent progressivement et se réorganisent.

Contrairement à l'os, pour lequel les cellules responsables des phases de formation (ostéoblastes) et de destruction (ostéoclastes) sont originaires de deux lignées cellulaires distinctes, dans l'émail, seuls les améloblastes matures créent un milieu propice à la dégradation des protéines (Roodman, 1998; Smith, 1998). C'est ainsi que la plus grande partie de la matrice organique de l'émail sécrétée durant le stade de formation est dégradée par des enzymes et ensuite éliminée, au cours du stade de maturation, pour permettre la croissance en épaisseur et en largeur des cristallites (Smith, 1998). Cependant, étant donné que la croissance rapide des cristallites génère des protons qui

acidifient le milieu (Lin *et al.*, 1994; Sasaki *et al.,* 1987), les améloblastes libèreraient du bicarbonate pour, d'une part, maintenir un pH neutre, prévenant ainsi la dissolution des cristallites, et d'autre part, assurer une activité enzymatique à un niveau optimal (discuté par Smith, 1998).

Cette classification est arbitraire et schématique et il est maintenant généralement admis que les améloblastes sont capables de sécréter et dégrader simultanément des protéines pendant une grande partie de leur vie, de la pré-sécrétion à la maturation. C'est ainsi que la sécrétion prédomine durant la phase de formation et que la dégradation est plus importante au cours de la maturation (Nanci et Smith, 1992).

**Les Protéines de l'émail**

Les amélogénines et les non-amélogénines sont les deux principales classes de protéines matricielles amélaires isolées (Fincham *et al.*, 1999; Nanci et Smith, 2000;). En très grande majorité dans la matrice organique (90%), les amélogénines (<31 kDa), sont basiques, hydrophobes et contiennent plusieurs isoformes dérivées de l'épissage différentiel de l'ARN messager. Ces protéines subissent peu de modifications post-traductionnelles mais possèdent des sites d'interaction avec le N-acetyl-D-glucosamine, qui pourraient jouer un rôle fonctionnel important. La classe des protéines non-amélogénines (>50 kDa) regroupe: l'améloblastine, également connue sous le nom de "sheathlin" et "améline", l'énaméline, une protéine sulfatée 65 kDa, et probablement la "tuftéline". Seule, cette classe de protéines semble être glycosylée (Cerny *et al.,* 1996; Fukae *et al.*, 1996; discuté par Smith, 1998).

L'améloblaste produit aussi des enzymes qui sont libérées dans la matrice extra-cellulaire et sont impliquées dans la modification et la dégradation extra-cellulaires des protéines de l'émail (Smith *et al.*, 1989a,b; Smith, 1998). Des métallo-protéinases, dont la MMP-20 (énamélysine), semblent tout d'abord impliquées dans la modification à court terme des protéines nouvellement sécrétées (Bartlett *et al.*, 1997; Fukae *et al.*, 1998). D'autres enzymes, de la famille sérine-protéinase, dont l'EMSP1, agiraient particulièrement durant la phase de maturation (Simmer *et al.*, 1998).

**Fonction des protéines de l'émail**

Les protéines de la matrice sont distribuées spécifiquement dans l'ensemble de la couche de l'émail (Nanci *et al.*, 1998). Les non-amélogénines sont concentrées près de la surface, au voisinage des améloblastes, dans la zone où les cristallites croissent en longueur. En revanche, la concentration des amélogénines est moindre près de la cellule, alors que ces protéines et/ou leurs produits de dégradation se retrouvent dans l'ensemble de l'émail en formation. En effet, les cristallites au stade initial de leur formation seraient entourés par des agrégats supra-moléculaires d'amélogénines que l'on appelle "nanosphères" (Fincham *et al.*, 1999). Compte tenu de cette répartition topographique et des caractéristiques biochimiques différentes entre les deux classes de protéines amélaires, une hypothèse fonctionnelle a été émise selon laquelle certaines non-amélogénines serviraient de promoteur et de guide pour la formation des cristallites, alors que les amélogénines en contrôleraient la croissance en épaisseur et préviendraient la fusion des cristallites pendant leur formation (Nanci et Smith, 2000).

**Analyse bibliographique des recherches antérieures.**

L'organe dentaire de l'incisive inférieure du rat, de par sa ressemblance morphogénique et structurelle avec la dent humaine (Warshawsky *et al.*, 1981), représente un excellent modèle pour étudier les divers évènements cellulaires et extra-cellulaires impliqués dans la formation des tissus calcifiés. Du fait de sa croissance continue compensatrice de l'abrasion régulière, l'incisive de rat permet également de retrouver toutes les étapes de la formation de l'émail dans une seule dent (Leblond et Warshawsky, 1979; Nanci et Smith, 1992).

Quelques modèles expérimentaux *in vivo* ont été développés, consistant en des injections ou des micro-injections de traceurs tels que l'albumine marquée au DNP ("dinitrophénol"), (Nanci *et al.*, 1996a), le HRP ("horseradish peroxidase") (Kallenbach, 1980; Sasaki *et al.*, 1983; Takano et Ozawa, 1980), le nitrate de lanthanum (Tanaka, 1980). Plusieurs études ont utilisé des médicaments qui inactivaient drastiquement la sécrétion des protéines, *in vivo* (Miake *et al.*, 1982; Nanci *et al.*, 1987; Nanci *et al.*, 1996b) et *in vitro* (Gonzales et Sartre, 1989; Lancaster *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989; Otani *et al.*, 1990). D'autres expériences consistaient en la création d'un abord chirurgical invasif près de l'organe de l'émail au stade de sécrétion/maturation, suivie par une micro-injection d'agents chimiques dans les tissus sous-jacents (Benson *et al.*, 1998; Eisenmann *et al.*, 1989; McKee et Warshawsky, 1984). Cependant, cette approche délabrante ne permettait, ni une étude dans des conditions physiologiques, ni un contrôle précis des doses nécessaires d'agent expérimental injecté *in situ*. En outre,

ces injections d'agents expérimentaux entraînaient des conséquences systémiques souvent toxiques pour l'animal.

La recherche se développe par ailleurs vers l'étude de la promotion des mécanismes responsables de la formation tissulaire. Nous savons aujourd'hui que nombre de facteurs de croissance sont impliqués dans la différenciation, l'activité et la mort cellulaire (Mohan et Baylink, 1991; Jilka *et al.*, 1998) et jouent un rôle dans le processus de réparation osseuse des tissus calcifiés (Bostrom, 1998) ainsi que dans la morphogenèse de la dent (Aberg *et al.*, 1997; Thesleff et Aberg, 2000). Nos connaissances tendent à tirer bénéfice de l'emploi de ces facteurs de croissance afin de simuler et stimuler les évènements impliqués dans le processus de minéralisation.

**Stratégie expérimentale**

Malgré de nombreuses connaissances sur le rôle régulateur de certaines molécules matricielles et des facteurs de croissance, il reste encore plusieurs incertitudes concernant les mécanismes moléculaires impliqués dans la formation des tissus dentaires calcifiés. Une approche expérimentale, récemment développée dans notre laboratoire, nous a permis une manipulation *in vivo* des évènements impliqués dans la formation des tissus dentaires (Vu *et al.*, 1999). Nous avons entrepris certaines expériences pour administrer différents agents expérimentaux à l'apex de l'incisive de rat ainsi qu'aux tissus avoisinants. Une fois la surface osseuse buccale de la branche mandibulaire de rats proprement exposée, nous avons localisé deux points de repère très importants: l'échancrure sigmoïde de la branche mandibulaire et la crête osseuse située en regard de

l'apex de l'incisive. A l'aide d'une pièce à main odontologique, une fenêtre chirurgicale est réalisée dans l'os alvéolaire. Celle-ci est localisée à environ 2mm de l'échancrure et est légèrement supérieure à la crête osseuse. Ensuite, une mini-pompe osmotique a été raccordée à la fenêtre osseuse par un cathéter pour l'administration des agents expérimentaux. Le grand intérêt de la mini-pompe réside dans la possibilité de délivrer les substances choisies de façon continue et contrôlée, pour une période de temps prédéterminée.

La sélection des substances que nous avons utilisées, a été déterminée à partir des résultats rapportés dans la littérature. Les améloblastes sont séparés de l'émail en voie de maturation par une lame basale (LB), dont la composition précise n'est pas encore bien déterminée (Nanci *et al.*, 1993), mais cependant reconnue riche en glycoconjugués (Nanci *et al.*, 1993). Elle a été caractérisée par microscopie électronique (Takano, 1979) mais aussi et surtout en utilisant des techniques de cytochimie avec des lectines (Nanci *et al.*, 1993). La LB disparaît pendant la phase de différenciation des améloblastes et une "autre" structure similaire se reforme ensuite, reproduite par les mêmes cellules au cours d'une étape plus avancée de leur développement. Nous avons donc choisi des agents qui inactivaient la glycosylation des protéines, afin de déterminer le rôle de la LB en terme d'adhésion des améloblastes à l'émail et/ou de filtration sélective au cours de la maturation (Nanci *et al.*, 1993). Il faut noter que dans certaines pathologies, comme l'hypocalcémie, la perméabilité de cette membrane est altérée et ceci mène alors à une présence anormale et massive dans l'émail d'une protéine plasmatique, l'albumine, ainsi qu'à une hypominéralisation de la matrice extra-cellulaire (Nanci *et al.*, 2000a).

Parmi les différents agents inhibiteurs existants, nous avons porté notre intérêt sur les molécules agissant sur la N-glycosylation des protéines puisque cette liaison est la plus fréquemment retrouvée dans les glycoprotéines des mammifères (Elbein, 1988). Il était par ailleurs important pour nos travaux que la/les substances envisagées soient très stables pour obtenir un effet conséquent au cours de l'administration avec la mini-pompe. L'inhibiteur de la formation des N-glycoprotéines que nous avons choisi est la tunicamycine (TM), un antibiotique qui a été utilisé surtout *in vitro*, pour déterminer le rôle de la glycosylation dans la formation de différents tissus. La tunicamycine peut causer certaines altérations neurologiques (Lin *et al.*, 1999; Pow et Morris, 1992) et affecter la formation de plusieurs organes (Ekblom *et al.*, 1979; Giraud et Franc, 1989; Kanai *et al.*, 1991; Yang et Hilfer, 1982; Webster *et al.*, 1988). Il a été montré qu'elle était également capable de provoquer une désorganisation des membranes basales dans différents organes (Kanai *et al.*, 1991), dont la dent (Thesleff et Pratt, 1980a,b). En outre, cette substance a des effets sur la différenciation et l'état des odontoblastes (Chardin *et al.*, 1989). Notre modèle expérimental a permis d'administrer, *in vivo*, des doses élevées de cet antibiotique, en agissant localement et spécifiquement sur l'organe de l'émail, évitant ainsi les effets systémiques.

Les secondes substances testées dans notre étude favorisaient la morphogenèse et la croissance de l'organe de l'émail ainsi que la réparation osseuse. Après une revue approfondie de la littérature sur les facteurs de croissance (Orsini et Nanci, 2000), il est apparu clairement que ces facteurs agissent en cascade et en synergie. Nous avons donc opté pour un mélange de facteurs dérivés des plaquettes porcines, afin de stimuler les tissus autour de l'apex de l'incisive du rat.

## Objectifs du projet

Le but principal de notre travail consistait à améliorer la technique chirurgicale pour accéder à la région apicale de l'incisive de rat et permettre l'administration d'agents expérimentaux. Le corollaire était d'examiner l'effet inhibiteur de la tunicamycine, distillée par la mini-pompe osmotique, sur la glycosylation et précisément son influence sur le respect de l'intégrité de la lame basale qui sépare les améloblastes de l'émail en phase de maturation. Nous avons également entrepris des études visant à analyser, dans les mêmes conditions d'accès expérimental, l'effet de mélanges de facteurs de croissance sur des cellules cibles de l'organe dentaire.

Le deuxième objectif de notre étude était de caractériser et de valider un anticorps polyclonal inédit et hautement spécifique, produit par le biais d'un système utilisant le jaune d'œuf de poulet, et préparé pour reconnaître dans les améloblastes et l'émail la principale isoforme de l'amélogénine.

## Méthodologie

Afin d'examiner les effets induits par la diffusion *in situ* d'agents expérimentaux sous contrôle d'une mini-pompe osmotique, à un rythme et pendant un temps déterminés, nous avons développé une méthodologie consistant en une analyse morphologique optique et électronique des changements structuraux. La cytochimie étant une sorte de "biochimie sur coupe", deux techniques de marquage à l'or colloïdal furent exploitées à l'échelle ultra-structurale: la cytochimie utilisant les lectines pour caractériser la

distribution des glycoconjugués de la lame basale et l'immuno-détection des protéines amélaires et de l'albumine au sein de la matrice amélaire.

**Organisation du mémoire**

L'introduction fait tout d'abord état des connaissances actuelles concernant la structure et les éléments matriciels de l'émail dentaire, pour ensuite expliquer et motiver nos objectifs d'étude. La deuxième partie du travail, présentée sous forme d'articles, rapporte les effets majeurs obtenus après l'administration de tunicamicyne, ainsi que la caractérisation d'un nouvel anticorps anti-amélogénine. Des annexes présentent ensuite une analyse et un argumentaire des différents aspects cognitifs et techniques de cette étude. Il détaille les éléments qui nourriront la discussion, l'évolution de projets expérimentaux ainsi que des résultats qui seront, très probablement, développés à la faveur de nouveaux travaux approfondis. Enfin la discussion développe les principaux résultats et extrapole les autres acquis préliminaires, en suggérant quelques voies prospectives de recherche.

# Chapitre 1

# Article I

**Localized Infusion of Tunicamycin in Rat Hemimandibles: Alteration of the Basal Lamina Associated with Maturation Stage Ameloblasts**

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**Abstract**

At the beginning of the maturation stage of amelogenesis, ameloblasts deposit a basal lamina (BL) at the interface between their apical surface and maturing enamel. This structure has been shown to be rich in glycoconjugates and proposed to exhibit adhesive and/or filtering functions. In order to clarify its role, we have applied a recently-developed surgical window model to administer locally tunicamycin (TM), an antibiotic which interferes with N-glycosylation. The hemimandibles of male Wistar rats were infused, using an osmotic minipump, with either TM or saline as a control. Lectin-gold cytochemistry was performed to reveal glycoconjugates in the BL. Immunogold labeling of enamel proteins and albumin was carried out to verify whether depletion of N-linked sugars in the BL affects the content and distribution of endogenous and exogenous proteins in the enamel layer. Under the influence of the drug, the BL became irregular and exhibited alterations in structural organization and composition. The number of *Helix pomatia* agglutinin binding sites was not significantly affected but their distribution was altered. The labeling density of wheat germ agglutinin over the BL was slightly reduced. Immunoreactivity for enamel proteins showed only a small decrease, but that of albumin, both between ameloblasts and within the enamel layer, increased significantly. No structural alterations were observed in the contralateral incisor and in other sampled tissues and organs. These results demonstrate that it is possible to achieve a localized administration of TM without systemic side effects and lend support to the proposal that the BL represents a specialized structure with filtering functions.

**Key words**: osmotic minipump, tunicamycin, basal lamina, glycoconjugates, enamel proteins, colloidal gold, lectin cytochemistry, immunocytochemistry, incisor, rat.

## Introduction

During the formative phase of amelogenesis, ameloblasts produce an extracellular matrix which regulates the formation of the extremely long enamel crystals (reviewed in Nanci and Smith 1992). Subsequently, during the maturation stage, most of this organic matrix is lost and the mineral content of the enamel layer increases to a level that is the highest among vertebrate mineralized matrices (reviewed in Smith 1998). The morphology of ameloblasts undergoes important changes throughout their life cycle (reviewed in Smith and Nanci 1995). In addition, a structure resembling a basal lamina (BL) appears at the interface between ameloblasts and the maturing enamel (Takano 1979; Nanci *et al.* 1993). Unlike typical basement membranes, this BL is enriched in glycoconjugates. In addition, immunolabeling for constituents such as laminin and type IV collagen is not as strong and convincing as that obtained over other dental basement membranes and remains to be clearly demonstrated (Nanci *et al.* 1993).

The BL associated with maturation stage ameloblasts has been proposed to provide the means for their hemidesmosomal attachment to the enamel surface (Takano 1979; Sawada *et al.* 1992; Nanci *et al.* 1993), and/or to act as a filter/barrier, controlling the influx and efflux of material during enamel maturation (Nanci *et al.* 1993). Although for a short period of time the enamel organ is leaky (reviewed in Smith 1998), it is generally believed that interstitial fluid molecules have a limited access into enamel (Chen *et al.* 1995; Nanci *et al.* 1996a). In this context, it has been shown that hypocalcemia alters the BL, leading to an accumulation of albumin (ALB) in maturing enamel and enamel hypoplasia (Nanci *et al.*

2000). It is thus likely that this specialized interface plays a critical role in enamel maturation.

To investigate the function of this glycoconjugate-rich BL, we have applied a recently developed experimental model to locally administer tunicamycin (TM), an antibiotic which interferes with glycosylation. The system consists of drilling a "window" in the alveolar bone overlying the apex of the rat incisor, and connecting to it an osmotic minipump (Vu *et al.* 1999). This model allows local infusion with relatively large concentrations of biological or chemical agents, over a defined period of time, without disturbing the whole-animal biology.

Tunicamycin inhibits the first step in the lipid carrier-dependent protein glycosylation (dolichol pathway), specifically preventing N-linked glycosylation (Takatsuki *et al.* 1975; Elbein 1988). It has been reported to affect morphogenesis and cell differentiation in various tissues and, notably, the organization of basement membranes (Hart and Lennarz 1978; Ekblom *et al.* 1979; Yang and Hilfer 1982; Giraud and Franc 1989; Kanai *et al.* 1991; Webster *et al.* 1993). Tunicamycin has been largely applied in vitro because it is highly toxic in vivo, causing major alterations of the nervous system (Gonzalez *et al.* 1981; Jago *et al.* 1983; Finnie and O'Shea 1988; Leaver *et al.* 1988; Pow and Morris 1992). To our knowledge only 3 studies have been reported in the tooth organ. This inhibitor of N-glycosylation alters basement membranes formation in tooth buds and blocks odontoblast differentiation, in vitro (Thesleff and Pratt 1980a,b). In vivo, it causes an accumulation of glycogen in some cells of the enamel organ and in young odontoblasts (Chardin *et al.* 1989).

In this study, the effect of TM on enamel maturation was examined at the light and electron microscopic level. Lectin-gold cytochemistry was performed to reveal and map the distribution of glycoconjugates. Immunodetection of enamel proteins (EPs) and ALB was carried out to verify whether N-linked sugar depletion in the BL structural components affects the content and distribution of endogenous and exogenous proteins in the enamel layer.

## Materials and Methods

### Surgical Procedure

Male Wistar rats weighing 100±10g (Charles River Canada; St-Constant, QC, Canada) were anesthetized with a 0.27 ml intraperitoneal (IP) injection of a 1:1:2 mixture of Hypnorm (fentanyl citrate and fluanisone; Janssen Pharmaceutica, Beerse, Belgium), Versed (midazolam; Hoffmann-LaRoche Limited, Mississauga, ON, Canada) and distilled water. An incision was made through the skin to access the muscle layer, according to an imaginary line joining the auditory meatus and the lip commissure. After separating the fibers of the masseter muscle and elevating the periosteum, the bony surface of the ramus was exposed. A slow-speed dental drill equipped with a carbide round burr size 0.14 (Brassler, Montreal, QC, Canada) was used to create a hole through the alveolar bone, under manual saline irrigation (Fig. 1A). The hole was drilled approximately 2 mm anterior to the posterior border of the ramus and slightly superior to the bony elevation overlying the apical end of the incisor. Another incision through the skin in the neck area was made to accomodate a 7-day Alzet osmotic minipump (model 2001D, Alza Corporation; Palo Alto, CA). The pump was tunneled into a subcutaneous pouch on the back of the animal and connected to the bony hole using a vinyl tubing and a metal catheter (Fig. 1B). The tubing was passed underneath the masseter muscle and through the neck area. Tissue adhesive Indermil (distributed by Sherwood Davis & Geck, St Louis, MO) and bone cement (Zimmer; Warsaw, IN) were used to help immobilize the metal catheter against the bone surface and maintain its tip in the hole. The animals were then sutured and the surgical site

was cleaned and disinfected with 70% ethanol. Some rats were administered Temgesic (buprenorphine hydrochloride; Reckitt & Colman Ltd., Hull, England) as an analgesic to control post-surgical pain. Five days after surgery, X-ray radiographs of the rat mandibles were taken to verify that the catheter was well in place (Figs 1C,D). Animal procedures and experimental protocols described above were in accordance with guidelines of the Comité de déontologie de l'expérimentation sur les animaux of Université de Montréal.

Preparation and Administration of Tunicamycin

Tunicamycin (Sigma Chemical; St Louis, MO) was first dissolved in 0.01M sodium hydroxide at pH 9 and then diluted to a final concentration of 10 μg/ml with 0.1 M phosphate buffered saline (PBS), pH 7.2 (Pow and Morris 1992). The solution was freshly prepared before use. Six rats were each implanted with an osmotic minipump filled with ~ 230 μl of TM solution. The minipumps were connected to a vinyl tubing, also filled with the drug, and soaked in sterile saline at 37°C for 1-3 hr before placement, as described above. Controls consisted of 3 rats implanted with the same minipumps filled with physiological saline (Sigma).

Tissue Processing

On the 8$^{th}$ day after placement of the minipumps, the animals were anesthetized with an IP injection of 0.25 ml of 20% chloral hydrate (Sigma) and sacrificed by intravascular perfusion through the left ventricle. The vasculature was prerinsed with lactated Ringer's

solution (Abbott Laboratories; Montreal, QC, Canada) for about 30 sec, followed by perfusion with a fixative solution consisting of 1% glutaraldehyde in 0.1 M sodium phosphate (PB), pH 7.2, for 20 min. Both hemimandibles were dissected out and immersed in the fixative overnight at 4°C. They were then washed in 0.1 M PB, pH 7.2, and decalcified either in 4.13% disodium ethylene tetraacetic acid (EDTA) for 21 days, at 4°C (Warshawsky and Moore 1967), or in a solution consisting of hexahydrate aluminum chloride, chloridric acid (10N), 88% formic acid (Plank and Rychlo 1952), diluted in 1:8 with distilled water, for 3 days, at 4°C. Segments of incisors containing the early and mid maturation stage were prepared using a molar reference line (Smith and Nanci 1989). Each segment was split in half along their length using a double-edged razor blade (Smith 1974) and washed for 2 hr in 0.1M PB. All hemisegments were then dehydrated in graded alcohols and embedded in LR White resin (London Resin, Berkshire, UK). The contralateral hemimandible, liver, duodenum, kidney, and parotid gland were similarly processed for embedding in LR White resin.

Each tooth segment was oriented for sectioning along its longitudinal axis. One µm-thick sections were cut with glass knives on a Reichert Jung Ultracut E ultramicrotome and stained with toluidine blue. Thin sections of selected areas were then prepared with a diamond knife and mounted on 200-mesh nickel grids having a carbon-coated Formvar film. The sections were then processed for postembedding lectin-gold cytochemistry for detection of glycoconjugates (reviewed in Benhamou 1989), and for colloidal gold immunocytochemistry (reviewed in Bendayan 1995) for detection of EPs and ALB. All grids were stained with 4% aqueous uranyl acetate and lead citrate for examination in a

JEOL JEM-1200EX-II transmission electron microscope operated at 60 kV.

Lectin Cytochemistry

*Helix pomatia* agglutinin (HPA), specific for N-acetyl-D-galactosamine (GalNAc), and wheat germ agglutinin (WGA, *Triticum vulgaris*), specific for N-acetyl-glucosamine (GlcNAc)/N-acetyl-neuraminic acid (NeuNAc) were used (Roth 1983; Nanci *et al.* 1993). Sections were first incubated for 1 hr with the native lectin (30 µg/ml 0.01M PBS; Sigma) and washed with 0.01M PBS. Binding sites were then revealed by incubating for 1 hr with anti-HPA or anti-WGA antibodies (Inter Medico, Markham, ON, Canada), diluted 1:500, followed by protein A-gold for 30 min. The protein A-gold was prepared as described in Bendayan (1995). Grids were then washed with PBS followed by distilled water. Controls consisted of incubating the tissue sections with the native lectins in the presence of 0.2M of their respective competing saccharide (Sigma). All the incubations were carried out at room temperature.

Immunocytochemistry

For the immunolabeling of the two main classes of EPs, amelogenin and nonamelogenin (see Fincham *et al.* 1999), sections were blocked by floating 15 min. on a drop of 0.01 M PBS containing 1% ovalbumin (Oval; Sigma). Briefly, for the amelogenin class, sections were transferred either on a drop a chicken egg yolk antibody (Gassman *et al.* 1990) raised against 24-kD rat amelogenin (AMELy) (Chen *et al.* 1995; Nanci *et al.* 1996b), diluted

1:150 for 3 hr, or against mouse recombinant 179 amelogenin isoform (M179y) (Orsini *et al.* 2000), diluted 1:100 for 3 hr. They were washed with PBS, refloated on PBS-Oval and then incubated for 1 hr with a rabbit anti-chicken IgG antibody (diluted 1: 2000) (Cappel Research Products; Scarborough, ON, Canada). Finally, grids were rewashed with PBS, blocked with PBS-Oval, and incubated with protein A-gold complex for 30 min. For ameloblastin (AMBN, a nonamelogenin), sections were incubated for 1 hr with anti-rat recombinant AMBN antibody diluted 1:20, as recently described (Nanci *et al.* 1998), followed by protein A-gold complex for 30 min. For the immunodetection of ALB, sections were incubated for 1 hr with rabbit anti-rat albumin (Cappel), diluted 1:80, followed by protein A-gold for 30 min. After immunolabeling, the grids were extensively washed with PBS followed by distilled water. Controls consisted of incubations with the secondary antibody and protein A-gold, or protein A-gold alone. All incubations were carried out at room temperature.

## Results

### Macroscopic Observations and Light Microscopy

Appropriate positioning of the bony window was confirmed on X-rays and by visual inspection at time of dissection (Figs. 1C and 1D). Continous exposure of the dental organ to TM for 7 days affected tooth eruption such that the treated incisor was about 0.5 to 1 mm shorter than the contralateral one (Fig. 2B). None of the rats implanted with saline minipumps showed a notable difference in length between the two incisors (Fig. 2A). In some cases, a slight erosion was noted surrounding the hole, probably due to the combined inflammatory effect of the adhesives used.

Histologically, in early to mid maturation, there was no significant alteration of the enamel organ (Fig. 3A). However, in some regions, groups of odontoblasts appeared to have degenerated (Fig. 3B) or to have lost their secretory polarity (Fig. 3C). In the latter case, the predentin layer was thickened (Fig. 3C). Dentin consistently exhibited a differential metachromatic staining pattern at about half its thickness (Figs. 3A and 3B). None of the above cell and matrix alterations were observed in control rats. Duodenum, kidney, liver and parotid cells of treated and control animals showed no significant structural alterations.

### Ultrastructural Observations

The BL in incisors from hemimandibles infused with saline (Figs. 4A and 5A), as well as

from contralateral teeth from both saline and TM infused rats showed structural characteristics similar to those previously described in the incisor of normal animals (Nanci *et al.* 1993). However, in TM-treated incisors, both its integrity and structure were affected. In some regions the BL was absent or difficult to distinguish (Figs. 4B, 4C and 6B), while in others it increased substantially in thickness (Figs. 6A and 8). Where enamel is almost EDTA soluble (Warshawsky and Smith 1974), the BL often ramified into the enamel layer (Fig. 5B) and showed an irregular surface (Fig. 11C).

Lectin Cytochemistry

In control rats, the BL showed a uniform and intense labeling with HPA (Figs. 4A and 5A). At the start of the maturation stage, the labeling over the BL in TM-treated rats appeared weaker than in controls, and was sometimes interrupted (Fig. 4B), but for the most part the labeling appeared to have the same density as in controls. In some cases, the labeling normally associated with the BL appeared to diffuse into the overlying enamel matrix (compare Fig. 5C with 5A). In regions where the BL was enlarged, HPA binding sites were found throughout the structure, sometimes showing a concentration at the interface with enamel (Fig. 6A).

In both control and TM-treated rats, labeling with WGA over the BL was weak and difficult to distinguish from that over the adjacent enamel (Figs. 5C and 5D). In TM-treated teeth, regions of altered BL did not show any accumulation of gold particles near the cell surface (Fig 6B). Qualitative estimation suggested that the overall WGA labeling over the BL and

enamel was somewhat weaker in TM-treated than in saline infused animals (Figs. 5C and 5D).

Immunolocalization of Enamel Proteins and Albumin

All three anti-EPs antibodies showed slightly weaker immunoreactivities over enamel from TM-treated incisors compared to saline infused teeth. Both anti-amelogenin antibodies used labeled the enamel matrix, however, they yielded different distribution patterns. Anti-AMELy showed an intense reaction throughout the enamel layer (Fig. 7A), whereas labeling with anti-AMEL M179y was more intense near the enamel surface (Fig. 7B). In TM-treated incisors, in regions where the BL appeared widened, immunoreactivity for amelogenin started farther away from the cell surface (Fig. 8). In general, labeling for AMBN over enamel was very weak and in treated rats almost absent in the region where rods are visible (Warshawsky and Smith 1974) (Fig. 7C). With all three antibodies to EPs there was little or no labeling over the BL in both control and treated rats (Figs. 7A and 8A).

In both control and TM-treated rats, albumin was immunodetected in the intercellular space along the baso-lateral surface of ameloblasts. However, in treated animals, accumulation of ALB between cells was more frequent and abundant (compare Fig. 9B with 9A, and Fig. 10B with 10A). There were very few, or no gold particles over enamel in control rats, but there was a weak and conspicuous labeling at the dentino-enamel junction (Figs. 11A and 11B). The number of gold particles over maturing enamel was significantly higher in TM-treated incisors, including at the dentino-enamel junction (Figs. 8B, 11C and 11D). Labeling

for ALB over enamel was patchy and more intense around regions where the BL was absent or significantly altered.

Control Incubations

Labeling was significantly reduced when sections were incubated with HPA or WGA in the presence of their respective competing saccharides, or when they were incubated with anti-HPA or anti-WGA only, followed by protein A-gold. In the case of incubations for EPs and ALB, controls showed an abolition of the selective labeling observed and in the presence of few, randomly distributed gold particles throughout the tissue sections.

## Discussion

The present study demonstrates that it is possible to achieve a localized administration of TM in the rat hemimandible without systemic side effects. Infusion of this antibiotic through a bony window using an osmotic minipump over a 7 day period causes alterations in both the structure and composition of the BL interposed between ameloblasts and maturing enamel.

Noncollagenous matrix proteins in mineralized tissues, such as bone sialoprotein (BSP) and osteopontin (OPN), are highly glycosylated (reviewed in Butler 1989; Midura and Hascall 1996). Indeed, a large portion of the molecular weight of these two proteins is accounted for by N-and O-linked carbohydrates. On the other hand, enamel matrix proteins are distinctively less glycosylated. Sugar residues are mainly present on nonamelogenins and are predominately N-linked in the case of enamelin (Fukae *et al.* 1996; Hu *et al.* 1998) and a 65 kDa sulfated enamel protein (Smith *et al.*, 1995), and O-linked in the case of ameloblastin (Cerny *et al.* 1996; Krebsbach *et al.* 1996; Hu *et al.* 1997; Uchida *et al.* 1997; Nanci and Smith 2000). However, a highly-glycosylated basal lamina forms at the interface between ameloblasts and enamel at the start of the maturation stage of amelogenesis (Nanci *et al.* 1993). It is rich in glycoconjugates comprising N-acetyl-D-galactosamine (GalNAc) and/or N-acetyl-glucosamine/N-acetyl-neuraminic acid (GlcNAc/NeuNAc). This compositional characteristic distinguishes the BL related to maturation stage ameloblasts from typical basement membranes who, with the exception of the glomerular basement membrane (Kanwar and Farquhar 1979), do not show such a high degree of glycosylation

(Roth 1993). It is likely that the maturation stage BL is a unique structure and that its functional properties are, at least in part, related to its glycosylation status.

In the present study, lectin-gold cytochemistry was applied to examine compositional alterations of the BL induced by inhibition of N-glycosylation. Under the influence of TM, the BL becomes irregular and/or is replaced by an interfacial region poor in EPs, but rich in glycoconjugates. The presence of a large number of HPA binding sites throughout this region suggests that it represents a widened BL. The density of HPA binding sites over the BL did not significantly differ in treated and control animals, indicating that this lectin recognizes glycoconjugates whose concentration is not affected by TM. On the other hand, the density of labeling of WGA seemed to decrease over both the BL and enamel in TM-treated incisors. This apparent reduction is consistent with the expectation that TM should prevent the incorporation of N-linked sugars, such as GlcNac.

A number of enzymes are involved in the processing and degradation of EPs (reviewed in Smith 1998). Tunicamycin is likely to have had an effect on the activity of enamel proteinases, as enzymes are generally glycosylated. This should have resulted in the delayed removal of EPs and a corresponding increase in density of labeling at certain times during enamel maturation. One possible explanation for why this was not observed is that the enamel being examined, and its constituting matrix proteins and enzymes, were in part formed prior to administration of the drug. While EPs are generally not believed to serve any major structural function, the BL on the other hand forms a dense meshwork which may be, in part, dependent on carbohydrate side chains for its integrity. Protein conformation

might be altered in the absence of glycosylation (Olden et al 1978; Elbein 1988), such that N-glycosylated BL components interact less efficiently, leading to a more diffuse structure or its absence. In the case of EPs, only some nonamelogenins are N-glycosylated and these represent a minor component of enamel. They are processed very soon after their secretion and their primary role is in the secretory stage where crystals actively elongate (Nanci *et al.* 1998). Any alteration of the few nonamelogenins still secreted during the maturation stage is likely to have a minor impact on enamel formation. Alternatively, sugar residues on EPs may have no significant role in their function. Although no major modification of maturing enamel structure was observed, defective dentin matrix production and mineralization were noted, suggesting that N-glycosylated matrix components play an important role in its formation.

The immunodetection of large amounts of ALB in the enamel matrix of TM-treated rats indicates that there has been an abnormal influx of this plasma protein following its administration. Ameloblasts do not synthesize and secrete ALB (Couwenhoven *et al.* 1989; Fincham *et al.*, 1999) and circulating ALB has limited access to the enamel layer in normal rats (McKee *et al.* 1986; Robinson *et al.* 1994; Chen *et al.* 1995; Nanci *et al.* 1996;), as well as in saline infused control rats. Thus, it is likely that the alteration of the BL may, at least in part, be responsible for its presence in enamel (Nanci *et al.* 2000). Similarly to proteoglycans in the kidney glomerular basement membrane (Kanwar and Farquhar 1979), glycoconjugates may confer on the BL charge-selective properties, which would likely be affected by the inhibition of N-glycosylation.

In conclusion, the rat hemimandible bony window model is well suited for studying locally the effect of drugs such as TM. This antibiotic affects both the structure and composition of the BL interposed between maturation stage ameloblasts and enamel. This structure has been proposed to have adhesive and/or any filtering functions. No significant detachment of the enamel organ was observed following 7 days of TM administration, but there was an abnormal presence of ALB in maturing enamel. While not excluding a potential adhesive role, the data thus lend support to the proposal that this BL is a specialized structure exhibiting filtering functions.

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**Literature Cited**

Bendayan M (1995) Colloidal gold post-embedding immunocytochemistry. *Prog Histochem Cytochem* 29:1-163.

Benhamou N (1989) Preparation and application of lectin-gold complexes. In Hayat MA (ed) *Colloidal Gold: Principles, Methods, and Applications*. Vol. 1, Academic Press, Inc., San Diego, 95-143.

Butler WT (1989) The nature and significance of osteopontin. *Connect Tissue Res* 23:123-136.

Cerny R, Slaby I, Hammarström L, Wurtz T (1996) A novel gene expressed in rat ameloblasts codes for proteins with cell binding domains. *J Bone Miner Res* 11:883-891.

Chardin H, Septier D, Lecolle S, Goldberg M (1989) Effect of tunicamycin on glycogen accumulation in the stratum intermedium and odontoblasts of rat incisor. *Cell Tissue Res* 256:519-527.

Chen W-Y, Nanci A, Smith CE (1995) Immunoblotting studies on artifactual contamination of enamel homogenates by albumin and other proteins. *Calcif Tissue Int* 57:145-151.

Couwenhoven RI, Davis C, Snead ML (1989) Mouse ameloblasts do not transcribe the albumin gene. *Calcif Tissue Int* 45:367-371.

Elbein AD (1988) Inhibitors of the biosynthesis and processing of N-linked oligosaccharides. *CRC Critical Reviews in Biochemistry* 16:21-49.

Ekblom PS, Nrdling L, Saxen L, Rasilo ML, Renkonen O (1979) Cell interactions leading to kidney tubule determination are tunicamycin sensitive. *Cell Differ* 8:347-352.

Fincham AG, Moradian-Oldak J, Simmer JP (1999) The structural biology of the developing dental enamel matrix. *J Struct Biol* 126:270-299.

Finnie JW, O'Shea JD (1988) Pathological and pathogenic changes in the central nervous system of guinea pigs given tunicamycin. *Acta Neuropathol* 75:411-421.

Fukae M, Tanabe T, Murakami C, Dohi N, Uchida T, Shimizu M (1996) Primary structure of the porcine 89-kDa enamelin. *Adv Dent Res* 10:111-118.

Gassmann M, Thömmes P, Weiser T, Hübscher U (1990) Efficient production of chicken egg yolk antibodies against a conserved mammalian protein. *FASEB J* 4:2528-2532.

Giraud A, Franc J-F (1989) Effects of tunicamycin and n-linked oligosaccharide-processing inhibitors on the morphology of cultured porcine thyroid cells. *Eur J Cell Biol* 48:128-134.

González CB, Swann RW, Pickering BT (1981) Effects of tunicamycin on the hypothalamus-neurohypophysial system of the rat. *Cell Tiss Res* 217:199-210.

Hart GW, Lennarz WJ (1978) Effects of tunicamycin on the biosynthesis of glycosaminoglycans by embryonic chick cornea. *J Biol Chem* 253:5795-5801.

Hu C-C, Fukae M, Uchida T, Qian Q, Zhang CH, Ryu OH, Tanabe T, Yamakoshi Y, Murakami C, Dohi N, Shimizu M, Simmer JP (1997) Sheathlin: Cloning, cDNA/polypeptide sequences, and immunolocalization of porcine enamel sheath proteins.

*J Dent Res* 76:648-657.

Hu CC, Simmer JP, Bartlett JD, Qian Q, Zhang C, Ryu OH, Xue J, Fukae M, Uchida T, MacDougall M (1998) Murine enamelin: cDNA and derived protein sequences. *Connect Tissue Res* 39:351-365.

Jago MV, Payne AL, Peterson JE, Bagust TJ (1983) Inhibition of glycosylation by corynetoxin, the causative agent of annual ryegrass toxicity: a comparison with tunicamycin. *Chem Biol Interactions* 45:223-234.

Kanai Y, Hayashi Y, Kawakami H, Takata K, Kurohmaru M, Hirano H, Nishida T (1991) Effect of tunicamycin, an inhibitor of protein glycosylation, on testicular cord organization in fetal mouse gonadal explants in vitro. *Anat Rec* 230:199-208.

Kanwar YS, Farquhar MG (1979) Presence of heparan sulfate in the glomerular basement membrane. *Proc Natl Acad Sci USA* 76:1303-1307.

Krebsbach PH, Lee SK, Matsuki Y, Kozak CA, Yamada KM (1996) Full-length sequence, localization, and chromosomal mapping of ameloblastin: a novel tooth-specific gene. *J Biol Chem* 271:4431-4435.

Leaver DD, Schneider KM, Rand MJ, Anderson RM, Gage PW, Malbon R (1988) The neurotoxicity of tunicamycin. *Toxicology* 49:179-187.

McKee MD, Martineau-Doizé B, Warshawsky H (1986) Penetration of various molecular-weight proteins into the enamel organ and enamel of the rat incisor. *Arch Oral Biol* 31:287-296.

Midura RJ, Hascall VC (1996) Bone sialoprotein - A mucin in disguise? *Glycobiology* 6:677-681.

Nanci A, Smith CE (1992) Development and calcification of enamel. In Bonucci E (ed) *Calcification in Biological Systems*, CRC Press, Inc., Boca Raton, 313-343.

Nanci A, Zalzal S, Kogaya Y (1993) Cytochemical characterization of basement membranes in the enamel organ of the rat incisor. *Histochemistry* 99:321-331.

Nanci A, Fortin M, Ghitescu L (1996a) Endocytotic functions of ameloblasts and odontoblasts: Immunocytochemical and tracer studies on the uptake of plasma proteins. *Anat Rec* 245:219-234.

Nanci A, Hashimoto J, Zalzal S, Smith CE (1996b) Transient accumulation of proteins at interrod and rod enamel growth sites. *Adv Dent Res* 10:135-149.

Nanci A, Zalzal S, Lavoie P, Kunikata M, Chen W-Y, Krebsbach PH, Yamada Y, Hammarström L, Simmer JP, Fincham AG, Snead ML, Smith CE (1998) Comparative immunochemical analyses of the developmental expression and distribution of ameloblastin and amelogenin in rat incisors. *J Histochem Cytochem* 46:911-934.

Nanci A, Mocetti P, Sakamoto Y, Kunikata M, Lozupone E, Bonucci E (2000) Morphological and immunocytochemical analyses on the effects of diet-induced hypocalcemia on enamel maturation in the rat incisor. *J Histochem Cytochem* 48:1043-1058.

Nanci A, Smith CE (2000) Matrix-mediated mineralization in enamel and the collagen-

based hard tissues. In Goldberg M, Boskey A, Robinson C (eds) *Chemistry and Biology of Mineralized Tissues*, American Academy of Orthopaedic Surgeons, Rosemont, 217-224.

Olden K, Pratt RM, Yamada KM (1978) Role of carbohydrates in protein secretion and turnover: Effects of tunicamycin on the major cell surface glycoprotein of chick embryo fibroblasts *Cell* 13:461-473.

Orsini G, Lavoie P, Smith CE, Nanci A (2001) Immunochemical characterization of chicken egg yolk antibody to secretory forms of rat incisor amelogenin. *J Histochem Cytochem* (in press) MS 5343-00-A.

Plank VJ, Rychlo A (1952) Eine schnellentkalkungsmethode. *Zentralbl Pathol* 89:252-254.

Pow DV, Morris JF (1992) Tunicamycin, puromycin and brefeldin A influence the subcellular distribution of neuropeptides in hypothalamic magnocellular neurones of rat. *Cell Tissue Res* 269:547-560.

Robinson C, Brookes SJ, Kirkham J, Shore RC, Bonass WA (1994) Uptake and metabolism of albumin by rodent incisor enamel *in vivo* and postmortem: Implications for control of mineralization by albumin. *Calcif Tissue Int* 55:467-472.

Roth J (1983) Application of lectin-gold complexes for electron microscopic localization of glycoconjugates on thin sections. *J Histochem Cytochem* 31:987-999.

Roth J (1993) Cellular sialoglycoconjugates: A histochemical perspective. *Histochem J* 25:687-710.

Sawada T, Yanagisawa T, Takuma S, Hasegawa H, Watanabe K (1992) Electron-immunocytochemical localization of laminin and type-IV collagen in the enamel organ of the rat incisor. *Acta Histochem Cytochem* 25:395-403.

Smith CE (1974) A method for preparing longitudinal semi-thin epon sections of entire rat incisors. *Arch Oral Biol* 19:1045-1048.

Smith CE, Nanci A (1989) A method for sampling the stages of amelogenesis on mandibular rat incisors using the molars as a reference for dissection. *Anat Rec* 225:257-266.

Smith CE, Chen W-Y, Issid M, Fazel A (1995) Enamel matrix protein turnover during amelogenesis: Basic biochemical properties of short-lived sulfated enamel proteins. *Calcif Tissue Int* 57:133-144.

Smith CE, Nanci A (1995) Overview of morphological changes in enamel organ cells associated with major events in amelogenesis. *Int J Dev Biol* 39:153-161.

Smith CE (1998) Cellular and chemical events during enamel maturation. *Crit Rev Oral Biol Med* 9:128-161.

Takano Y (1979) Cytochemical studies of ameloblasts and the surface layer of enamel of the rat incisor at the maturation stage. *Arch Histol Jpn* 42:11-32.

Takatsuki A, Kohno K, Tamura G (1975) Inhibition of biosynthesis of polyisoprenyl sugars in chick embryo microsomes by tunicamycin. *Agric Biol Chem* 39:2089-2091.

Thesleff I, Pratt RM (1980a) Tunicamycin-induced alterations in basement membrane formation during odontoblast differentiation. *Dev Biol* 80:175-185.

Thesleff I, Pratt RM (1980b) Tunicamycin inhibits mouse tooth morphogenesis and odontoblast differentiation in vitro. *J Embryol Exp Morphol* 58:195-208.

Uchida T, Murakami C, Dohi N, Wakida K, Satoda T, Takahashi O (1997) Synthesis, secretion, degradation and fate of ameloblastin during the matrix formation stage of the rat incisor as shown by immunocytochemistry and immunochemistry using region-specific antibodies. *J Histochem Cytochem* 45:1329-1340.

Vu D-D, Daniel NG, Nanci A (1999) In vivo model for the experimental manipulation of calcified tissues: A surgical approach for accessing the odontogenic organ and associated tissues in the rat incisor. *J Histochem Cytochem* 47:1-14.

Warshawsky H, Moore G (1967) A technique for the fixation and decalcification of rat incisors for electron microscopy. *J Histochem Cytochem* 15:542-549.

Warshawsky H, Smith CE (1974) Morphological classification of rat incisor ameloblasts. *Anat Rec* 179:4.

Webster EH, Hilfer SR, Searls RL, Kornilow J (1993) Effect of tunicamycin on maturation of fetal mouse lung. *Am J Physiol Lung Cell Mol Physiol* 265:L250-L259.

Yang J-JW, Hilfer SR (1982) The effect of inhibitors of glycoconjugate synthesis on optic cup formation in the chick embryo. *Develop Biol* 92:41-53.

**Figure legends**

**Figure 1**: (A) Micrograph illustrating the surgically-created bony window (arrow) on the buccal aspect of the hemimandible. (B) For delivery of experimental agents, a metal catheter (cat) is fitted into the bony hole and the vinyl tubing linking it to the osmotic minipump is passed under the posterior portion (asterisks) of the masseter muscle (M). X-ray radiographs of the (C) rat head (submandibular view) and (D) hemimandible (lateral view) showing the relative position of the bony window. Such radiographs are routinely used to confirm that the metal catheter is retained in place.

**Figure 2**: Micrographs showing the incisors in a rat infused for 7 days with (A) saline and (B) tunicamycin (TM). In saline controls, the incisor on the infused side (*) is of the same length as the contralateral one. Tunicamycin results in a shortening of the treated incisor (arrows), suggesting that the drug has slowed down its eruption.

**Figure 3**: Light micrographs illustrating typical histological alterations observed following tunicamycin administration. (A) During the early to midmaturation stage, ameloblasts (Am) and enamel (E) do not exhibit any significant changes. However, both dentin (D) and odontoblasts (Od) are affected by the drug. (A, B). At about the middle of the dentin layer there is a differentially-staining region (arrowheads) and the dentin formed beyond this point appears somewhat less metachromatic. In some regions, groups of odontoblasts (B) appear to have degenerated (asterisks) or (C) to have lost their secretory polarity, releasing matrix components all around them (arrows). P, pulp; PD, predentin; PL, papillary layer.

**Figure 4**: Cytochemical preparations with *Helix pomatia* agglutinin (HPA) for the detection of N-acetyl-D-galactosamine sugar residues. (A) In control rats infused with saline, the basal lamina (BL) interposed between the ameloblasts and the maturing enamel layer is intact and intensely labeled. Enamel shows very few gold particles. (B, C) In tunicamycin (TM)-treated animals, the basal lamina is frequently interrupted (arrows) and the labeling associated with it appears to have diffused into the enamel layer. ly, lysosome-like element.

**Figure 5**: Micrographs from sections of control and tunicamycin (TM)-treated rats incubated with (A, B) *Helix pomatia* agglutinin (HPA) and (C, D) wheat germ agglutinin (WGA). In control animals, the basal lamina (BL) forms a uniform, planar structure that labels intensely with HPA and weakly to moderately with WGA. Tunicamycin treatment alters both the structure and composition of the basal lamina. In some cases, (B) it sends ramifications (arrows) into the enamel layer while in others (D) it simply appears less distinct. The labeling intensity with HPA does not seem to be altered by inhibition of N-glycosylation (compare A with B) but that of WGA is slightly reduced (compare C with D).

**Figure 6**: The maturation stage basal lamina (BL) is regionally affected during tunicamycin (TM) administration. In some regions (A) it is thicker while in others (B) it is absent (A). In some cases, incubation with *Helix pomatia* agglutinin (HPA) shows a heterogeneous distribution of gold particles over the enlarged BL region (B). When the BL is absent, enamel matrix extends within the membrane infoldings (arrows) of ruffle-ended ameloblasts and labeling with wheat germ agglutinin does not show a higher density near the cell surface. cp, cell process.

**Figure 7**: Immunocytochemical preparations from tunicamycin (TM)-treated rats incubated with antibodies raised against (A) 24 kDa rat amelogenin (AMELy), (B) mouse recombinant 179 amelogenin isoform (M179y) and (C) rat recombinant ameloblastin (AMBN). Anti-AMELy, which recognizes various amelogenin fragments (see Chen *et al.*, 1995), yields and intense immunoreactivity throughout the enamel layer. However, anti-M179y (see Orsini *et al.* 2001) and anti-AMBN (see Nanci *et al.*, 1998), both of which recognize essentially intact molecules, give a weak labeling. BL, basal lamina.

**Figure 8**: Immunocytochemical preparations from tunicamycin (TM)-treated rats in a region of the incisor where the maturation stage basal lamina (BL) is thickened. (A) Incubation with anti-mouse recombinant 179 amelogenin isoform antibody (M179y) results in very few gold particles over the basal lamina region (arrows) and a moderate to weak labeling over enamel (B). On the other hand, incubation with anti-rat albumin (ALB), a plasma protein not normally found in enamel (see figure 11A), reveals an important reactivity over both basal lamina region and enamel.

**Figure 9**: (A) In saline-infused control rats, some albumin (ALB) can be immunodetected along the baso-lateral surfaces (arrows) of maturation stage ameloblasts. (B) However, large pools of this plasma protein can be found between ameloblasts (arrows) in some regions of the enamel organ in tunicamycin (TM)-treated incisors, suggesting that the permeability of the enamel organ is affected by tunicamycin. mvb, multivesicular body, N, nucleus.

**Figure 10**: Electron micrographs, from sections immunolabeled for albumin (ALB), showing the apical portion of ameloblasts. (A) In control incisors, few gold particles are observed over the lateral intercellular space (arrows) and over enamel (asterisks). Note that both in control and treated rats, the BL sometimes appears as an electron-lucent region. (B) Tunicamycin (TM) treatment frequently causes an accumulation of albumin in this space. Particularly in regions adjacent to these accumulations, the basal lamina (BL) as well as the enamel shows an intense immunoreactivity.

**Figure 11**: Comparative immunocytochemical preparations illustrating (A) in control rats, the paucity of labeling for albumin (ALB) over enamel near the ameloblast surface and (B) the presence of a weak but consistent reactivity at the dentino-enamel junction (arrows). (C, D) There is a major influx of this plasma protein under the effect of tunicamycin (TM), both (C) near the cell surface and (D) at the dentino-enamel junction. (C) The basal lamina (BL), at the interface between ameloblast and enamel, appears thicker and irregular.

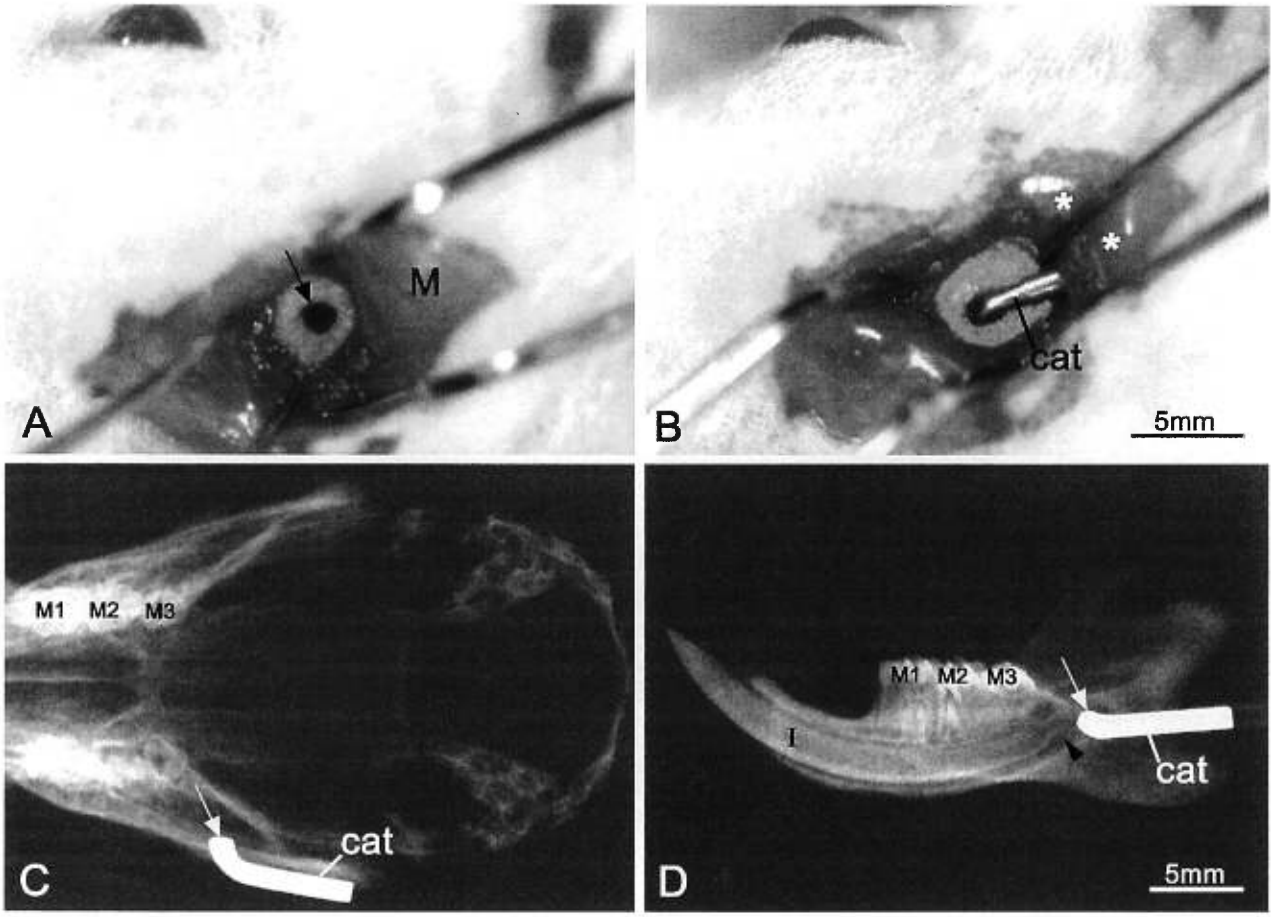



Figure 1

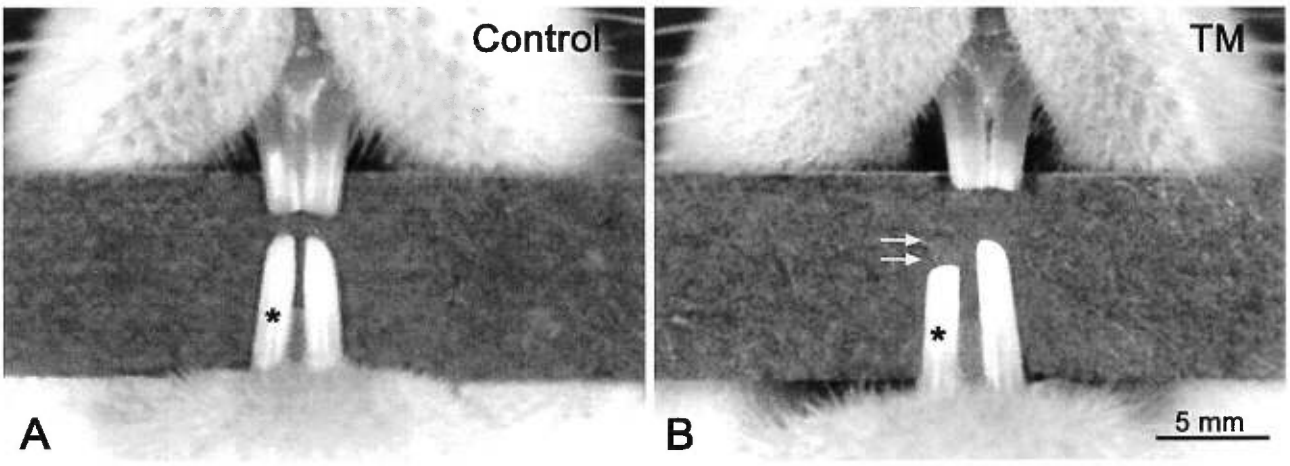
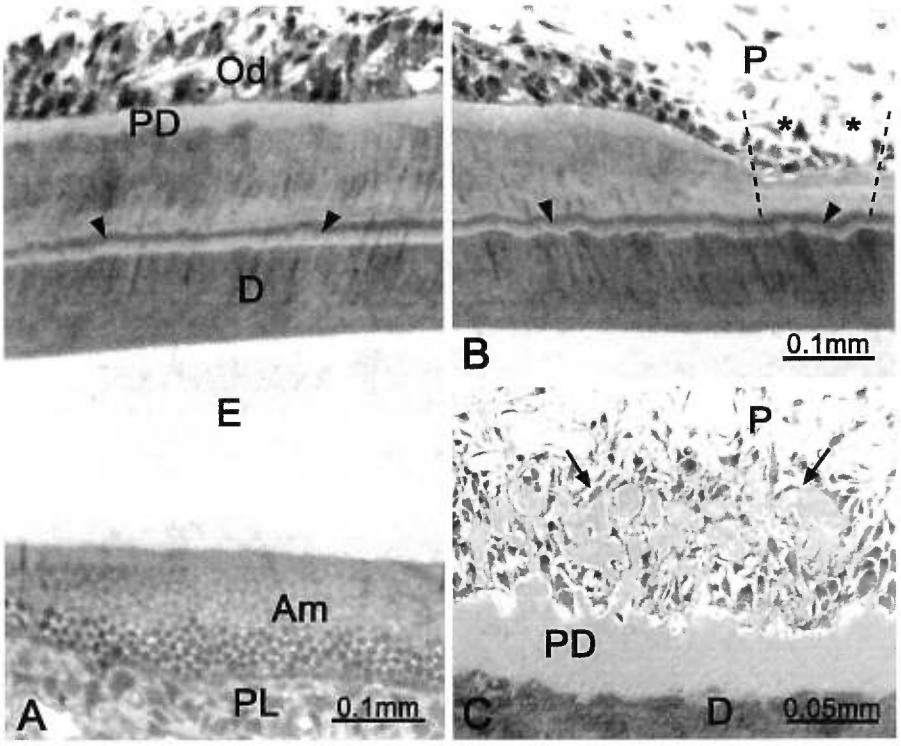



Figure 2

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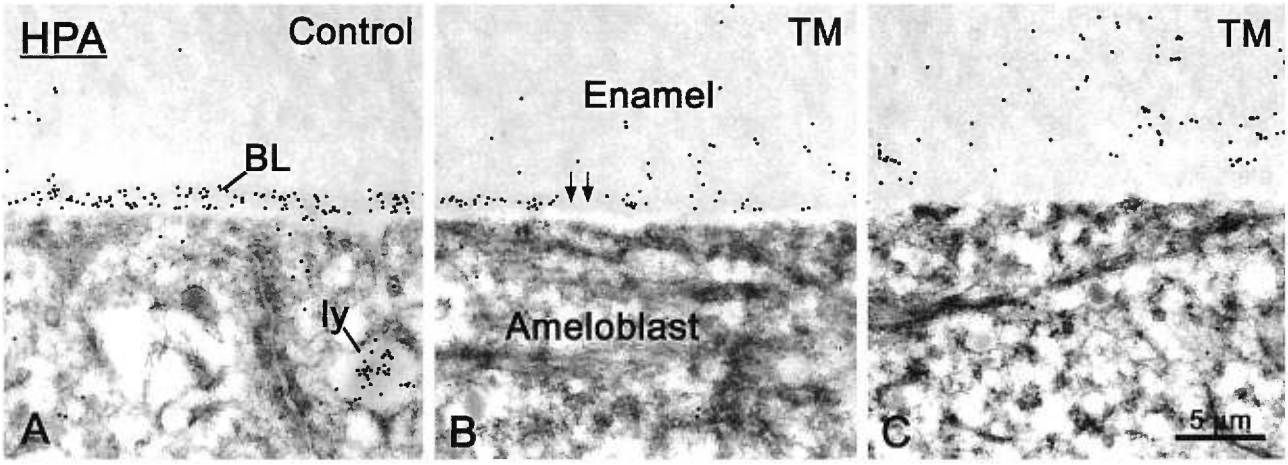


Figure 4

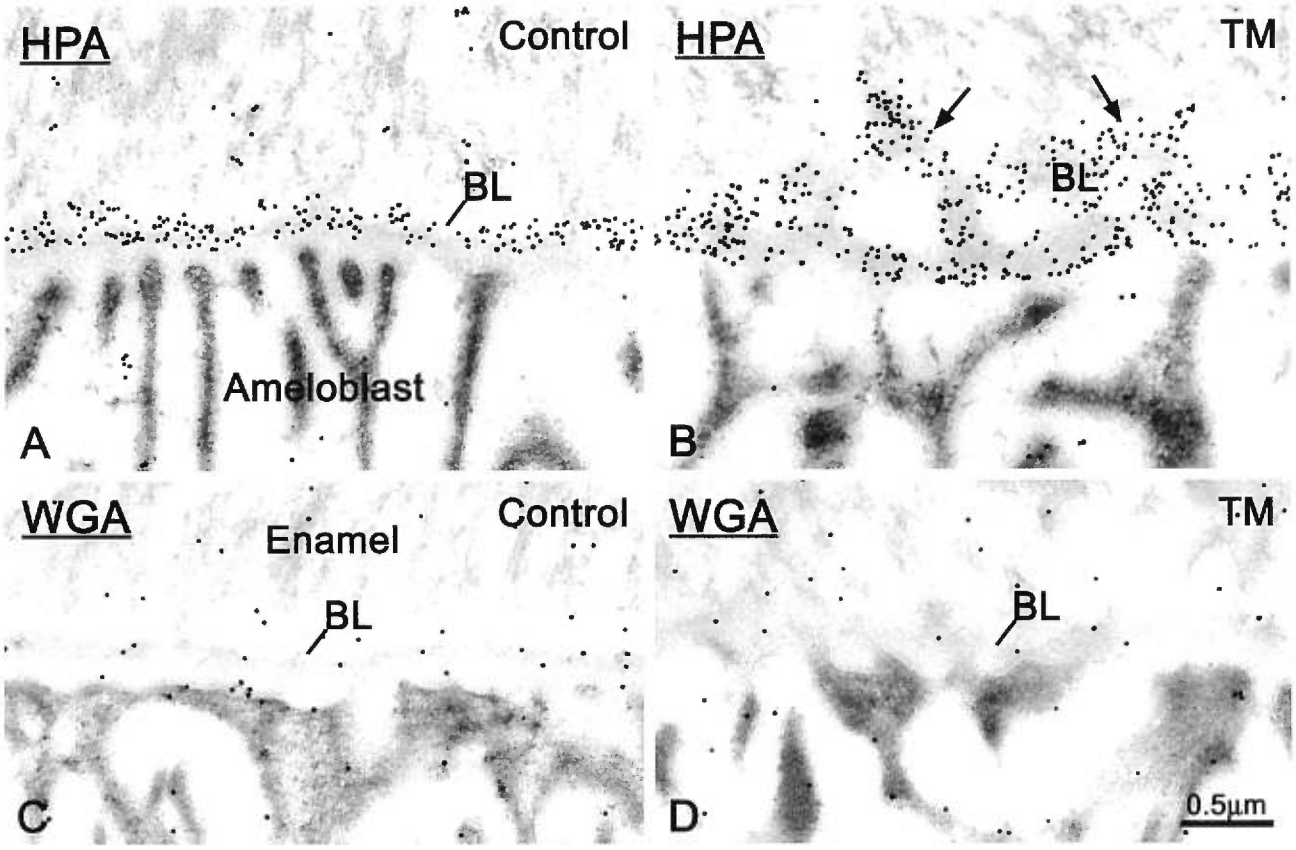


Figure 5

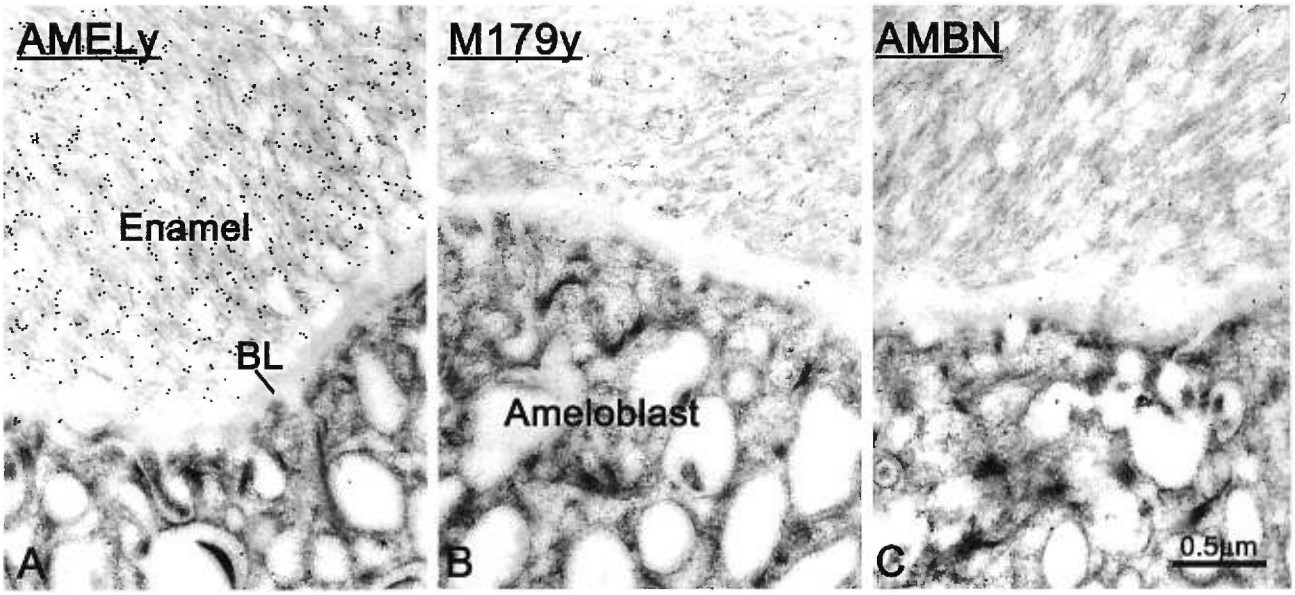


Figure 7

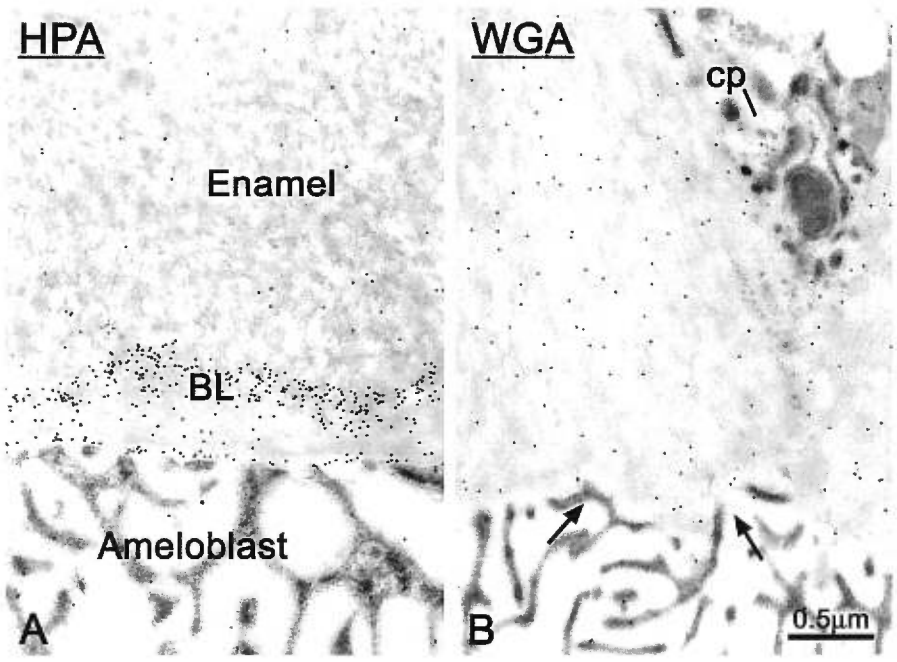


Figure 6

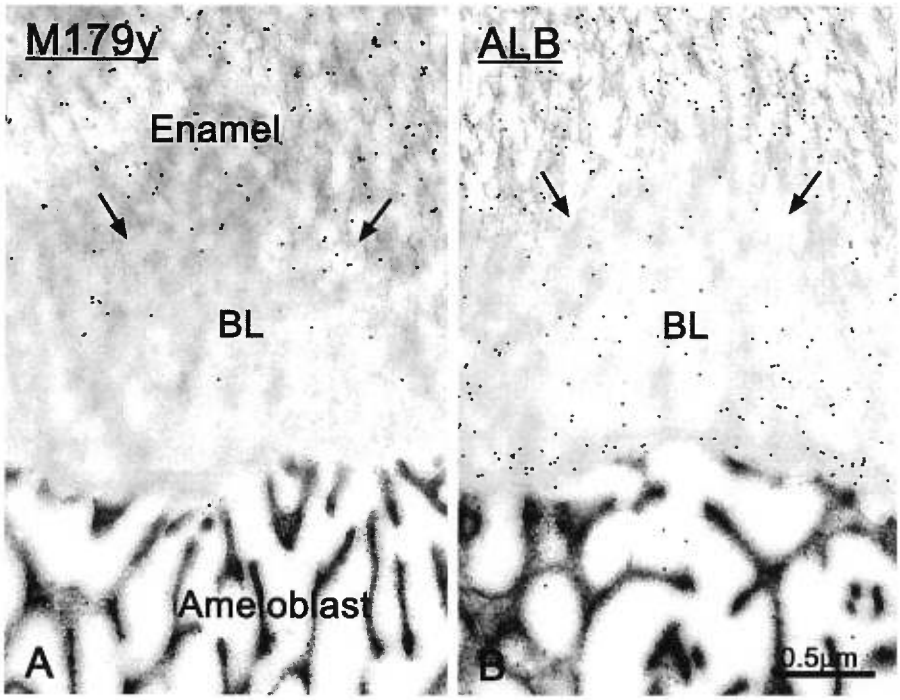


Figure 8

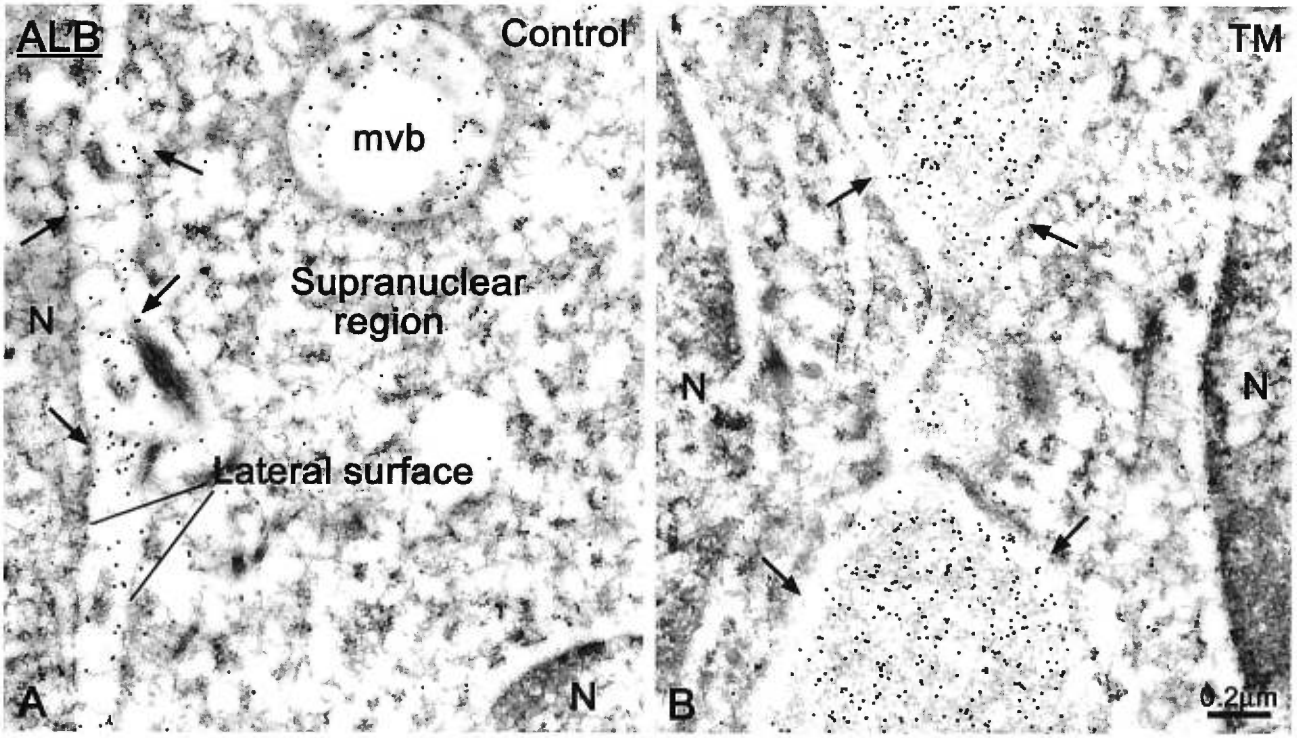


Figure 9

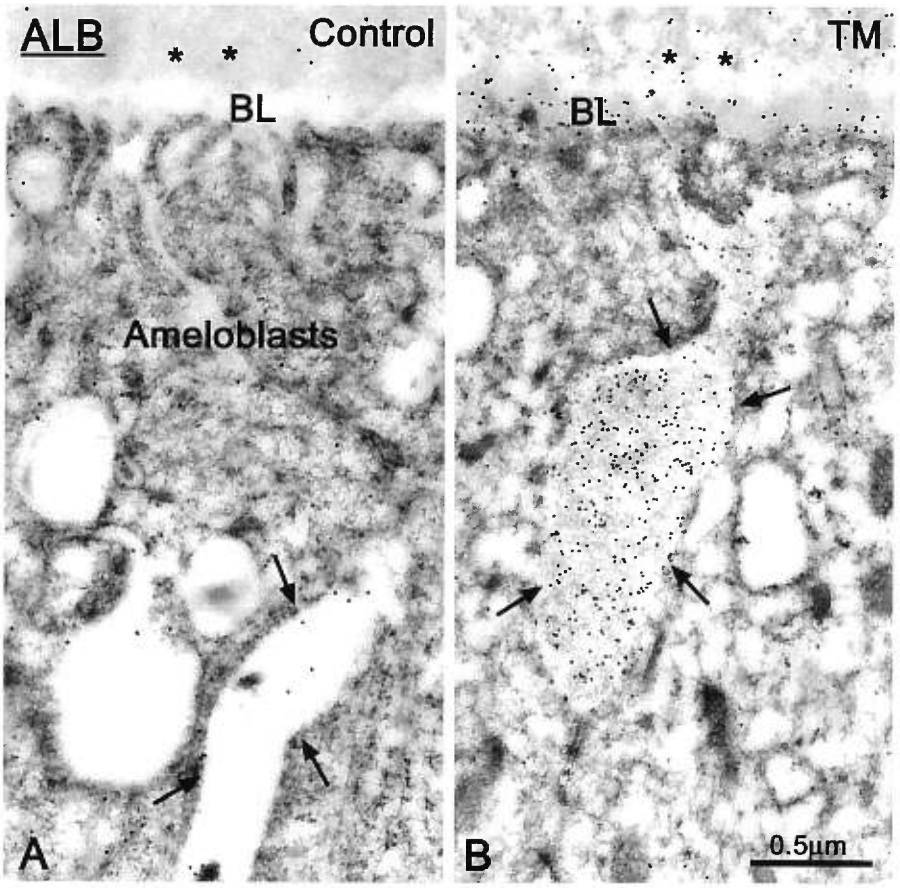


Figure 10

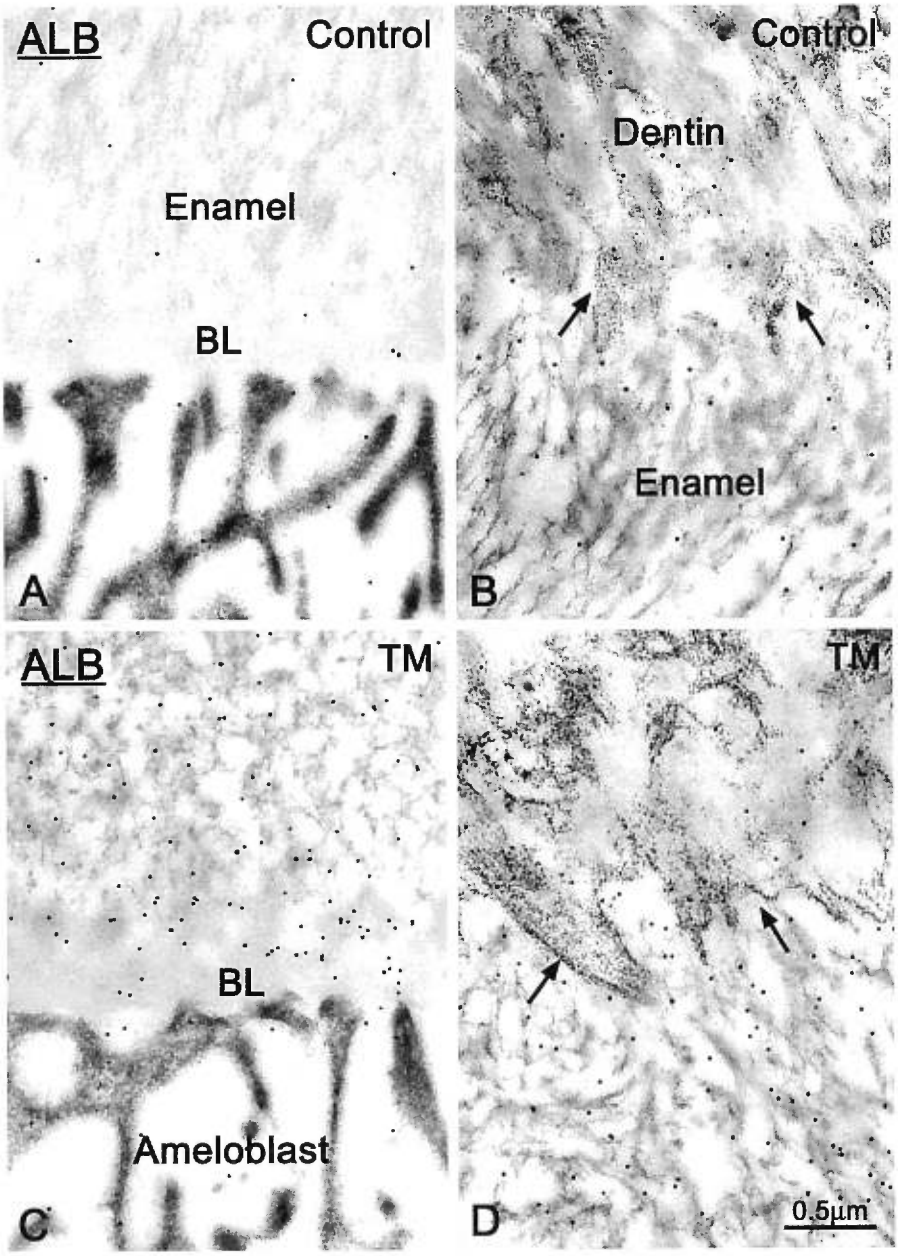


Figure 11

# Chapitre 2

# Article II

**Immunochemical Characterization of a Chicken Egg Yolk Antibody to Secretory Forms of Rat Incisor Amelogenin.**

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**Abstract**

Amelogenins represent the major component of the organic matrix of enamel and consist of several intact and degraded forms. A precise knowledge of their respective distributions throughout the enamel layer could provide some insight into their functions. To date there exists no antibody that selectively detects the secretory forms of amelogenin. In this study, we have used the chicken egg yolk system to generate an antibody to recombinant mouse amelogenin. Immunoblots of whole homogenates from rat incisor enamel organs and enamel showed that the resulting antibody (M179y) recognized proteins corresponding to the 5 known secretory forms of rat amelogenin. Immunogold cytochemistry demonstrated that reactivity was restricted to ameloblasts and enamel. Secretory forms of amelogenin persisted in significant amounts throughout the enamel layer. The density of labeling was highest over the surface portion of the enamel layer but enamel growth sites within this region showed a localized paucity of gold particles. Immunoreactivity was lowest over the mid portion of the layer and increased moderately near the dentino-enamel junction. These results indicate that intact forms of amelogenin likely have a more complex distribution within the enamel layer than heretofore suspected.

**Key words**: Polyclonal antibody, Amelogenin, Secretory forms, Immunoblotting, Immunocytochemistry

## Introduction

During enamel formation, ameloblasts produce a number of matrix proteins that are believed to promote and regulate mineral ion deposition into unique and extremely long apatite crystals. Amelogenins represent the major secretory product of these epithelial-derived cells (reviewed in Fincham *et al.* 1999; Nanci and Smith 2000). It is generally accepted that all mammalian ameloblasts produce several amelogenin variants from a single gene (reviewed in Simmer and Snead 1995). Most of these amelogenin isoforms result from differential splicing of the mRNA (Lau *et al.* 1992). Molecular, metabolic and mass analyses have helped clarify possible derivative relationships between newly secreted, intact amelogenins and their degradation products (Smith and Nanci 1996; Chen *et al.* 2000; Fincham *et al.* 1999). However, there is still uncertainty when it comes to identifying these components in gels or immunocytochemical preparations. Indeed, despite the availability for some time of several polyclonal, monoclonal and anti-peptide antibodies, none selectively reveal just the intact versions of nascent amelogenin.

Over the past few years, we have successfully used the chicken egg yolk system (Lösch *et al.* 1986; Gassmann *et al.* 1990) to produce polyclonal antibodies to enamel proteins and other calcified tissue matrix proteins (Chen *et al.* 1995; Nanci *et al.* 1996). One advantage of this system is that chickens can yield high titer antibodies against conserved mammalian antigens (Gassmann *et al.* 1990).

The aim of this study was to determine whether this system could produce an antibody selective for secretory forms of amelogenin. While such an antibody is beneficial for

biochemical characterizations, it would be particularly useful for immunocytochemical mapping of the temporo-spatial distribution of the protein. This information is essential for understanding its function as well as its implication in pathological alterations.

## Materials and Methods

All animal handling and experimental procedures were approved by the Comité de déontologie de l'expérimentation sur les animaux of Université de Montréal.

### Preparation of Chicken Egg Yolk Polyclonal Antibody

A mouse recombinant amelogenin (M179, lacking the N-terminal methionine and the $Ser^{16}$-phosphate group found on the main native mouse amelogenin, M180 isoform; Simmer *et al.* 1994) was purified, resuspended in 10 mM PBS, and emulsified in Quil A saponin adjuvant. A polyclonal antibody was raised in chickens and purified from egg yolks using the procedure of Gassmann *et al.* (1990). Briefly, 50 μg of the purified protein was injected into the pectoral muscle of egg laying hens, followed by a second injection of the same amount 15 days later. Eggs were collected before injections (preimmune controls) as well as for 30 days following the first injection of antigen. This antibody is referred to as M179y.

### Sample Preparation for Immunoblotting

Male Wistar rats weighing 100-150 g (Charles Rivers Canada; St-Constant, QC, Canada) were anesthetized with Metofane (methoxyfluorane; Janssen Pharmaceutica, North York, ON, Canada) and decapitated. The hemimandibles were dissected out and the enamel organ was partially exposed by cracking off some of the covering alveolar bone. They were immediately plunged into liquid nitrogen and maintained in it for a minimum of 5 hours before freeze-drying for at least 48 hours at – 80°C on a 12-liter

cascade lyophilizer system (Labconco; Kansas City, MO). The enamel organ with adhering labial connective tissue and enamel were then transected on each incisor into a series of 5 sequential strips relative to the secretory and maturation stages of amelogenesis using a molar reference line (Smith and Nanci 1989). The 2 secretory stage strips were 2.5 mm long while the 3 maturation ones were about 2 mm long. Each strip, was placed in a separate sterile 1.5-ml screw-top microfuge vial and proteins were extracted directly into 100 µl of a sample preparation buffer containing 62.5 mM Tris (pH 6.8), 2 % SDS, 15% glycerol, 5% β-mercaptoethanol, and 0.005% bromophenol blue. The vials were immersed in a boiling water bath for 5 minutes, cooled and stored at 4°C.

Immunoblotting

Twenty µl of extraction fluid from each vial was applied to separate lanes of standard format (16 cm X 14 cm X 1 mm) 12% polyacrylamide slab gels. Broad range molecular weight marker proteins (Bio-Rad; Mississauga, ON, Canada) were also loaded in 1 lane of each gel. Proteins were separated by electrophoresis at 20 mA per gel constant current using a discontinuous buffer system (Laemmli 1970). They were then electrotransferred from the gels onto 0.45-um pore size nitrocellulose membrane and probed with M179y followed by alkaline phosphatase-labeled anti-chicken IgG antibody (Cappel Research Products; Scarborough, ON, Canada), as described previously (Chen *et al.* 1995).

Tissue Processing for Immunohistochemistry

Male Wistar rats weighing 100 ± 10 (Charles Rivers Canada) were anesthetized with chloral hydrate (0.4 mg/g bw) and sacrificed by intravascular perfusion with a fixative solution consisting of 1% glutaraldehyde in 0.1 M sodium phosphate (PB), pH 7.2. The hemimandibles were removed and further immersed in the fixative overnight at 4°C. They were then washed in 0.1 M PB, pH 7.2, and decalcified for 21 days in 4.13% EDTA, at 4°C (Warshawsky and Moore 1967). Segments of incisors from the secretory and early to midmaturation stages were prepared using a molar reference line (Smith and Nanci 1989). They were then dehydrated in graded alcohols and processed for embedding in LR White resin (London Resin, Berkshire, UK). Thin sections were cut with a diamond knife, mounted on Formvar-carbon-coated nickel grids, and processed for postembedding protein A-gold immunocytochemistry (reviewed in Bendayan 1995).

Immunocytochemistry

Sections were floated for 15 min on a drop of 0.01 M phosphate buffered saline (PBS) containing 1% ovalbumin (Oval; Sigma Chemical; St Louis, MO). They were transferred for 3 hr onto a drop of M179y diluted 1:100, washed with PBS, refloated on PBS-Oval, and then incubated for 1 hr with a rabbit anti-chicken IgG antibody (diluted 1: 2000) (Cappel Research Products). Finally, they were again washed with PBS, refloated on PBS-Oval, and incubated with protein A-gold complex for 30 min. After immunolabeling, the grids were extensively rinsed with PBS followed by distilled water.

Controls consisted of incubations with preimmune antibody followed by the secondary antibody and protein A-gold, secondary antibody and protein A-gold, or protein A-gold alone. All incubations were carried out at room temperature. Grids were stained with 4% aqueous uranyl acetate and lead citrate for examination in a JEOL JEM-1200EX-II transmission electron microscope operated at 60 kV.

Quantitative Analysis of Immunocytochemical Labeling

Sections from secretory, early and mid maturation stage of amelogenesis (see Warshawsky and Smith 1974), from a minimum of 2 rats were examined. For each stage, the enamel layer was partitioned into three regions. Region 1 was a randomly selected area close to the apical surface of ameloblasts (N= 478); region 2 was situated near the middle of the enamel layer (N= 396); region 3, was at the dentino-enamel junction (N= 528). For the examination of labeling density, the numbers of gold particles were counted in all 3 regions within a window precalibrated to 150.4 $\mu m^2$. ANOVA and Post-Hoc Comparisons of means including the Tukey HSD test for unequal N were performed with alpha = 0.05, using version 5.5A of Statistica for Windows (Statsoft Inc., Tulsa, OK). Power tests of differences between means were done using version 1.01 I of GraphPad StatMate (GraphPad Software, San Diego, CA). The lowest sampling number overall (N= 66) was in the middle region of the enamel layer in early maturation (see Fig. 8, EMAT, Mdl).

## Results

### Immunoblotting

M179y revealed a band near 27 kDa in the S1 enamel organ cell extracts and an additional weakly-stained band near 29 kDa in the M1 sample (Fig. 1). No immunoreactive proteins were discernible in the M2 and M3 cell extracts. Enamel extracts, on the other hand, showed three bands: 23, 27 and 29 kDa from S1 to M1 (Fig. 1). Additional faint bands were present near 30 kDa in S2 and M1, and near 31 kDa only in S2 samples.

### Immunolabeling

Numerous gold particles were observed over the saccules of the Golgi apparatus (Fig. 2) and secretory granules in Tomes' processes (Fig. 3) of secretory stage ameloblasts. Enamel was intensely immunoreactive except at rod (Fig. 3) and interrod (Fig. 4) enamel growth sites where few particles were present. Early to mid maturation stage ameloblasts still showed immunoreactivity over the Golgi apparatus and occasional secretory granules found in these cells (Fig. 5). The overall density of labeling over enamel gradually decreased towards late maturation, however the general distribution was similar throughout (compare Fig. 6A with 6B). In the regions sampled, no immunoreactivity was seen in other cells of the enamel organ or in odontoblasts.

Statistical analyses of the three stages of amelogenesis confirmed that there was a general decline in the density of M179y labeling from secretory to early and mid maturation stages (Fig. 7, SEC to EMAT, $p < 0.0000$; EMAT to MMAT, $p < 0.0152$). When considering the 3 regions in which the enamel layer was partitioned, all the stages showed a higher density of labeling over the surface portion of the enamel layer (region 1) than over the middle part of the enamel layer (region 2) (Fig. 7, Near AM to Middle, $p < 0.0000$; Fig. 8, AM to Mdl for all stages, $p < 0.0000$). An increase in the density of labeling near the DEJ (region 3) was detected in secretory and midmaturation stage samples (Fig. 8; SEC, Mdl to DEJ, $p < 0.0000$; MMAT, Mdl to DEJ is not significant). Early maturation stage samples in contrast showed fairly uniform density of labeling for most of the thickness of the enamel layer except near the surface where the density of labeling was higher (Fig. 8; Mmat, AM to Mdl or DEJ, $p < 0.0000$).

In all cases, control incubations resulted in a major reduction of the labeling and in the presence of few randomly distributed gold particles throughout the tissue sections.

**Discussion**

Using the chicken egg yolk system (Lösch *et al.* 1986; Gassmann *et al.* 1990; Schmidt *et al.* 1993), we have produced a polyclonal antibody that reveals an intensely-reactive protein band at 27 kDa and a very faint one near 29 kDa in rat incisor enamel organ extracts and 5 bands (23, 27, 29, 30 and 31 kDa) in enamel extracts. Immunocytochemistry only showed reactivity in the protein synthetic organelles of ameloblasts and enamel matrix, demonstrating that the epitopes recognized by the M179y antibody are present only on secretory products produced by these cells. Indeed, the proteins revealed by immunoblots correspond very closely to the secretory products identified previously by direct metabolic radiolabeling in the rat (Smith and Nanci 1996). In addition, their relative staining intensities by immunoblotting are remarkably similar to the signals obtained on fluorographs at 1 hour following injection of $^{35}$S-methionine (Smith and Nanci 1996). The predominance of the 27 kDa band in cell extracts is consistent with this being the major secretory form of amelogenin in rat incisors (see Smith and Nanci 1996; Chen *et al.* 2000). The other secretory forms are most probably present in too small quantities in cell extracts to be resolved by the alkaline phosphatase blotting method we have used. Indeed, even with high-energy $^{35}$S-methionine, radiolabeling is mostly associated with the 27 kDa protein. This interpretation is further supported by the appearance of a very faint band near 29 kDa in the M1 cell extract only at the time when it stains most intensely in enamel samples. The absence of staining of fragments derived from postsecretory degradation further suggests that the antibody is directed against intact forms or ones that have undergone little C-terminal processing.

One past interpretation given to radioautographic findings of a sharp increase of labeling over areas of enamel that initially show almost no radioactivity is that protein fragments cleaved from parent amelogenins diffuse into deeper regions of the enamel layer (discussed in Smith *et al.* 1989). This view predicts that amelogenins in deeper (older regions) of enamel essentially consist of fragments which are becoming smaller over time. The presence of substantial amounts of M179y immunoreactivity throughout the entire thickness of the enamel layer suggests that this is not necessarily the case and intact and/or relatively intact amelogenins are found even in the oldest enamel, at least in the case of rat incisors. In secretory stage enamel, about 53% of sampled gold particles were found over the surface portion of the enamel layer while 47% were accounted for by counts over regions near the middle of the layer and near the dentino-enamel junction. The latter percentage is surprisingly close to the estimated 40% of total counts in radiolabeling studies originally released as secretory forms by ameloblasts and which seem to move deeper into the enamel layer (Smith *et al.* 1989). Hence, rather than protein fragments "moving in the wrong direction", this study suggest intact amelogenins move continuously toward the DEJ.

It has for long been assumed that amelogenins are uniformly distributed throughout the enamel layer, a belief consistent with the notion that enamel proteins are arranged in a thixotropic gel which allows free mixing of all components (Eastoe 1979). Immunocytochemical results with the present anti-amelogenin antibody does not support this assumption and suggest that the distribution of amelogenins is more complex than expected. Two patterns of immunolabeling were observed over enamel. Firstly, as has

been shown previously with other anti-amelogenin antibodies (Nanci *et al.* 1996, 1998), there is a paucity of gold particles both at rod and interrod growth sites where enamel crystals actively elongate. Secondly, there is a difference in density of labeling across the enamel layer among the three representative regions of the enamel layer sampled which is most dramatic during the secretory stage (Fig. 8, SEC, $p < 0.0000$ between all locations analyzed). Although such a difference has not been detected in pulse label radioatographic studies (see Nanci *et al.* 1989; Smith *et al.* 1989; Smith and Nanci 1996), it could be inferred from the reported randomization behavior of the main wave of radiolabeled enamel proteins that accumulation over time could potentially lead to its establishment. Indeed, judging from the approximate thickness (40-60 μm) of the secretory stage enamel layer sampled, the immunolabeling gradient observed could have taken up to 4.5 days to establish itself (in the rat incisor it takes 7.5 days to form the full 100 μm enamel thickness; Smith and Nanci 1996).

The persistence of secretory forms of amelogenin throughout the enamel layer has some important functional implications. Since, amelogenin fragments adsorb less efficiently to enamel crystals (Ryu *et al.* 1998), one would expect that the presence of relatively large amounts of apparently intact amelogenin in the deeper regions of forming enamel may have an impact on the rate at which the crystals will grow. This may not be the case in certain species in which there is no well-defined temporal segregation of formative and degradative events. In the pig, for instance, biochemical analyses have shown that forming enamel near the dentino-enamel junction comprises mostly fragments (Bartlett and Simmer 1999).

In conclusion, we have prepared a chicken egg yolk antibody which appears to recognize only secretory forms of amelogenins. This antibody has revealed a more complex distribution of parent amelogenins than previously suspected and represents a potentially useful tool for studying their functional relationship with enamel crystals.

**Acknowledgments**

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**Literature Cited**

Bartlett JD, Simmer JP (1999) Proteinases in developing dental enamel. *Rev Oral Biol Med* 10:425-441.

Bendayan M (1995) Colloidal gold post-embedding immunocytochemistry. *Prog Histochem Cytochem* 29: 1-163.

Chen W-Y, Nanci A, Smith CE (1995) Immunoblotting studies on artifactual contamination of enamel homogenates by albumin and other proteins. *Calcif Tissue Int* 57: 145-151.

Chen W-Y, Bell AW, Simmer JP, Smith CE (2000) Mass spectrometry of native rat amelogenins: Primary transcripts, secretory isoforms, and C-terminal degradation. *J Dent Res* 79: 840-849.

Eastoe JE (1979) Enamel protein chemistry. Past, present and future. *J Dent Res* 58: 753-763.

Fincham AG, Moradian-Oldak J, Simmer JP (1999) The structural biology of the developing dental enamel matrix. *J Struct Biol* 126: 270-299.

Gassmann M, Thömmes P, Weiser T, Hübscher U (1990) Efficient production of chicken egg yolk antibodies against a conserved mammalian protein. *FASEB J* 4: 2528-2532.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage $T_4$. *Nature* 227: 680-685.

Lau EC, Simmer JP, Bringas PJr, Hsu DDJ, Hu C-C, Zeichner-David M, Thiemann F, Snead ML, Slavkin HC, Fincham AG (1992) Alternative splicing of the mouse amelogenin primary RNA transcript contributes to amelogenin heterogeneity. *Biochem Biophys Res Commun* 188: 1253-1260.

Lösch U, Schranner I, Wanke R, Jürgens L (1986) The chicken egg, an antibody source. *J Vet Med* B33: 609-619.

Nanci A, Ahluwalia JP, Pompura JR, Smith CE (1989) Biosynthesis and secretion of enamel proteins in the rat incisor. *Anat Rec* 224: 277-291.

Nanci A, Hashimoto J, Zalzal S, Smith CE (1996) Transient accumulation of proteins at interrod and rod enamel growth sites. *Adv Dent Res* 10: 135-149.

Nanci A, Zalzal S, Lavoie P, Kunikata M, Chen W-Y, Krebsbach PH, Yamada Y, Hammarström L, Simmer JP, Fincham AG, Snead ML, Smith CE (1998) Comparative immunochemical analyses of the developmental expression and distribution of ameloblastin and amelogenin in rat incisors. *J Histochem Cytochem* 46: 911-934.

Nanci A, Smith CE (2000) Matrix-mediated mineralization in enamel and the collagen-based hard tissues. In Goldberg M, Boskey A, Robinson C (eds) *Chemistry and Biology of Mineralized Tissues*, American Academy of Orthopaedic Surgeons, Rosemont, 217-224.

Ryu OH, Hu CC, Simmer JP (1998) Biochemical characterization of recombinant mouse amelogenins: Protein quantitation, proton absorption, and relative affinity for enamel crystals. *Connect Tissue Res* 39: 207-214.

Schmidt P, Erhard MH, Schams D, Hafner A, Folger S, Lösch U (1993) Chicken egg antibodoes for immunohistochemical labeling of growth hormone and prolactin in bovine pituitary gland. *J Histochem Cytochem* 41: 1441-1446.

Simmer JP, Lau EC, Hu C-C, Aoba T, Lacey M, Nelson D, Zeichner-David M, Snead ML, Slavkin HC, Fincham AG (1994) Isolation and characterization of a mouse amelogenin expressed in *Escherichia coli*. *Calcif Tissue Int* 54: 312-319.

Simmer JP, Snead ML (1995) Molecular biology of the amelogenin gene. In: *Dental enamel, formation to destruction* (Robinson C, Kirkham J, Shore R, eds), Boca Raton: CRC Press, Inc, 59-83.

Smith CE, Nanci A (1989) A method for sampling the stages of amelogenesis on mandibular rat incisors using the molars as a reference for dissection. *Anat Rec* 225: 257-266.

Smith CE, Pompura JR, Borenstein S, Fazel A, Nanci A (1989) Degradation and loss of matrix proteins from developing enamel. *Anat Rec* 224: 292-316.

Smith CE, Nanci A (1996) The protein dynamics of amelogenesis. *Anat Rec* 245: 219-234.

Warshawsky H, Moore G (1967) A technique for the fixation and decalcification of rat incisors for electron microscopy. *J Histochem Cytochem* 15: 542-549.

**Figure Legends**

**Figure 1**: Immunoblot of whole enamel organ cells and enamel extracts probed with chicken egg yolk M179y antibody . Lanes S1-M3 represent contiguous strips dissected from the same incisor. Standard broad-range molecular weight marker proteins stained with Ponceau S are shown on the left side of the figure. Cell extracts only show 1 major immunoreactive band whereas 5 bands are revealed in the enamel samples. The most intensely-stained band is at 27 kDa and corresponds to the major amelogenin secreted by rat incisor ameloblasts. S, secretory stage; M, maturation stage.

**Figure 2**: Immunocytochemical preparation illustrating the labeling detected over the Golgi apparatus of a secretory stage ameloblast with the M179y antibody. The absence of gold particles over mitochondria (m) and the nucleus (N) indicates a very low level of background labeling. rER, rough endoplasmic reticulum.

**Figure 3**: Cross-cut view of the distal portion of a Tomes' process (Tomes) and its associated forming enamel rod. Secretory granules (sg) in the process are labeled by the M179y antibody. Both the rod and surrounding interrod enamel show intense reactivity. Note, however, the paucity of gold particles near the cell surface, in the area (arrows) corresponding to the rod growth site (RGS).

**Figure 4**: Immunocytochemical preparation illustrating the increasing gradient of labeling over a forming interrod enamel prong. The area (arrows) at the extremity of the prong where enamel crystallites actively elongate, the interrod growth site (IRGS),

shows substantially fewer gold particles. dpTP, distal portion of Tomes' process; ppTP, proximal portion of Tomes' process.

**Figure 5**: Micrographs from early maturation stage specimens labeled with M179y antibody. Ameloblasts at this stage still exhibit immunoreactivity over the Golgi apparatus (Golgi) and secretory granules (sg). A significant amount of labeling for secretory forms of amelogenin is still observed over enamel. ly, lysosome-like element.

**Figure 6**: Preparations from the secretory (A) and mid-maturation (B) stage comparing the labeling obtained with M179y antibody over enamel in the region near the dentino-enamel junction (arrows). During the secretory stage, there is a concentration of gold particles over this region. By mid-maturation, the labeling is significantly reduced and the accumulation of particles at this site is no longer readily apparent visually but can still be detected by quantitative analyses (see figure 7). There are almost no gold particles over dentin, confirming the specificity of the antibody.

**Figure 7**: Box plots of mean density of immunolabeling (± SD) by stage (top) and by sampling location within the enamel layer (bottom). There is a significant decrease in labeling density for secretory forms of amelogenins as the developing enamel ages (matures) ($p < 0.0000$ for SEC to MMAT). Regional analyses indicate that secretory forms are in higher concentration in the surface portion of the enamel layer than in deeper areas of the enamel ($p < 0.0000$ for Near AM to Middle). They also appear to slightly accumulate near the dentino-enamel junction (DEJ) (the difference is not

statistically significant however in these data compared to Middle). AM, ameloblasts; SEC, secretory stage; EMAT, early maturation stage; MMAT, midmaturation stage.

**Figure 8**: Composite box plots of mean density of immunolabeling (± SD) for all stages and the 3 regional locations across the thickness of the enamel layer. All stages show the same basic pattern in distribution of labeling with counts being highest over the surface portion of the enamel layer. Both secretory stage enamel (SEC) and mid-maturation stage enamel (MMAT) show a trend for higher counts over the portion of the enamel layer near the dentino-enamel junction (DEJ) than in the middle (Mdl) of the enamel layer (only the difference between Mdl and DEJ is significant at $p < 0.0000$ in SEC). AM, near ameloblasts; EMAT, early maturation stage.

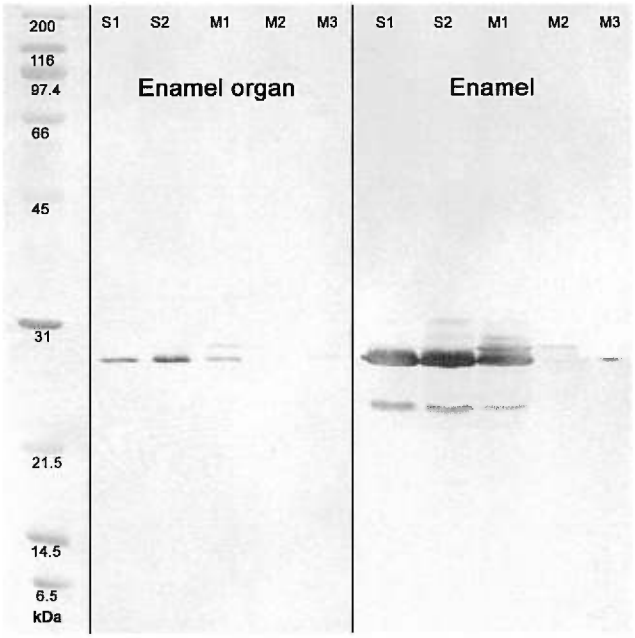



Figure 1

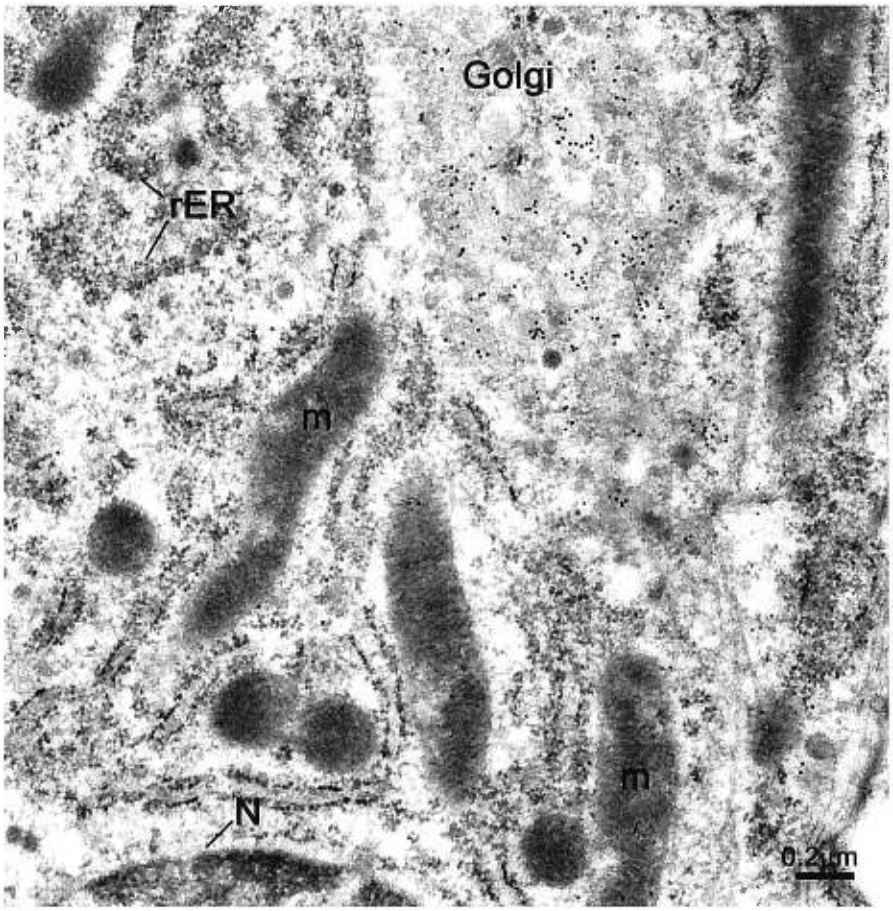



Figure 2

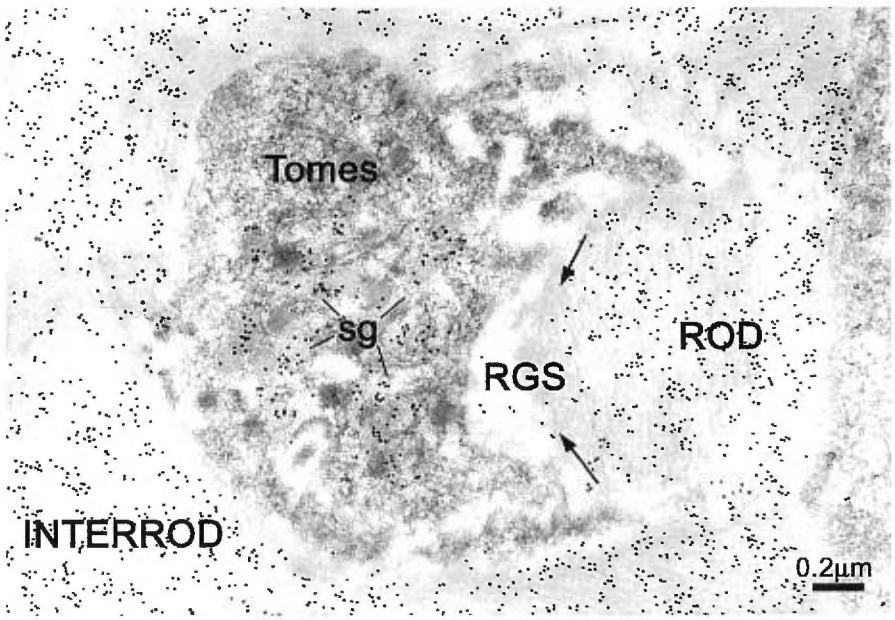



Figure 3

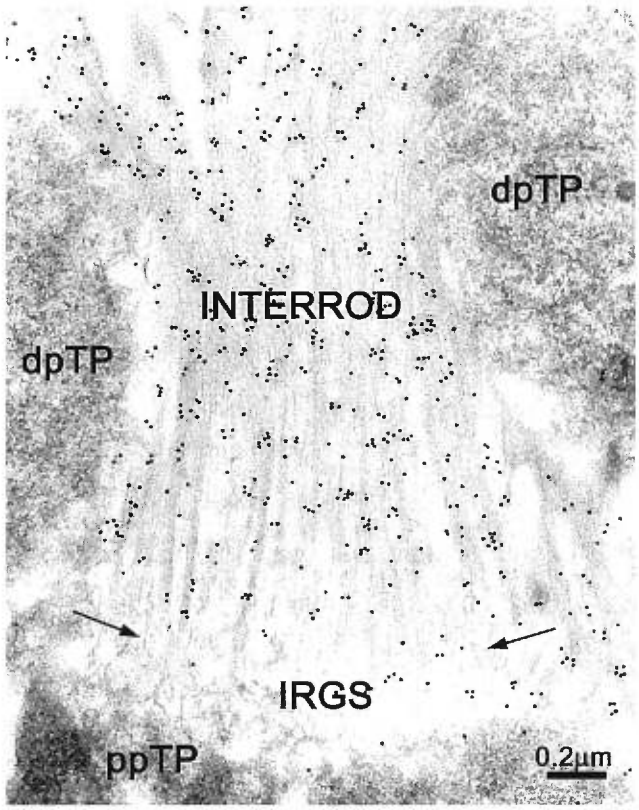



Figure 4

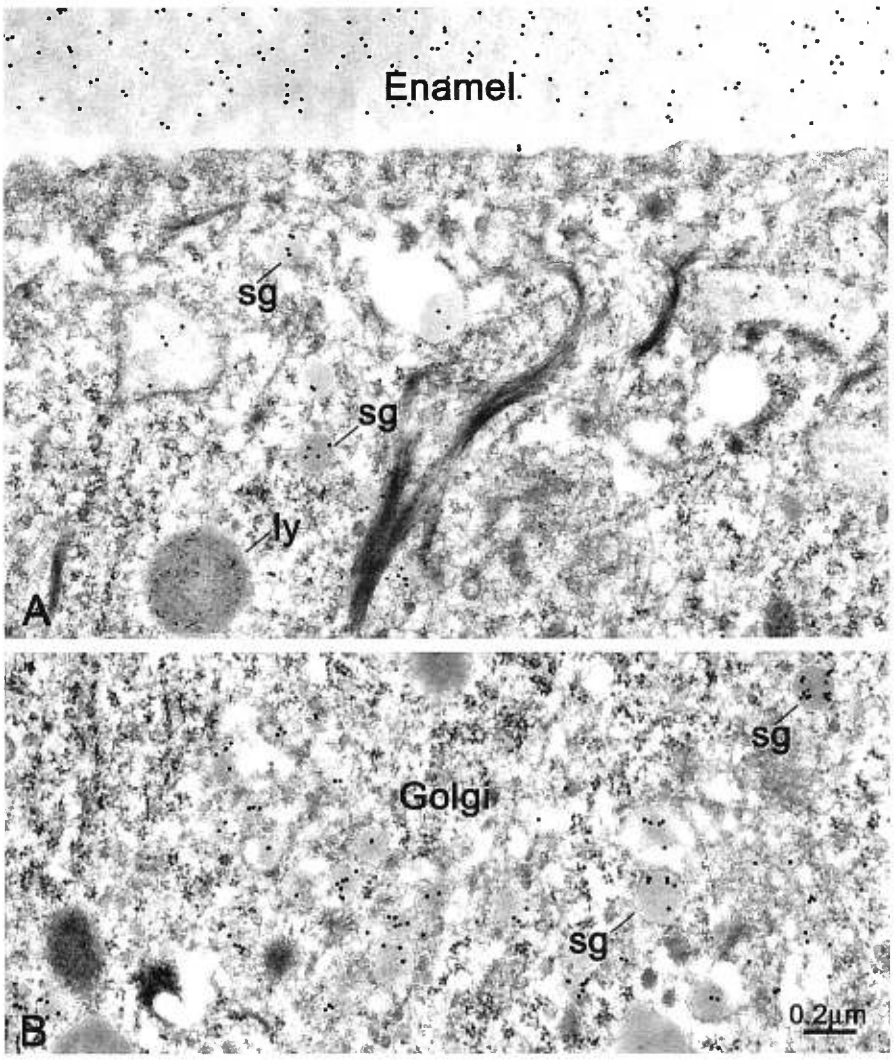



Figure 5

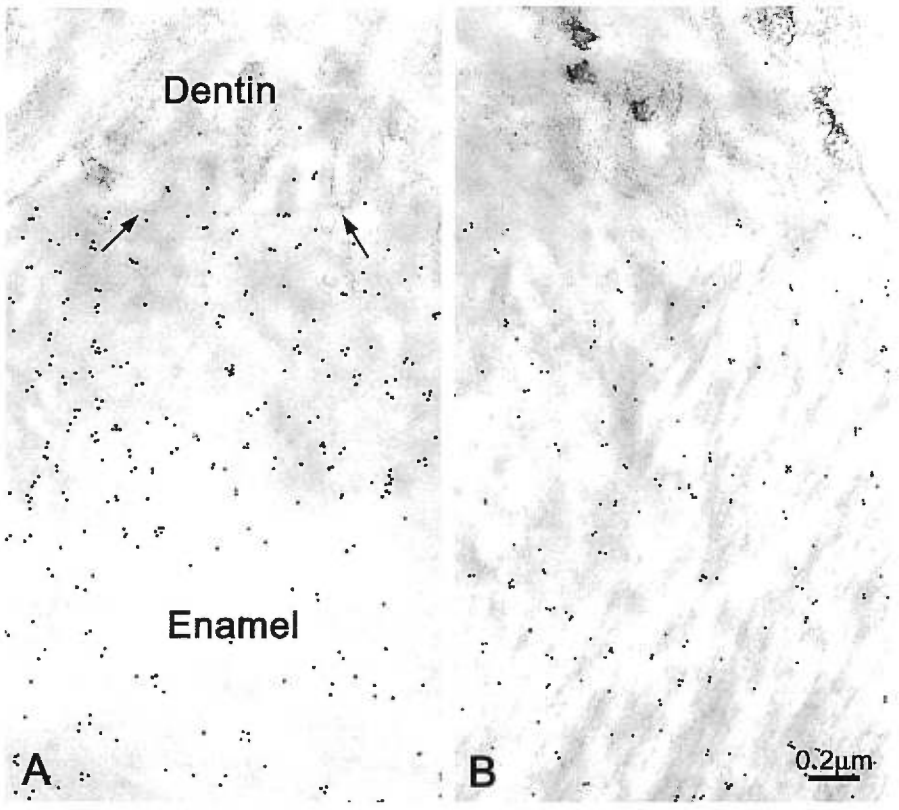
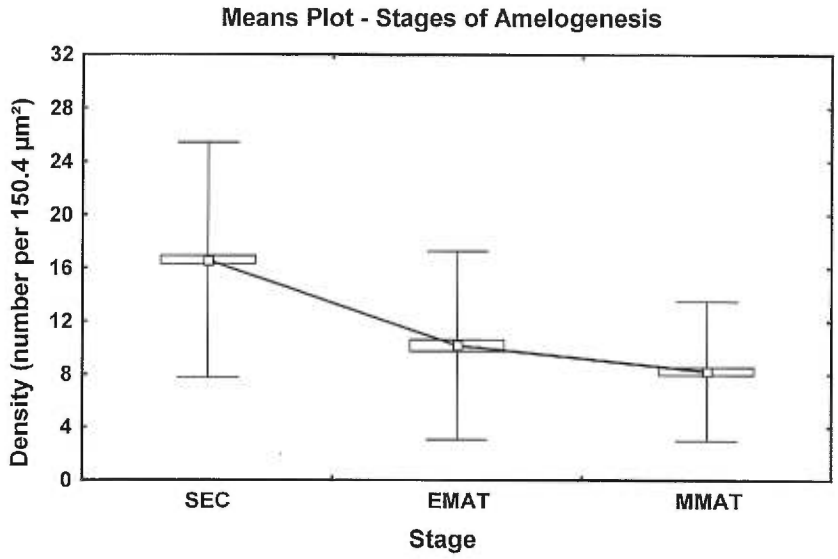



Figure 6



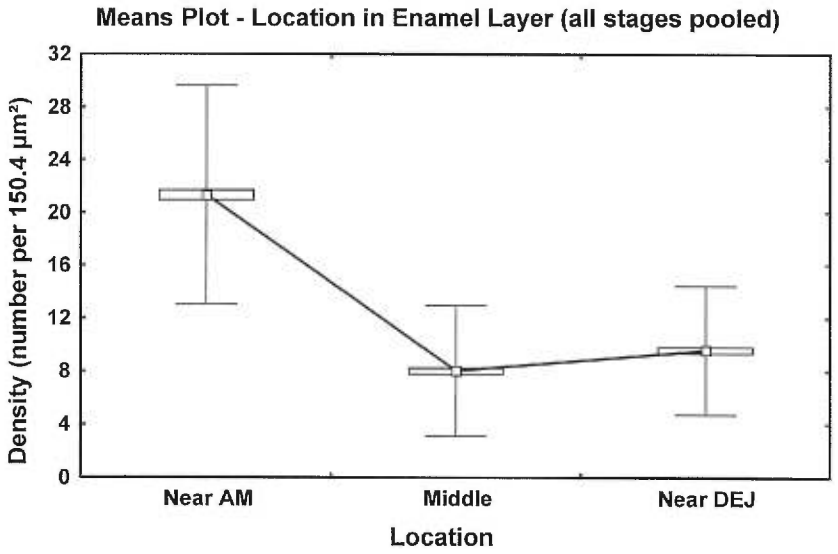




Figure 7

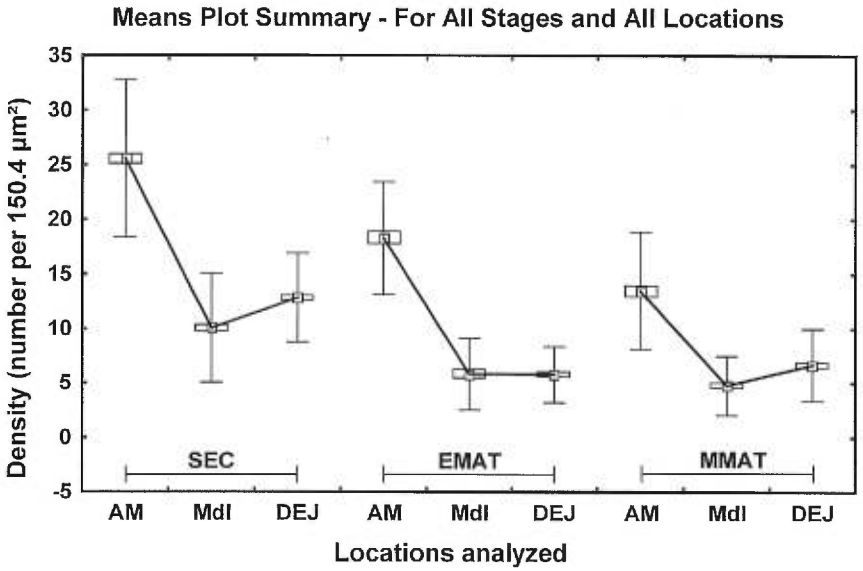



Figure 8

# Chapitre 3

As is the case of most scientific endeavors, discovery does not occur in a single moment, nor necessarily in the originally planned sequence of events. It is a combination of factors that together determine the development of the body of work. "There is a cyclic process at work which alternates between periods of hypothesis and insight, followed by periods of intensive investigation, and ending in a time of summing up or synthesis" (Moss, 1972).

The purpose of the material annexed in this chapter is to present additional results derived during progression of my research program and to discuss pertinent background information. Annex I reviews enamel formation and basement membranes. Annex II examines glycoproteins inhibitors and in more detail tunicamycin. Additional information on experimental procedure, technical considerations and results not included in chapters 1 and 2 are also presented. Annex III reports preliminary findings using growth factors and outlines future perspectives for the application of our model that may help advance our understanding of calcified tissues formation, and that may also lead to some strategies for the treatment of pathological alterations and repair of hard tissues.

# Annex I

# Amelogenesis and Basement membranes

**Amelogenesis**

Enamel development has been traditionally divided into 3 well-defined stages: the presecretory, secretory, and maturation stage (reviewed in Warshawsky, 1985; Nanci and Smith, 1992). During the presecretory stage of amelogenesis, ameloblasts differentiate and develop the organelles necessary for protein synthesis and secretion. Secretory stage ameloblasts are actively involved in the production of enamel proteins (EPs) and participate in the formation of the full thickness of the enamel layer. Ultimately, during the maturation stage, the majority of the previously deposited organic matrix is lost and the mineral content of the enamel layer increases dramatically. However, recent biochemical, immunocytochemical and radioautographic evidence have shown that ameloblasts secrete and degrade EPs throughout amelogenesis, and that the difference in secretory activities between stages essentially reflects the types and the quantities of proteins that are produced at any moment in time (reviewed in Nanci and Smith, 1992).

There are two major categories of EPs: (1) the smaller (22 to 30 kDa), proline-rich, hydrophobic amelogenins, and (2) the relatively large (48 to 70 kDa), hydrophilic nonamelogenins, which are acidic and show posttranslational modifications (reviewed in Fincham *et al.*, 1999; Robinson *et al.*, 1998). Amelogenins comprise the largest amount (>90%) of total EPs released by ameloblasts during the secretory stage (Simmer and Fincham, 1995; Nanci *et al.,* 1998). In addition to amelogenins and nonamelogenins, there is clear evidence that ameloblasts release several types of proteinases, including

metalloproteinases (Overall and Limeback, 1988; DenBesten *et al.* 1989; Fukae et al. 1998; Bartlett et al. 1997) and one group from serine proteinase family, including EMSP1 (Simmer *et al.* 1998). It is suspected their actions may be organized as a cascade, such that different enzymes act on different level and time of degradation (reviewed in Smith, 1998).

Ameloblasts undergo profound morphological changes during the maturation stage of amelogenesis (Smith and Nanci, 1995; Smith, 1998). Typically, this stage starts with post-secretory transition of the tall, columnar secretory stage cells into shorter maturation stage ameloblasts (Kallenbach, 1974; Warshawsky and Smith, 1974; Smith and Nanci, 1995).). The apical surface of maturation stage ameloblasts completes several ruffle to smooth-ended transitions during the period of time it takes enamel to mature, a process referred to as "modulation" (Smith *et al.*, 1987; Smith and Nanci, 1995). The predominant morphology of ameloblasts during the maturation stage is ruffle-ended (reviewed in Smith, 1998). Finally, the ameloblasts atrophy and regress to create a thin epithelial covering the mature enamel called the reduced EO (Warshawsky and Smith, 1974).

**Basement membranes**

Basement membranes (BMs) are a special form of extracellular matrix widely distributed in almost all organs of the body. These BMs lie adjacent to cells, which

secrete them and are associated with almost all linings of epithelial origin of the body (Alberts *et al.*, 1994a).

During tooth organogenesis, BMs play a significant role in tissue interactions and cytodifferentiation (Slavkin, 1990). Presecretory stage ameloblasts are separated from the underlying odontoblasts and predentin by a BM which is destroyed just prior to when they start secreting EPs (Sawada and Nanci, 1995; Kjoelby *et al.*, 1996; Meyer *et al.*, 1996). At the end of the secretory stage of amelogenesis, the cells form a basal lamina (BL) between the apices of ameloblasts and the maturing enamel (Takano, 1979; Nanci *et al.*, 1993). It is a unique structure which exists not between an epithelium and the connective tissue as can be expected, but between an epithelium (a layer of ameloblasts) and the enamel surface (an epithelial product) (Takano, 1979).

Composition of basement membranes

Glycoproteins are the major constituents of BMs; they are highly insoluble due to covalent and noncovalent interactions among them (reviewed in Timpl, 1996; Paulsson, 1992). While most BMs share a common set of structural components, these may be organized in significantly different ways (Abrahamson, 1986; Timpl, 1996). The main constituents of BMs are: isoforms of collagen type IV, proteoglycans, and noncollagenous glycoproteins such as the laminin and nidogen (reviewed in Aumailley and Smyth, 1998; Bernfield *et al.*, 1992; Burgeson *et al.*, 1994; Timpl and Brown, 1994; Ekblom *et al.*, 1998); fibronectin (Laurie *et al.*, 1983); anchoring elements, such as collagen type VII (reviewed in Brukner-Tuderman *et al.*, 1999).

Regarding the EO, the BM present during its early development (presecretion) is "typical" and contains laminin, type IV collagen, and proteoglycans (Sawada *et al.*, 1992; Meyer *et al.*, 1995). On the other hand, the BL related to maturation stage ameloblasts appears to be a specialized structure, enriched in certain glycocomponents, but in which "typical" BM constituents have so far not been consistently revealed (discussed in Nanci *et al.*, 1993).

Function of basement membranes

Several important biological functions are ascribed to BMs (reviewed in Alberts *et al.*, 1994a; Timpl, 1996). BMs are responsible for the proper maintenance and compartmentalization of tissue architecture, and their status determines repair after injury. They are thought to provide adequate anchorage for cells and thus maintain their polarized and differentiated state. They can control cell migration and invasion and are selective barriers in the filtration of macromolecules. Very importantly, they are implicated in epithelial mesenchymal-interactions and cell differentiation during embryonic development (Bernfield *et al.*, 1992; Marinkovich *et al.*, 1993; Xu *et al.*, 1990). In the tooth, development of cementum, dentin and enamel all depends on such interactions in which the BM is an important mediator (Meyer *et al.*, 1995; Ruch *et al.*, 1983; Sahlberg *et al.*, 1999; Slavkin, 1990; Thesleff *et al.*, 1991). On the other hand, the BL associated with maturation stage ameloblasts may serve to maintain these cells closely apposed to the surface of enamel layer. It has also been proposed to possess filtration properties, but its precise function is still not clear (Nanci *et al.*, 1993; see chapter 1).

**Why the incisor tooth of the rat?**

The continuously erupting rat incisor represents a model system for studying the cell biology of odontogenesis. The loss of tooth substance, due to the incisal attrition, is balanced by continuous cell division and production of new matrices at the growing (or apical) end. All stages of development can be found in a single tooth (Leblond and Warshawsky 1979) and these have been mapped with great precision in the 100 g rat (Smith and Nanci, 1989). Despite some obvious differences between rodent and human teeth, including limited eruption, size, time of formation, it is widely believed that there is a fundamental similarity in the basic structure and mode of formation of teeth in all species (Warshawsky *et al.*, 1981).

# Annex II

# Glycoproteins inhibitors studies

**Background**

Many important proteins in higher organisms contain carbohydrate side chains attached to the polypeptide. The carbohydrate chains may consist of oligosaccharides (2-10 carbohydrate residues) or short polysaccharides (usually 10-25 residues, although some are as large as 150 residues). In mammals, carbohydrates can be either N-glycosides (linked to the amido nitrogen of asparagine) or O-glycosides (linked to the hydroxyl oxygen of serine, threonine or, rarely, hydroxylysine). A single glycoprotein may have multiple chains, some of which are O-glycosides and some which are N-glycosides. It has been reported that N-glycosides are more commonly found in mammalian glycoproteins than O-glycosides (Elbein,1988).

Formation of N-linked glycoproteins involves synthesis of dolichol phosphate, attachment of sugars to this lipid, transfer of oligosaccharide to protein, processing of the oligosaccharide, and movement of the glycoprotein through the various cellular compartments. There are a number of inhibitors that block individual steps in the formation of lipid-linked oligosaccharides and others that prevent various processing reactions from occurring (reviewed in Elbein, 1988). The consequences of the protein not being glycosylated may vary widely, depending on the glycoprotein in question. In many cases, the absence of glycosylation causes marked alterations in the properties and fate of the molecule. However, in other cases, lack of carbohydrate has little or no effect on the protein in question.

**Tunicamycin**

Tunicamycin (TM) was originally isolated by Tamura and associates and was shown to be inhibitory towards Gram-positive bacteria, yeast, fungi, protozoa, enveloped viruses, and mammalian cells in culture (Takatsuki *et al.*, 1971). It is an antibiotic composed of uracyl, fatty acid, and two glycosidically (N and O) linked sugars (Takatsuki *et al.* 1975; Ito *et al.* 1980). Tunicamycin is produced as a mixture of at least ten homologous antibiotics that can be separated from each other by chromatography (Ito et al., 1980; Duskin and Mahoney, 1982). The site of inhibition of TM is the first reaction in the lipid-linked saccharide pathway (Takatsuki and Tamura, 1982). Tunicamycin binds very tightly to the enzyme which catalyzes this reaction, and it is only displaced with some difficulty by high substrate concentration (reviewed in Elbein, 1988). It was also shown to block protein glycosylation in vivo by the same mechanism as demonstrated in vitro. Suggested working concentrations range between 0.5- 5 μg/ml for inhibition of protein glycosylation in vitro or 0.1-10 μg/ml for inhibition of protein glycosylation in vivo (chemical specifications from Sigma). Tunicamycin is a highly toxic substance. The letal dose in mice is 6.5 mg/kg (oral); 1.8 mg/kg (subcutaneous or intravenous).

Tunicamycin has been mostly employed to block glycosylation in vitro (Chang and Korolev 1996; Lin *et al.*, 1999). On the other hand, only few studies have been carried out in vivo (Finnie and O'shea, 1988; Gonzales, 1981; Jago *et al.*, 1983; Leaver *et al.*, 1988; Michaels, 1980; Pow and Morris, 1992). In general, a significant difference between in vitro and in vivo inhibition of protein synthesis was observed. Biochemically, it has been shown that TM in culture systems reduces the carbohydrate

content of many glycoproteins in mammalian cells (Olden *et al.*, 1978). However, in some *in vivo* experiments, the ultrastructural and cytochemical findings showed minimal alterations (Michaels, 1980). Some in vitro studies have shown that TM interferes with the formation of many organs such as lung (Webster *et al.*, 1993), optic vesicle (Yang and Hilfer, 1982), testis (Kanai *et al.*, 1991), thyroid (Giraud and Franc, 1989), kidney (Ekblom *et al.*, 1979). A salient point emerging from all these investigations is the principal effect of TM on BM organization. The involvement of glycoconjugates of the BM in morphogenesis has been recently described for several organs (Durbeej and Ekblom, 1997; Ekblom *et al.*, 1998; Relan and Schuger, 1999; Schuger, 1997), including teeth (Thesleff and Pratt, 1980a,b). Tunicamycin may interfere with the ordered organization of constituents resulting in disintegration of the BM (Kanai *et al.*, 1991). The results of our work are consistent with these findings (see chapter 1).

## Experimental procedure

### Surgical procedure

In chapter 1, for journal editorial considerations, the surgical protocol for preparation of the bony window was briefly described. This section provides a comprehensive view of our experimental procedure. Previous studies have established the appropriate position of the bony window, identifying the posterior border of the ramus and the bony elevation overlying the apical end of the incisor as reliable reference points for drilling the hole (Vu *et al.*, 1999). Because of the confined and narrow space separating the alveolar bone

and the underlying EO, inappropriate position of the hole can either damage the apical end of the incisor or perforate the thin alveolar bone of the rat mandible. The rat were anesthetized and the vestibular surface of the right mandibular ramus was exposed as follows: an incision about 8 mm long was made through the skin along an imaginary line joining the auditory meatus and the lip commissure, to access the muscle layer. The masseter fibers were separated along their longitudinal axis with a scalpel blade and kept retracted with a plastic ring made from a slice of an embedding BEEM capsule size 1 (Marivac; Halifax, NS, Canada). A periosteal separator was used to elevate the periosteum and expose the underlying bony surface. The bony hole was drilled approximately 2 mm from the posterior border of the ramus, and its position estimated using a 1.8 mm dental Woodson condenser (Brassler, Montreal, QC, Canada). A slow-speed dental drill was used first with a carbide round burr (Brassler) size 010 and then a size 014 burr. Saline irrigation was used during drilling. Subsequently, to accomodate the selected Alzet osmotic minipump (Alza Corporation; Palo Alto, CA), another incision was made through the skin of the posterior area of the neck. The pump was tunneled into a subcutaneous pouch created with a hemostat and connected to the bony hole using a vinyl tubing, about 4 cm long, and a metal catheter, made from a 20G1 syringe needle (Becton-Dickinson; Rutherford, NJ) (Fig 1A). Passing the minipump catheter below the masseter muscle and immobilization the metal tip with Indermil glue and bone cement (see chemical specifications in chapter 1) did not always result in a firm anchorage. Sometimes the catheter tip detached from the hole, especially when the hole was not big enough to fully accommodate the catheter. To confirm the positioning and stability of the catheter in the bony window, latero-lateral and submandibular X-rays of the rats were made a few days after surgery, using bite wings dental films and

radiographic tube's impulses of 12. None of the rats with a detached catheter was included in our studies.

Bone erosion around the window was sometimes observed, probably due to inflammation. A study was therefore undertaken to examine the cause of this problem. The results showed that either excessive use of glue or the uncontrolled leaking of the liquid monomer of the bone cement induced bone erosion. Precise fit of the catheter into the hole is considered to be most important for its retention, whereas glue and cement provide additional support. Thus, in the final set of experiments, care was taken to use minimal amounts of these agents. After surgical placement of the minipump, the animals were sutured, using resorbable Plain gut 4-0 (Ethicon Inc., Somerville, NJ) for muscle, and 4-0 Silk (Sherwood Davis & Geck, Wayne, NJ) for skin (Fig1B). The surgical site was then cleaned and disinfected with 70% ethanol.

In order to evaluate any effect of TM treatment on the eruption rate of the incisor, a slight nick was made with a dental disc on the enamel of the two incisors, incisally to the free border of the gingiva. After 7 days, in rats implanted with saline minipumps, this line was displaced at the tip of the tooth and sometimes was no longer visible, consistent with the known eruption rate of approximately 650 μm/day (Smith and Warshawsky, 1975). On the other hand, the majority of TM-treated rats still showed the nick on the tooth and/or the incisor was shorter than the contralateral untreated tooth.

**Tunicamycin preparation, minipumps filling and working conditions**

Initial experiments were carried out to test the stability of TM and the effect of concentration and infusion time. For each study, we freshly prepared TM solutions. The minipumps were filled very slowly, using sterile conditions, paying particular attention to avoid creation of air bubbles, which can cause pumping rate fluctuations. ALZA Company suggests to place the prefilled pumps in 0.9% saline for at least 4 hours at 37°C before implantation so that the pumping rate will attain a steady state and release starts upon placement. Although this protocol was followed for 1 day pumps, for pumps of longer duration we only did a 1 hour preincubation.

Initially, concentrations of 0.5 μg/ml and 1.0 μg/ml were used to fill 1 and 3 day osmotic minipumps. In some cases, 1:20.000 i.u./ml heparin was added to the TM solution to avoid clotting around the catheter. However, solutions containing heparin and those without, produced similar histological results, and, therefore, we have opted not to continue using heparin. When a concentration of 0.5 μg/ml was used, the effect obtained on tooth tissues was not consistent. Thus, a concentration of 1.0 μg/ml infused for 3 days was tested. In this case, some morphological changes, especially at the level of the BL associated with maturation stage ameloblasts, were observed. To obtain a more consistent effect and because at this dose there were no major changes in the EO structure and the cells were generally in a good state, we felt that the rats could tolerate a higher dose of TM. Thus, the TM concentration was increased to 10 μg/ml and drug exposure extended to 7 days. This protocol was adopted for the final studies, since it caused a definite and constant effect on the BL, without major signs of toxicity. In some

cases, there were localized alterations in odontoblasts and ameloblasts. However, no alterations in other organs such as liver, duodenum, kidney, parotid and brain were observed. This suggested that very little drug leaked into the general circulation and that its effect was mostly local.

**Tissue processing**

The procedure briefly described in chapter 1 was followed. The decalcification agent EDTA (Warshawsky and Moore, 1967) is already well-known and has been extensively used in our laboratory, without any significant effect on immunolabeling. On the other hand, the decalcification with a solution consisting of hexahydrate aluminum chloride, chloridric acid, formic acid and distilled water (see specifications chapter 1; Plank and Rychlo, 1952) has been less documented, especially regarding its compatibility with immunocytochemistry. The advantage of using the latter agent is the speed at which it acts, allowing rapid evaluation of specimens. This rapid method of decalcification did not interfere with the immuno- and lectin- gold cytochemical studies and, therefore, was routinely applied for all our screening studies.

**Technical considerations**

**Light microscopy analysis**

Light microscopy was used to analyze and select the tooth organ areas which showed alterations in treated animals and verify that such alterations were not present in control

rats infused with saline. Firstly, the position of the hole and its relation with the apical end of the incisor was examined. Only rats in which the bony window was situated a short distance away from the EO (Fig 2A) were included in our studies. In those animals where the hole was created anteriorly to the reference points, major damage was caused to the incisor. However, incisally to the site of damage, the EO appeared to develop normally, as reported in previous studies (Vu *et al.*, 1999).

Secondly, presecretory and secretory stage ameloblasts were examined for any sign of alteration. Control rats implanted with saline filled minipumps exhibited no alterations. Tunicamycin treatment frequently induced structural modifications of ameloblasts, particularly in the secretory stage (Fig 2B). In addition, some areas of enamel near the dentino-enamel junction stained less intensely, suggesting that there was less matrix at these sites (Fig 2C). There were also focal areas where odontoblasts were absent (Fig 2B). Odontoblastic cells seemed to be more affected than ameloblasts.

Thirdly, we examined we concentrated our attention on the early and midmaturation stage of amelogenesis. Saline-infused rats showed no signs of abnormality. Treated rats, on the other hand, always presented a slight irregularity in the appearance of the BL interposed between maturation stage ameloblasts and maturing enamel. The findings observed in early and mid maturation stage have been discussed in chapter 1.

**Electron microscopy analysis of the secretory stage in TM-treated incisors**

At the ultrastructural level, it was readily apparent that both the organization and shape of secretory stage ameloblasts were disrupted. The Golgi apparatus appeared structurally

normal but the shape and orientation of Tomes' processes was altered. There was an intense immunolabeling over the Golgi apparatus with various anti-amelogenin antibodies (Fig 3A), however, there were very few secretory granules in Tomes' processes (Fig 3B). Taken together these data suggest that protein synthetis was not significantly affected, but that either formation and/or translocation of secretory granules were inhibited. Sometimes the newly-secreted enamel matrix was thin and contained cell debris. There were areas near the dentin-enamel junction (DEJ) showing less matrix, suggesting accelerated matrix degradation of these sites.

**Conclusion**

In conclusion, our studies show that the surgical window approach is a reliable method to administer locally a toxic pharmacological agent, without any significant systemic side effects. Tunicamycin delivered in this manner did not induce any major alteration of the enamel, consistent with the fact that this extracellular matrix, compared to other calcified tissues, is poor in N-linked glycoconjugates. However, it affected the integrity of the BL, which separates ameloblasts from maturing enamel. The data on secretory stage ameloblasts suggest that TM may affect the enamel protein handling by these cells and possibly their short-term extracellular processing.

# Annex III

# Growth factors studies

**Background**

The "bony window" surgical model is well adapted for local administration of biological agents such as antibodies, extracellular matrix (ECM) molecules and various growth factors (GFs). In the following set of experiments, we have infused a mixture of GFs to elucidate its effects on tooth and especially on bone formation.

Bone is a living, dynamic tissue with substantial capacity for regeneration. It is continuously remodeled and involved in calcium homeostasis, continuously ongoing in the formation and resorption processes (Parfitt, 1987). The systemic hormonal regulation of bone is well understood. However, local control still remains to be defined with precision, but is receiving increasing attentions. Calcified tissues are known to contain GFs and these are believed to locally influence their formation (Canalis *et al.*, 1988). Growth factors regulate general cellular activities and organ morphogenesis as well as formation and resorption of bone (Mohan and Baylink, 1991; Jilka, 1998; Kuboki *et al.*, 1997). These local factors can be synthesized by bone-forming cells, although some cytokines are secreted by stromal cells and by cells of the immune or hematopoietic system (Canalis *et al.*, 1993; Manolagas and Jilka, 1995). They can influence cells of the same class (autocrine factors) or cells of another class within the tissue (paracrine factors), by binding to cell-surface receptors (Lin and Lodish, 1993; Massague, 1992). Growth factors are part of a cascade and generally act in synergy (Lynch *et al.*, 1989). Their activity is regulated by a "feed-back" mechanism, in which the quantity of the released factor will modulate its production, according to the biological needs, by changes in synthesis, activation, receptor binding, and binding proteins (reviewed in

Bostrom, 1998). In experimental models, GFs have been shown to promote fracture healing, osseointegration of biomaterials, periodontal repair/regeneration, and to control bone loss in osteopathies (Linkhart *et al.*, 1996; Lynch *et al.*,1991; Rutheford *et al.*, 1993). They also act as morphogenes mediating inductive interactions throughout tooth development (Aberg *et al.*, 1997; Thesleff and Aberg, 2000; St. Amand *et al.*, 2000).

Although osteoblasts and other cells produce many GFs and cytokines, we will focus on the following systems: transforming growth factorß (TGFß) superfamily (which includes Bone Morphogenetic Proteins, "BMPs"); fibroblast growth factors (FGFs); insulin-like growth factors (IGFs); and platelet derived growth factors (PDGFs). However, a number of other factors, such as: interleuchines, epidermal growth factors (EGFs), TGFα, granulocyte/macrophage (GM) colony-stimulating factors (CSFs), tumor necrosis factor (TNFs), leukemia inhibitory factors (LIFs) and prostaglandins may play a role in bone metabolism, as well as in morphogenetic processes (reviewed in Marks and Hermey, 1996; Lian *et al.*, 1999).

**The TGFß system**

Transforming growth factorß superfamily includes: "transforming growth factor-betas" (TGFßs), activins/inhibins and BMPs (Alberts *et al.*, 1994b; Centrella *et al.*, 1994). Its various constituents have been implicated in a multitude of cellular functions, including regulation of cell proliferation, modulation of extracellular matrix synthesis, promotion of wound healing (Lian *et al.*, 1999; Toyono *et al.*, 1997). TGFß signals are transmitted from the cell-surface to the nucleus by the SMAD proteins pathway (reviewed in Hill,

1999). The effects of TGF-ßs (from -ß1 to -ß5) appear to be highly dependent upon bone cell source, dose applied, and local environment. The best known member of this group is TGFß1 and it has been implicated in tissue repair, periodontal regeneration and bone induction (Giannobile, 1996; Ripamonti *et al.*, 1997). In recent years, several studies have demonstrated a strong relationship between expression of TGFß1 and differentiation processes during embryogenesis and development of periodontium (Gao *et al.*, 1998; Kingsley, 1994).

**The BMP system**

The BMPs can induce cartilage and bone formation and are now of considerable interest as therapeutic agents for healing fractures and periodontal bone defects, and for inducing bone growth around implants and prostheses (Cochran *et al.*, 1999; Lindholm, 1996; Reddi and Cunningham, 1993; Ripamonti, 1996; Ripamonti and Reddi, 1997; Urist, 1994; Wozney, 1992, 1995). In addition to their capacity to induce ectopic bone formation, BMPs have widespread signaling functions throughout embryogenesis and have been conserved during evolution (Kingsley, 1994; Hogan, 1996). At least 15 BMPs (including the related family of growth and differentiation factors, "GDFs") have been cloned, and all of them belong to the TGFß superfamily, except for BMP-1 (reviewed in Reddi, 1998). The BMP-2, -3, -4, -5 and -7 are parts of signaling networks regulating tooth initiation and shape development (Vaahtokari *et al.*, 1996), and may be involved in the induction and formation of dentin, enamel and cementum (Aberg *et al.*, 1997; Ripamonti *et al.*, 1996). Recombinant BMPs (rhBMPs) have been shown to stimulate the formation of reparative dentin (Nakashima, 1994; Rutheford *et al.*, 1994b), and to be

osteoinductive in many experimental systems (Hedner and Linde, 1995; Kobayashi *et al.*, 1999; Ripamonti *et al.*, 1996; Sirgurdsson *et al.*, 1995; Wozney, 1995; Yoshida *et al.*, 1999), however the degree of their efficacy is still debated.

**The FGF system**

Fibroblast growth factors comprise a family of structurally related proteins, at the present time numbering eighteen (Hu *et al.*, 1999; Wang *et al.*, 2000). They are expressed in specific spatial and temporal patterns and involved in developmental processes such as angiogenesis, wound healing, and tumorigenesis (reviewed in Radomsky *et al.*, 1998). The most studied FGFs are acidic FGF (aFGF) and basic FGF (bFGF), which play a crucial role in the bone healing process, especially bFGF (Nakamura *et al.*, 1998). Depending on the dose and duration of treatment, they have been shown to stimulate or inhibit the terminal differentiation of osteoblasts (Nagai *et al.*, 1995). Moreover, they can influence osteoclastogenesis and bone resorption (Hurley and Follorkiewicz, 1996; Hurley *et al.*, 1992). The role of bFGF on periodontal fibroblasts activity, cementogenesis and tooth organogenesis has not yet been well elucidated (reviewed in Giannobile, 1996).

**The IGF system**

Insulin-like growth factors are polypeptides which act as local regulators of cell metabolism. Two IGFs have been well characterized, IGF-I and IGF-II (Hill *et al.*, 1995). Recent findings demonstrate that the local actions of IGFs may be controlled

by multiple components of the IGF regulatory system, including IGF variants, IGF receptors, IGF binding proteins (IGFBP), and extracellular IGFBP proteases (Hayden *et al.*, 1995; Mohan, 1993). IGFs stimulate bone formation in vitro and in vivo (reviewed in Linkhart *et al.*, 1996). They also directly enhance formation of osteoclasts from precursor cells. A recent in vitro report has demonstrated the expression of IGFs and IGFBPs during tooth morphogenesis (reviewed in Takahashi *et al.*, 1997), particularly increasing enamel extracellular matrix formation. The IGF signaling pathway may act during amelogenesis, by controlling transcriptional regulation of specific EPs like amelogenin and ameloblastin (Takahashi *et al.*, 1997).

**The PDGF system**

Platelet-derived growth factor was initially isolated from blood platelets and is considered important in the early phases of wound repair (reviewed in Canalis and Rydziel, 1996). Its various forms may also act as a systemic or local regulator of tissue growth. PDGFs can promote bone and cartilagine formation (Howes *et al.*, 1988). The principal effect of this factor has been demonstrated on cells from periodontal tissues, by enhancing periodontal repair and furcation defects regeneration (reviewed in Giannobile, 1996; Park *et al.*, 1995). Indeed, PDGF is chemotactic for periodontal ligament fibroblasts (PLFs) and promotes collagen and total protein synthesis (Matsuda *et al.*, 1992).

**Combinations of GFs**

From recent reports, it appears evident that combinations or "cocktails" of various GFs generally result in greater activity, to either regenerate bone or to specifically act on dental and periodontal tissues. In calvarial organ cultures, the combination of IGF-I and TGF-ß1, or IGF-I and PDGF increased bone matrix apposition more than TGF-ß1, PDGF or IGF-I individually (reviewed in Giannobile, 1996). In vivo studies have shown that the PDGF/IGF-I combination promoted new bone, cementum, and periodontal ligament (Lynch *et al.*, 1991; Rutherford *et al.*, 1994a; Howell *et al.*, 1997). Few studies have tested the role of other factors such as bFGF and IGF-II, and other combinations with varying concentrations remain to be tested.

**Methods of delivery GFs**

In order to establish the efficacy of GF based therapies, concentration, dose, and carriers have to be taken in consideration. The effective use of GFs for tissue repair requires that they be administered in a way which provides localized and controlled release of the factor and establishes an environment that promotes tissue regeneration (Radomsky *et al.*, 1998). Carrier-mediated delivery using natural or artificial matrices can be used (Radomsky *et al.*, 1998; Winn *et al.*, 1998; Jiang *et al.*, 1999). Covalent attachment to surfaces (Ito *et al.*,1991, 1993, 1998; Liu *et al.*, 1993; Zheng *et al.*, 1994) and uptake from tissue fluids (using "self-sufficient" biomaterials) have been also suggested (Ripamonti *et al.*, 1999; Nanci *et al.*, 2000b).

Recent studies of GFs actions *in vivo* have concentrated on development of improved delivery systems and conditions for treating bone defects as well as for investigating calcified tissue development and repair. The above described "bony window" model represents a valid system to investigate these aspects.

**Experimental procedure**

Minipump implantation was performed in male Wistar rats as described in chapter 1. The substance used to fill the minipumps was a solution containing a cocktail of GFs derived from porcine platelets (Theratechnologies Inc., Ville Saint Laurent, QC, Canada). TGFß1,TGFß2 and various isoforms of PDGF are the major components of this cocktail. The minipumps were filled using the prepared mixture and then soaked in 0.9% saline for 3 hours at 37°C. The infusion time was varied, using 1-3 and 7 days pumps. Controls consisted of saline infused rats. Some animals were first implanted with saline minipumps, which at 3 days were then substituted with GFs filled ones. In order to exchange the minipumps, the back incision on the neck area was reopened. The vinyl tube connected to the bony hole was left in place and the saline filled minipump was substituted with the one containing the GFs mixture.

**Technical considerations**

Because of the complexity of the produced effects, only LM observations were carried out. The drill site region was evaluated in GFs treated rats and compared to the similar

region in saline infused animals. TEM analyses and evaluation of GFs on the tooth EO will be the subject of future studies.

Briefly, the cellular events occurring during bone repair comprise three stages (Bostrom, 1998): (1) bleeding and coagulation followed by proliferation of mesenchymal cells; (2) differentiation of mesenchymal cells into cartilage- and/or bone-forming cells; and (3) repair and remodeling of the bone tissue. The second stage of the repair process determines the nature of the regenerated tissue and is essential for bone tissue recovery. It is at this stage that mesenchymal stem cells interact with the GFs released from the damaged bone to induce stem cell proliferation and differentiation into bone-forming cells (Charles, 1992; Diegelmann, 1997). GFs will also act on the remaining differentiated osteogenic cells, by controlling their activity.

At the macroscopic level, there was an increase in bone volume in hemimandibles infused with GFs (Fig 4A). In some cases, slight bone erosion was present around the bony hole (Fig 4B). There was stimulation of bone formation at all infusion intervals investigated, but it was particularly strong when the GFs mixture was infused for 7 days. Histologically, in both GFs and saline infused rats, there were some signs of an inflammatory reaction at the drill site (Fig 5A). GFs-treated rats showed a pronounced healing of the bony hole, characterized by abundant bone trabeculae, the presence of numerous mitotic cells and prominent Golgi regions in osteoblasts (Fig 5B). They also showed signs of bone remodeling, as evidence from the abundance of osteoclasts (Fig 6C). Colonies of hemopoietic cells with newly generated marrow and intense vascular

activity were also observed. On the other hand, only small amounts of newly formed bone were seen in the saline infused rats.

**Conclusion**

This set of experiments represents another approach (carrier strategy) to deliver GFs. The preliminary results obtained indicate that the cocktail of GFs used may serve as a potential therapeutic agent for bone repair. The experiments also show the flexibility of the infusion model, particularly as regards exchange of minipumps after their surgical placement. In particular, it will be interesting to examine the inductive role that GFs have during tooth morphogenesis and development. The positive results obtained with the GFs mixture warrant further studies such as a time-course and dosage effect, as well as the analysis of its function on dental tissues.

**Initial studies and future prospectives for gene transfer studies**

Gene therapy is a powerful tool for the treatment of a wide variety of diseases (Prince, 1998). It can be defined as the introduction of nucleic acids into cells, which can result in a therapeutic benefit to the individual (Gottschalk and Chan, 1998). The success of gene therapy is largely based on the delivery system for transfer of genetic information to a specific tissue through the use of recombinant technology. Gene delivery vehicles can basically be divided into viral (transduction) and non viral (transfection) systems.

The feasibility of human gene transfer has been demonstrated with retrovirus and adenovirus (Feng *et al.*, 1997; Massie *et al.*, 1998), plasmid liposomes (Lieberman *et al.*, 1998), herpes simplex virus (Fink *et al.*, 1996), naked DNA (Budker *et al.*, 1996), adeno-associated virus, electroporation, gene gun or particle bombardment (reviewed in Prince, 1998).

Nowadays, few studies have explored gene transfer for the treatment of oral diseases. However, just like for other medical indications, gene therapy may become an integral tool in dental practice early in the 21st century (Baum *et al.*, 1998). The major application to date has been in the use of gene vectors either to prevent or to retard the growth of oral carcinomas (Shillitoe, 1998; Cardinali *et al.*, 1998). Some authors reported in vivo gene transfer to salivary glands of animals (Delporte *et al.*, 1998; O'Connell et al., 1998; He et al., 1998). In a recent article, Taichman *et al.* (1998) discussed the use of gene transfer for periodontal therapy. Since the use of gene therapy in orthopaedics is already ongoing (Evans and Robbins, 1995), it is predictable that gene transfer will also be efficient for the treatment of oral diseases. Potential applications are the repair of genetic defects and the reduction of bone loss during periodontal disease. The strategy will be to introduce a selective vector, which contains appropriate promoter and enhancer elements, carrying the gene that encodes for the factor to transfer. It would be best if the expression of the selected substances were exclusively directed towards the cell population to be targeted. No specific promoters have yet been found for odontogenic cells, but some data suggest that such promoters could be developed (Reynolds *et al.*, 1999). The experimental surgical model applied here represents a valid

method to evaluate vectors and the effect of both gene knock-in and knock-out on hard tissue formation in the rat hemimandible. A recent report has demonstrated the applicability of the window technique as a gene transfer model (Daniel *et al.*, 2000).

In conclusion, the bony window system should provide an excellent in vivo approach of acting on the genes responsible for the production of target growth factors and matrix proteins. This could lead to greater biologic responsiveness, since the endogenously synthesized protein may continue to exert its effect so far an extended time (Niyibizi *et al.*, 1998). The choice of the most appropriate gene delivery vehicle is still in course of study (Reynolds *et al.*, 1999; Riew *et al.*, 1998). All the present efforts are directed in finding an efficient and safe method which could result in therapeutic levels of gene expression of GFs for the necessary period of time, in the target cell population. Despite known difficulties with transfecting and/or transducting cells in vivo, the preliminary data are very encouraging, but much more remains to be done.

# Figures

**Figure legends**

**Figure 1**: (A) Micrograph illustrating the metal catheter (Cat) fitted into the bony window (arrow) and the vinyl tubing (Vt) connected to the osmotic minipump (Mp). (B) After inserting the minipump (Mp) through a subcutaneous pouch in posterior area of the neck (*), the animal is sutured.

**Figure 2**: Light micrographs from tunicamycin (TM)-treated incisors illustrating (A) the hole drilled through the mandibular bone, (B) histological alterations in the ameloblasts (Am) and odontoblasts (Od) layers, and their associated extracellular matrices, and (C) localized areas of hypoplastic enamel (arrowheads) near the dentino-enamel junction. D, dentin. E, enamel.

**Figure 3**: (A) The antibody raised against the mouse recombinant 179 amelogenin isoform (M179y) shows intense labeling over the Golgi apparatus of secretory stage ameloblasts from tunicamycin (TM)-treated rats. (B) However, Tomes' processes exhibit an abnormal shape and contain few secretory granules (sg). ly, lysosome-like element.

**Figure 5**: (A) The bone of the hemimandible infused with growth factors (GFs) is thicker than that of saline-infused controls. (B) Sometimes there is a slight erosion of the bone surrounding the drill site, resulting from the inflammatory response induced by the adhesives used to retain the metal catheter.

**Figure 6**: Light micrographs from growth factors (GFs)-treated hemimandibles illustrating the presence (A) of inflammatory cells in the bony hole (arrows), (B) of abundant newly-formed bone trabeculae surrounding the drill site (*), and (C) of numerous osteoclasts (Ocl), suggesting active bone remodeling.

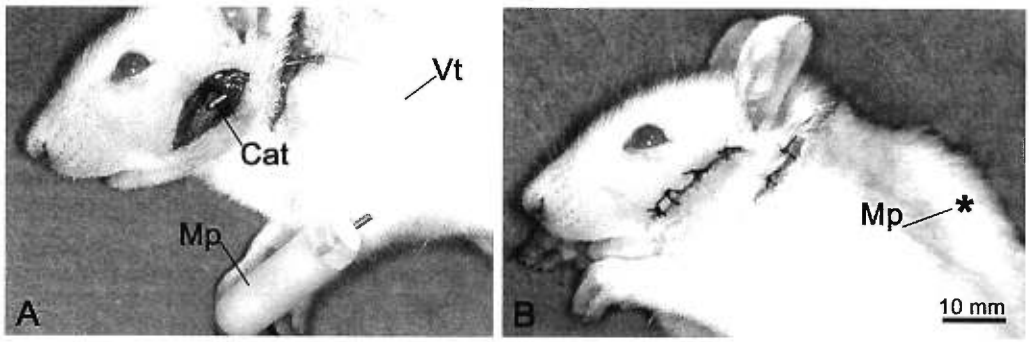



Figure 1

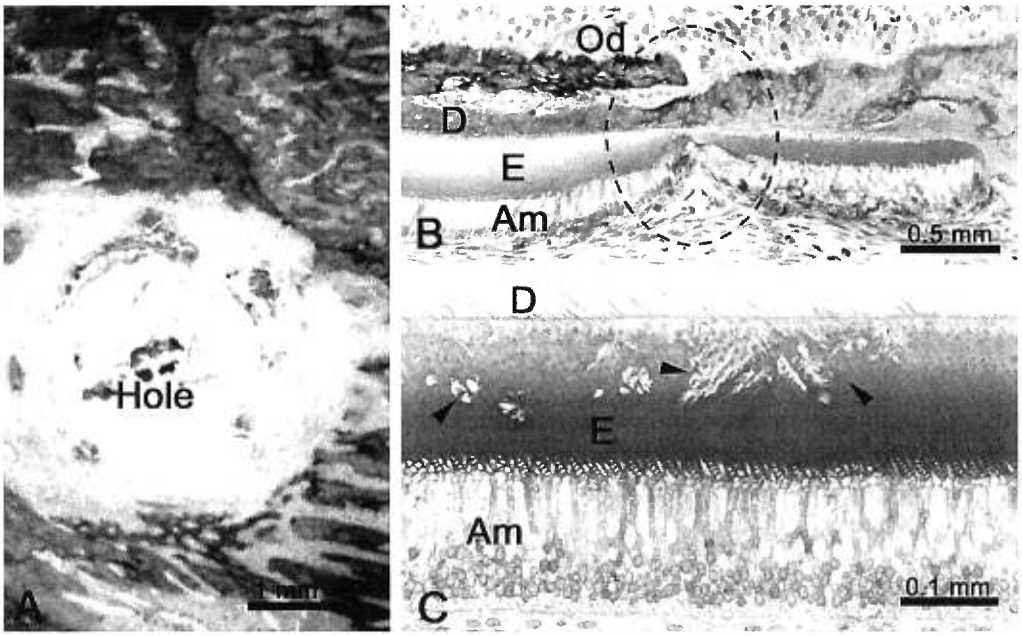



Figure 2

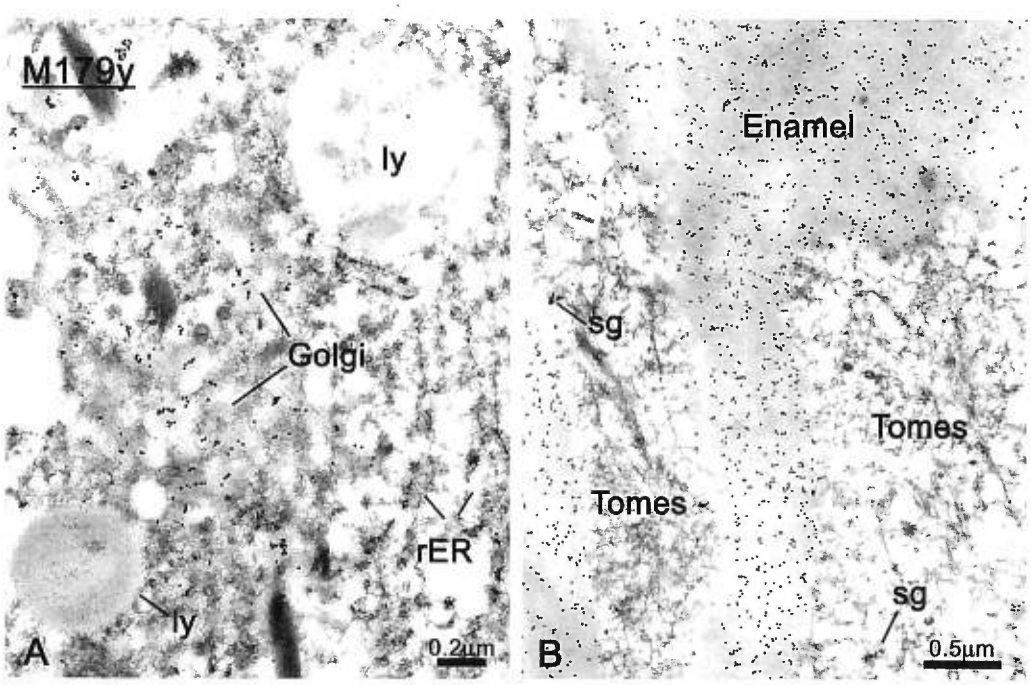



Figure 3

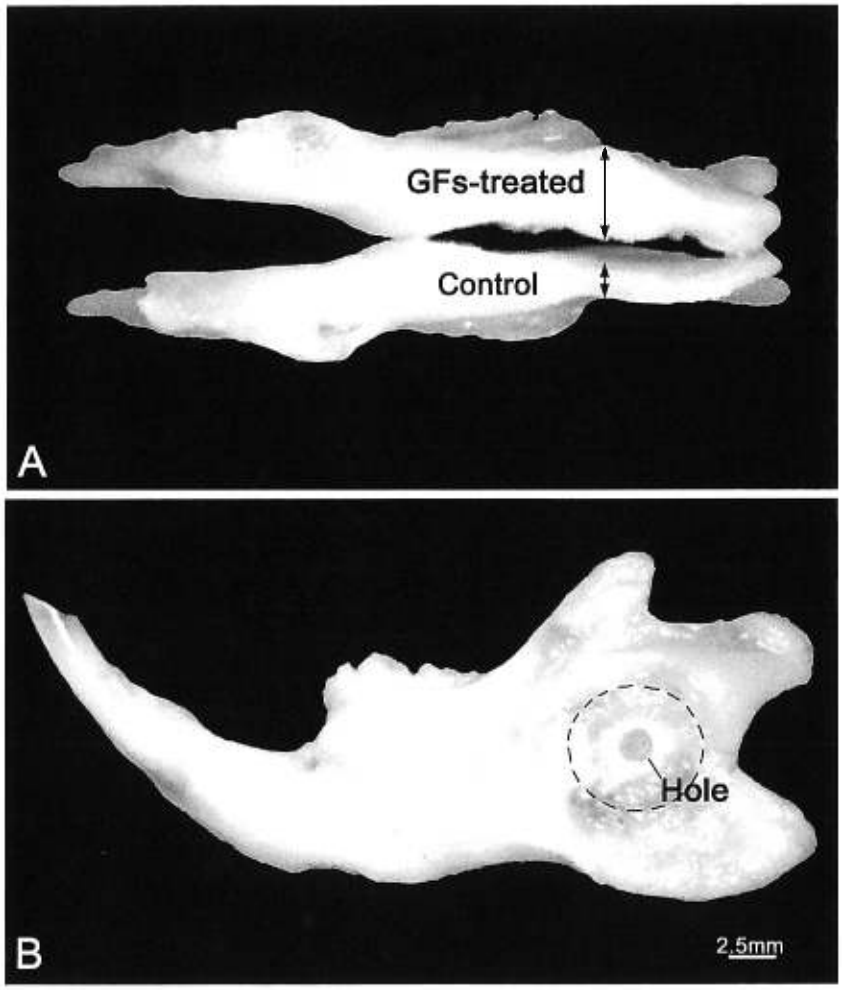



Figure 4

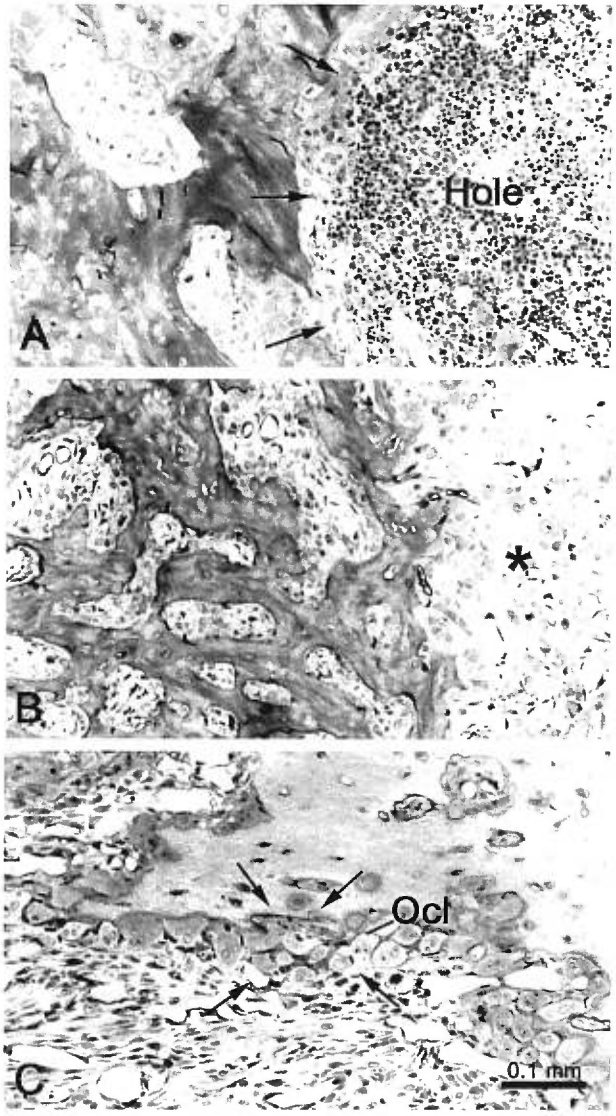



Figure 5

# Discussion

**Evaluation de la technique chirurgicale**

L'approche chirurgicale pour accéder à l'organe odontogénique et aux tissus associés de l'incisive de rat est novatrice à plus d'un titre. Son caractère technique est original, et elle permet d'étudier l'impact d'une substance bio-active, injectée *in vivo,* sur un tissu calcifié, sans effet systémique.

Quelques expériences ont été précédemment menées pour tenter d'influer directement sur les événements cellulaires et matriciels qui président à la formation de l'incisive de rat. Ainsi, à la faveur d'une ablation d'un bloc osseux, certains auteurs ont tenté d'étudier au niveau apical, l'activité cellulaire lors du remodelage osseux et de l'éruption dentaire (Berkovitz, 1971a,b; Berkovitz et Shore, 1978; Redondo *et al.*, 1995). D'autres auteurs créèrent une voie d'accès trans-osseuse labiale, afin d'obtenir un accès direct aux tissus sous-jacents (McKee et Warshawsky, 1984; Eisenmann *et al.*, 1989). Cependant, à cause d'une détérioration tissulaire inhérente à la technique elle-même, il fallait attendre que les éléments lésés migrent en dehors de la voie d'accès pour tirer profit de cette approche invasive. Cet abord, entraînant une rupture de l'intégrité de l'organe de l'émail d'une part, et situé trop à distance de l'apex d'autre part, ne permet dans certains cas ni d'observer, ni d'influer sur les évènements cellulaires et moléculaires du développement de la dent.

Dans notre étude, la fenêtre osseuse vestibulaire créée au niveau de l'apex de l'incisive de rat, alliée à l'administration contrôlée, dans le temps et dans l'espace, d'agents expérimentaux spécifiques par le biais d'une mini-pompe osmotique glissée sous la peau

de l'animal, permet de cibler très spécifiquement l'action de molécules bio-actives en respectant l'environnement physiologique de l'organe de l'émail. Précisément, notre étude a permis de systématiser et affiner les différentes procédures du protocole opératoire. Nous avons ainsi clairement défini les repères pour la trépanation osseuse ainsi qu'élucidé les causes d'inflammation rémanente en périphérie de l'accès trans-cortical. En effet, afin de limiter l'emploi de colle et de ciment acryliques responsables de cette réponse tissulaire, il faut obtenir une congruence entre le trou dans l'os et le cathéter. Nous avons pu montrer en outre que la rétention et la stabilisation de ce dernier sont grandement améliorées en le glissant sous les muscles masticateurs.

**Effet de la tunicamycine sur l'organe de l'émail**

Le chapitre 1 démontre qu'il est possible d'administrer localement, sans effet systémique, un agent pharmacologique dans une hémi-mandibule de rat. Il est ainsi montré dans ce travail que la diffusion à travers une fenêtre osseuse, de l'inhibiteur de la N-glycosylation, la tunicamycine (TM), cause des altérations de la lame basale (LB) située entre les améloblastes et l'émail en voie de maturation.

Certaines protéines non-collagéniques importantes de la matrice extra-cellulaire des tissus minéralisés, telles la «bone sialoprotéine» (BSP) et l'ostéopontine (OPN), sont hautement glycosylées (Butler, 1989; Midura et Hascal, 1996) et on peut aujourd'hui suggérer que cette glycosylation jouerait un rôle majeur dans le processus de minéralisation (discuté par Nanci et Smith, 2000). La LB qui sépare les améloblastes de l'émail en phase de maturation, est riche en glyco-conjugués comprenant des N-acétyl-

D-galactosamine (GalNAc) et/ou des N-acétyl-glucosamine/N-acétyl-acide neuraminique (GlcNAc/NeuNAc) (Nanci et al., 1993). C'est pourquoi le choix de la molécule expérimentalement perfusée s'est porté sur la TM, qui a été antérieurement employée de manière à étudier sur nombre de tissus et cellules, le rôle des protéines N-glycosylées dans la différenciation tissulaire et l'organisation des membranes basales (Kanai *et al.*, 1991; Yang and Hilfer, 1982; Webster *et al.*, 1993). La composition particulière et originale de la LB lui confère très certainement des fonctions propres. Au niveau ultra-structural, la LB, sous influence de TM, présente un aspect irrégulier. L'analyse en cytochimie par lectine à l'or colloïdal montre que la concentration de l'agglutinine *helix pomatia* (HPA) n'est pas affectée sur la LB et sur l'émail, alors que la concentration de l'agglutinine du germe de blé *triticum vulgaris* (WGA), est légèrement réduite, aussi bien sur la LB que dans l'émail. Ceci confirme la notion que TM préviendrait l'incorporation de sucres liés en position N, provoquant des modifications structurelles et compositionelles de la LB.

Nous n'avons toutefois pas pu clairement montrer dans notre étude par immuno-cytochimie, l'influence de TM sur les protéines amélaires et sur l'activité des enzymes. Ceci est tout d'abord probablement la conséquence de la formation en partie de l'émail avant l'administration de TM, et tiendrait également au fait que seules les non-amélogénines sont N-glycosylées et représentent en fait une part mineure des protéines de l'émail. Enfin, les glycoconjugués confèreraient à la LB des propriétés de filtration sélective qui pourraient être affectées par inhibition de la N-glycosylation. Ceci explique probablement la grande quantité d'albumine rencontrée dans la matrice amélaire et liée à l'altération de la LB consécutive à l'administration de TM.

Nous avons également débuté l'étude de l'effet de TM sur les améloblastes en phase de sécrétion, et sur les odontoblastes. *In vitro*, des études antérieures ont montré que cet antibiotique entraînait une inhibition de la différenciation des odontoblastes (Thesleff et Pratt 1980a,b). Dans notre expérience, l'étude histologique a démontré sporadiquement une dégénérescence des odontoblastes et, en phase de sécrétion, des changements de la structure des améloblastes. Nous avons aussi pu observer des altérations dans la couche d'émail. En effet, certaines zones proches de la jonction amélo-dentinaire semblaient contenir moins de matrice métachromatique, suggérant une maturation avancée. Les études ultra-structurales et d'immuno-cytochimie ont révélé le fait que les éléments sécrétoires de la cellule semblaient toujours fonctionnels, même si les prolongements de Tomes ne possédaient que peu ou pas de grains de sécrétion. La TM pourrait donc influer sur le processus de translocation des produits amélaires vers les sites de sécrétion. En outre, cet antibiotique affecterait la dégradation partielle des protéines amélaires au cours de la phase de sécrétion.

**Caractérisation d'un nouvel anticorps apte à détecter les formes sécrétoires de l'amélogénine**

Le chapitre 2 évalue la possibilité de fabriquer, par le biais d'un système utilisant le jaune d'œuf de poulet (Gassmann *et al.*, 1990), un anticorps polyclonal qui reconnaît dans les améloblastes et dans l'émail les cinq principales isoformes de l'amélogénine. Il est ainsi montré que l'anticorps produit, appelé M179y, ne révèle aucun des produits de dégradation. A l'échelle ultra-structurale, M179y marque l'appareil de Golgi et les granules de sécrétion, que ce soit aux stades de sécrétion ou de maturation.

Contrairement aux études précédentes qui montraient une répartition uniforme de l'amélogénine au sein de la couche d'émail (Nanci *et al.*, 1998), M179y décrit un gradient de marquage qui est intense à la surface de l'émail, puis décroît en profondeur et augmente près de la jonction amélo-dentinaire.

Ces résultats nous amènent à envisager une distribution des protéines de l'émail plus complexe que l'on pouvait le supposer jusqu'ici. L'anticorps polyclonal M179y se montre par conséquent être un outil d'investigation d'une exceptionnelle spécificité pour comprendre la dynamique des amélogénines sécrétées par les améloblastes, tant au niveau morphologique que biochimique, et pour étudier leur distribution par rapport aux cristaux de l'émail.

**Administration de facteurs de croissance**

Le modèle chirurgical utilisé au cours de cette étude présente également l'avantage d'administrer *in situ* des agents biologiques tels que les facteurs de croissance, sans effet systémique avéré. Ceux-ci sont retrouvés dans les tissus calcifiés et influencent localement leur formation (Canalis *et al.*, 1988). Ils régulent l'activité cellulaire et la morphogenèse autant que la formation et la résorption osseuses. Ils favorisent en outre la réparation osseuse, l'ostéo-intégration de biomatériaux, la régénération et la réparation parodontale, et contrôlent la perte osseuse dans certaines pathologies (Giannobile, 1996; Linkhart *et al.*, 1996; Lynch *et al.*, 1991; Rutherford *et al.*, 1993).

Le mélange de facteurs de croissance utilisé possède un potentiel thérapeutique dans la réparation osseuse, tels qu'en attestent nos résultats préliminaires positifs. Tous les spécimens ont démontré une augmentation de masse osseuse. Histologiquement, une apposition osseuse est mise en évidence près de la perforation alors qu'une aire de fort remaniement osseux entoure la fenêtre osseuse. Compte tenu de tous ces résultats encourageants, il serait intéressant d'approfondir l'observation au niveau ultra-structural et d'examiner le rôle inducteur de ce mélange de facteurs de croissance sur la morphogenèse et le développement dentaire.

**Etude prospective**

A la faveur de notre approche chirurgicale originale, il serait aujourd'hui opportun d'utiliser la thérapie génique pour introduire dans les cellules cibles un vecteur spécifique portant le gène codant pour des protéines matricielles ou le facteur de croissance à transférer. Etant entendu que la transduction dépend largement du vecteur utilisé, notre modèle s'avèrerait fort utile pour évaluer l'efficacité des divers vecteurs. En ce qui concerne le choix du vecteur le plus approprié pour introduire des informations géniques dans les tissus calcifiés, la littérature est toujours à la recherche d'une méthode efficace (Evans et Robbins, 1995; Fang *et al.*, 1996; Goldstein et Bonadio, 1998; Riew *et al.*, 1998). Tous les efforts tendent à trouver un vecteur qui aboutirait à l'expression de gènes en toute sécurité, sur une période déterminée, et ciblée sur une population cellulaire précise.

# Conclusion

Le modèle expérimental exposé ici est une méthode privilégiée pour évaluer *in vivo* l'action de substances expérimentales sur un tissu calcifié. La possibilité de cibler l'action d'une molécule dans des conditions physiologiques, sans interférence systémique, et ainsi obtenir une réponse biologique de grande sensibilité, va permettre de mieux comprendre la cascade de phénomènes qui président à la formation d'un tissu calcifié.

Nous avons illustré dans notre travail la possibilité d'administrer localement un produit pharmacologique toxique au niveau systémique et l'intérêt des analyses ultra-structurales à l'or colloïdal par cytochimie utilisant des lectines et des anticorps. Ainsi, ces moyens d'étude ont mis en évidence l'importance de la lame basale sur la maturation amélaire et ont démontré comment un défaut d'intégrité de celle-ci perturbe le contenu matriciel de l'émail. Dans ce même esprit, l'élaboration d'un nouvel anticorps anti-amélogénine représente un outil extrêmement performant pour localiser des protéines nouvellement sécrétées et leur distribution dans la matrice extra-cellulaire de l'émail. Dès lors, notre étude permet également d'envisager des développements prometteurs lors de l'administration des facteurs des croissance, apportant des informations utiles quant à la compréhension de la morphogenèse et la réparation des tissus calcifiés. Enfin, à la faveur des résultats obtenus, nous suggéront que le modèle expérimental utilisé peut être avantageusement exploité pour la transduction virale.

# Remerciements

Je souhaiterais remercier les personnes suivantes:

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# Références bibliographiques

Aberg T, Wozney J, Thesleff I (1997) Expression patterns of bone morphogenetic proteins (Bmps) in the developing mouse tooth suggest roles in morphogenesis and cell differentiation. *Dev Dyn* 210: 383-396.

Abrahamson DR (1986) Recent studies on the structure and the pathology of basement membranes. *J Pathol* 149: 257-278.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1994a) Cell junctions, cell adhesion, and extracellular matrix, in: *Molecular Biology of the Cell.* 3rd ed, Garland Publ., Inc. New York & London 19: 988-993.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1994b) Signaling via enzyme-linked cell-surface receptors, in: *Molecular Biology of the Cell.* 3rd ed, Garland Publ., Inc. New York & London 15: 759-771.

Aumailley M, Smyth N (1998) The role of laminins in basement membrane function. *J Anat* 193: 1-21.

Bartlett JD, Simmer JP, Xue J, Margolis HC, Moreno EC (1997) Molecular cloning and mRNA tissue distribution of a novel matrix metalloproteinase isolated from porcine enamel organ. *Gene* 183: 123-128.

Baum BJ, Atkinson JC, Baccaglini L, Berkman ME, Brahim JS, Davis C, Lancaster HE, et al. (1998) The mouth is a gateway to the body: gene therapy in 21st-century dental practice. *J Calif Dent Assoc* 26: 455-60.

Bendayan M (1995) Colloidal gold post-embedding immunocytochemistry. *Prog Histochem Cytochem* 29: 1-163.

Benhamou N (1989) Preparation and application of lectin-gold complexes. In: *Colloidal Gold: Principles, Methods, and Applications*, Vol. 1 (Hayat MA, ed), San Diego: Academic Press, Inc, 95-143.

Benson GP, Zaki AE, Eisenmann DR (1998) The use of autoradiography and cycloheximide to determine the origin of enamel proteins in the maturation stage ameloblasts of the rat incisor. *Arch Oral Biol* 43: 771-777.

Berkovitz BKB (1971a) The healing process in the incisor tooth socket of the rat following root resection and exfoliation. *Arch Oral Biol* 16: 1045-1054.

Berkovitz BKB (1971b) The effect of root transection and partial root resection on the unimpeded eruption rate of the rat incisor. *Arch Oral Biol* 16: 1033-1043.

Berkovitz BKB, Shore RC (1978) High mitochondrial density within peripheral nerve fibers of the periodontal ligament of the rat incisor. *Arch Oral Biol* 23: 207-213.

Bernfield M, Kokenyesi R, Kato M, Hinkes MT, Spring J, Gallo RL, Lose EJ (1992) Biology of the syndecans: A family of transmembrane heparan sulfate proteoglycans. *Annu Rev Cell Biol* 8: 365-393.

Bostrom MPG (1998). Expression of bone morphogenetic proteins in fracture healing. *Clinic Orthop* 355: S116- S123.

Boyde A (1989) Enamel. In: *Handbook of microscopic anatomy*, volume V/6: teeth (Oksche A, Vollrath L, eds), Berlin: Springer-Verlag, 310-473.

Brukner-Tuderman L, Hopfner B, Hammami-Hauasli N (1999) Biology of anchoring fibrils: lessons from dystrophic epidermolysis bullosa. *Matrix Biol* 18:43-54.

Budker V, Zhang G, Knechtle S, Wolff JA. Naked DNA delivered intraporally expresses efficiently in hepatocytes (1996). *Gene Ther* 3: 593-598.

Burgeson RE, Chiquet M, Deutzmann R, Ekblom P, Engel J, Kleinman H, Martin GR, Meneguzzi G, Paulsson M, Sanes J, Timpl R, Tryggvason K, Yamada Y, Yurchenco PD (1994) A new nomenclature for the laminins. Matrix 14: 209-211.

Butler WT (1989) The nature and significance of osteopontin. *Connect Tissue Res* 23:123-136.

Canalis E, McCarthy TL, Centrella M (1988) Growth factors and the regulation of bone remodeling. *J Clin Invest* 81: 277-281.

Canalis E, Pash J, Varghese S (1993) Skeletal growth factors. *Crit Rev Eukarot* Gene Expr 3: 155-166.

Canalis E, Rydziel S (1996) Platelet-derived growth factor and the skeleton, in Bilezikian JP, Raisz LG, Rodan GA (eds): *Principles of Bone Biology*. Academic Press 44: 619-626.

Cardinali M, Jakus J, Shah S, Ensley JF, Robbins KC, Yeudall WA (1998) p21 (WAF1/Cip1) retards the growth of human squamous cell carcinomas in vivo. *Oral Oncol* 34: 211-8.

Centrella M, Horowitz MC, Wozney JM, McCarthy TL (1994) Transforming growth factor-β gene family members and bone. *Endocrine Rev* 15: 27-39.

Chang J, Korolev V (1996) Specific toxicity of tunicamycin in induction of programmed cell death of sympathetic neurons. *Experimental Neurology* 137: 201-211.

Cerny R, Slaby I, Hammarström L, Wurtz T (1996) A novel gene expressed in rat ameloblasts codes for proteins with cell binding domains. *J Bone Miner Res* 11: 883-891.

Chardin H, Septier D, Lecolle S, Goldberg M (1989) Effect of tunicamycin on glycogen accumulation in the stratum intermedium and odontoblasts of rat incisor. *Calcif Tiss Res* 256: 519-527.

Charles CN, Lane JM (1992) Newest factors in fracture healing. *Clin Orthop* 277: 297-311.

Chen W-Y, Nanci A, Smith CE (1995) Immunoblotting studies on artifactual contamination of enamel homogenates by albumin and other proteins. *Calcif Tissue Int* 57: 145-151.

Cochran Dl, Schenk R, Buser D, Wozney JM, Jones AA (1999) Recombinant human morphogenetic protein-2 stimulation of bone formation around endosseous dental implants. *J Periodontol* 70: 139-150.

Daniel N G, Massie B, Cohen E A, and Nanci A (2000) Targeting of the odontogenic organ and periodontal tissues with gene tranfer vectors. *J Dent Res* 79 (sp. Iss), 474. 2000. Abstract.

Delporte C, Miller G, Kagami H, Lillibridge CD, O'Connell BC, Atkinson JC, Baum BJ (1998) Safety of salivary gland-administered replication-deficient recombinant adenovirus in rats. *J Oral Pathol Med* 27: 34-8.

DenBesten PK, Heffernan LM, Treadwell BV, Awbrey BJ (1989) The presence and possible functions of the matrix metalloproteinase collagenase activator protein in developing enamel matrix. *Biochem Pharmacol* 264: 917-920.

Diegelmann RF (1997) Cellular and biochemical aspects of normal and abnormal wound healing: an overview. *J Urol* 157: 298-302.

Duksin D, Mahoney WC (1982) Relationship of the structure and biological activity of the natural homologues of tunicamycin. *J Biol Chem* 257: 3105-3109.

Durbeej M, Ekblom P (1997) Dystroglycan and laminins: glycoconjugates involved in branching epithelial morphogenesis *Exp Lung Res* 23: 109-118.

Eisenmann DR, Chen J, Lehman G, El-Moneim Zaki A (1989) Studies on the influx of [$^{3}$H]-histidine and $^{45}$Ca through a surgical opening to rat incisor ameloblasts and adjacent enamel. *Arch Oral Biol* 34: 93-102.

Ekblom P, Nordling P, Saxén L, Rasilio M, Renkonen O (1979) Cell interactions leading to kidney tubule determination are tunicamycin sensitive. *Cell Differ* 8: 347-352.

Ekblom M, Falk M, Salmivirta K, Durbeej M, Ekblom P (1998) Laminin isoforms and epithelial development. *Ann NY Acad Sci* 857: 194-211.

Elbein AD (1988) Inhibitors of the biosynthesis and processing of N-linked oligosaccharides. In: *CRC Critical Reviews in Biochemistry*, 21-49.

Evans CH, Robbins PD (1995) Possible orthopaedic applications of gene therapy. *J Bone Joint Surg* 77A: 1103-1114.

Fang J, Zhu Y-Y, Smiley E, Bonadio J, Rouleau JP, Goldstein SA, McCauley LK, Davidson BL, Roessler BJ (1996) Stimulation of new bone formation by direct transfer of osteogenic plasmid genes. *Proc Natl Acad Sci USA* 93: 5753-5758.

Feng M, Jackson WH, Goldman CK, Rancourt C, Wang M, Dusing SK, Siegal G, Curiel D (1997) Stable in vivo gene transduction via a novel adenoviral/retroviral chimeric vector. *Nat Biotechnol* 15: 866-870.

Fincham AG, Moradian-Oldak J, Simmer JP (1999) The structural biology of the developing dental enamel matrix. *J Struct Biol* 126: 270-299.

Fink DJ, De Luca NA, Goins WF, Glorioso JC (1996) Gene transfer to neurons using herpes simplex virus-based vectors. *Annu Rev Neurosci* 19: 265-287.

Finnie JW, O'Shea JD (1988) Pathological and pathogenic changes in the central nervous system of guinea pigs given tunicamycin. *Acta Neuropathol* 75: 411-421.

Frank RM, Nalbandian J (1967) Ultrastructure of amelogenesis. In: *Structural and chemical organization of teeth* (Miles AEW, ed), New York: Academic Press, 399-466.

Fukae M, Tanabe T, Murakami C, Dohi N, Uchida T, Shimizu M (1996) Primary structure of the porcine 89-kDa enamelin. *Adv Dent Res* 10: 111-118.

Fukae M, Tanabe T, Uchida T, Lee SK, Ryu OH, Murakami C, Wakida K, Simmer JP, Yamada Y, Bartlett JD (1998) Enamelysin (Matrix metalloproteinase-20): Localization in the developing tooth and effects of pH and calcium on amelogenin hydrolysis. *J Dent Res* 77: 1580-1588.

Gao J, Symons AL, Bartold (1998) Expression of transforming growth factor-beta 1 (TGF-ß1) in the developing periodontium of rats. *J Dent Res* 77: 1708-1716.

Gassmann M, Thömmes P, Weiser T, Hübscher U (1990) Efficient production of chicken egg yolk antibodies against a conserved mammalian protein. *FASEB J* 4: 2528-2532.

Giannobile WV (1996) The potential role of growth and differentiation factors in periodontal regeneration. *J Periodontol* 67: 545-553.

Giraud A, Franc J-M (1989) Effects of tunicamycin and n-linked oligosaccharide-processing inhibitors on the morphology of cultered porcine thyroid cells. *Eur J Cell Biol* 48: 128-134.

Goldberg M, Boskey AL (1996) Lipids and biomineralizations. *Prog Histochem Cytochem* 31: 1-187.

Goldstein SA, Bonadio J (1998) Potential role for direct gene transfer in the enhancement of fracture healing. *Clin Orthop Rel Res* 355: 154-162.

Gonzalez C, Satre M (1991) Endocytosis in *Dictyostelium discoideum* amoebae. Inhibition by cycloheximide and other inhibitors of protein synthesis. *J Cell Sci* 99: 535-543.

González CB, Swann RW, Pickering BT (1981) Effects of tunicamycin on the hypothalamus-neurohypophysial system of the rat. *Cell Tiss Res* 217: 199-210.

Gottschalk U, Chan S (1998) Somatic gene therapy: present situation and future perspective. *Arzneim-Forsch/ Drug Res* 48: 1111-1120.

Hayden JM, Mohan S, Baylink DJ (1995) The insulin-like growth factor system and the coupling of formation to resorption. *Bone* 17 : 93S-98S.

He X, Goldsmith CM, Marmery Y, Wellner RB, Parlow AF, Nieman LK, Baum BJ (1998) Systemic action of human growth hormone following adenovirus-mediated gene transfer to rat submandibular glands. *Gene Therapy* 1998 5: 537-541.

Hedner E, Linde A (1995). Efficacy of bone morphogenetic protein (BMP) with osteopromotive membranes. An experimental study in rat mandibular defects. *Eur J Oral Sci* 103: 236-341.

Hill CS (1999) The Smads. *Int J Biochem Cell Biol* 31: 1249-1254.

Hill PA, Reynolds JJ, Meikle MC (1995) Osteoblasts mediate insulin-like growth factor-I and –II stimulation of osteoclast formation and function. *Endocrinol* 136: 124-131.

Hogan BLM (1996) Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev* 10: 1580-1594.

Howell TH, Fiorellini JP, Paquette DW, Offenbacher S, Giannobile WV, Lynch SE (1997) A phase I/II clinical trial to evaluate a combination of recombinant human platelet-derived growth factor-BB and recombinant human insulin-like growth factor-1 in patients with periodontal disease. *J Periodontol* 68: 1186-1193.

Howes R, Bowness JM, Grotendorst GM, Marting GR, Reddi AH (1988) Platelet-derived growth factor enhances demineralized bone matrix-induced cartilage and bone formation. *Calcif Tissue Int* 42: 34-38.

Hu MC, Wang YP, Qiu WR (1999) Human fibroblast growth factor-18 stimulates fibroblast cell proliferation and is mapped to chromosome 14p11. *Oncogene* 18: 2635-2642.

Hurley MM, Kessler M, Gronowicz G, Raisz LG (1992) The interaction of heparin and basic fibroblast growth factor on collagen synthesis in 21-Day fetal rat calvariae. *Endocrinol* 130: 2675-2681.

Hurley MM, Florkiewicz RZ (1996) Fibroblast growth factor and vascular endothelial cell growth factor families, in Bilezikian JP, Raisz LG, Rodan GA (eds): *Principles of Bone Biology*. Academic Press 45: 627-645.

Ito Y, Chen GP, Imanishi Y (1998) Micropatterned immobilization of epidermal growth factor to regulate cell function. *Bioconjug Chem* 9: 277-282.

Ito Y, Inoue M, Liu SQ, Imanishi Y (1993) Cell growth on immobilized cell growth factor. VI. Enhancement of fibroblast cell growth by immobilized insulin and/or fibronectin. *J Biomed Mater Res* 27: 901-907.

Ito Y, Liu SQ, Imanishi Y (1991) Enhancement of cell growth on growth factor-immobilized polymer film. *Biomaterials* 2: 449-453.

Ito T, Takatsuki A, Kawamura K, Sato K, Tamura G (1980) Isolation and structures of components of tunicamycin. *Agric Biol Chem* 44: 695-671.

Jago MV, Payne AL, Peterson JE, Bagust TJ (1983) Inhibition of glycosylation by corynetoxin, the causative agent of annual ryegrass toxicity: a comparison with tunicamycin. *Chem Biol Interactions* 45: 223-234.

Jiang D, Dziak R, Lynch SE, Stephan EB (1999) Modification of an osteoconductive anorganic bovine bone mineral matrix with growth factors. *J Periodontol* 70: 834-839.

Jilka RL, Weinstein RS, Bellido T, Parfitt AM, Manolagas SC (1998) Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines. *J Bone Min Res* 13: 793-802.

Kallenbach E (1974) Fine structure of rat incisor ameloblasts in transition between enamel secretion and maturation stages. *Tissue Cell* 6: 173-190.

Kallenbach E (1980) Access of horseradish peroxidase (HRP) to the extracellular spaces of the maturation zone of the rat incisor enamel organ. *Tissue Cell* 12: 165-174.

Kanai Y, Hayashi Y, Kawakami H, Takata K, Kurohmaru M, Hirano H, Nishida T (1991) Effect of tunicamycin, an inhibitor of protein glycosylation on testicular cord organization in fetal mouse gonadal explants in vitro. *Anat Rec* 230: 199-208.

Kingsley DM (1994) What do BMPs do in mammals? Clues from the mouse short-ear mutation. *Trends Genet* 10: 16-21.

Kjoelby M, Thesleff I, Sahlberg C, Fejerskov O, Josephsen K (1996) Degradation of the dental basement membrane during mouse tooth development *in vitro*. *Int J Dev Biol* 38: 455-462.

Kobayashi M, Takigushi T, Suzuki R, Yamaguchi A, Deguchi K, Shionome M, Miyazawa Y, Nishihara T, Nagumo M, Hasegawa K (1999) Recombinant human bone morphogenetic protein-2 stimulates osteoblastic differentiation in cells isolated from human periodontal ligament. *J Dent Res* 78: 1624-1633.

Kuboki Y, Sasaki M, Saito A, Takita H, Kato H (1997) Regeneration of periodontal ligament and cementum by BMP-applied tissue engineering. *Eur J Oral Sci* 106 (Suppl.1): 197-203.

Lancaster CA, Fryer PR, Griffiths S, Mason RM (1989) Ultrastructural changes accompany inhibition of proteoglycan synthesis in chondrocytes by Cyclofenil diphenol. *J Cell Sci* 92: 271-280.

Laurie GW, Leblond CP, Martin GR (1983) Light microscopic immunolocalization of type IV collagen, laminin, heparan sulfate proteoglycan, and fibronectin in the basement membranes of a variety of rat organs. *Am J Anat* 167: 71-82.

Leaver DD, Schneider KM, Rand MJ, Anderson RM, Gage PW, Malbon R (1988) The neurotoxicity of tunicamycin. *Toxicology* 49: 179-187.

Leblond CP, Warshawsky H (1979) Dynamics of enamel formation in the rat incisor tooth. *J Dent Res* 58B: 950-975.

Lian JB, Stein GS, Canalis E, Gehron Robey P, Boskey AL (1999) Bone formation: osteoblast lineage cells, growth factors, matrix proteins, and mineralization process, in Favus MJ (ed): *Primer on the metabolic bone diseases and disorders of mineral metabolism*. Lippincott W & W 3, 14-29.

Lieberman JR, Le LQ, Wu L, Finerman GAM, Berk A, Witte ON, Stevenson O (1998) Regional gene therapy with a BMP-2-producing murine stromal cell line induces heterotopic and orthotopic bone formation in rodents. *J Orthop Res* 16: 330-339.

Lin HM, Nakamura H, Noda T, Ozawa H (1994) Localization of $H^+$-ATPase and carbonic anhydrase II in ameloblasts at maturation. *Calcif Tissue Int* 55: 38-45.

Lin HY, Lodish HF (1993) Receptors for the TGF-β superfamily: multiple plypeptides and serine/threonine kinases. *Trends Cell Biol* 3: 14-19.

Lin TY, Wang SM, Fu WM, Chen YH, Yin HS (1999) Toxicity of tunicamycin to cultured brain neurons: Ultrastructure of the degenerating neurons. *Journal of Cellular Biochemistry* 74: 638-647.

Lindholm TS, Viljanen VV, Mattila M (1996) Thirty years of bone morphogenetic protein research, in Lindholm TS (ed): *Bone Morphogenetic Proteins: Biology, Biochemistry and Reconstructive Surgery*. Austin, R.G. Landes Company (Academic Press) 1: 3-27.

Linkhart TA, Mohan S, Baylink D (1996) Growth factors for bone growth and repair: IGF, TGFβ and BMP. B*one* 19: 1S-12S.

Lippincott-Schwartz J, Yuan LC, Bonifacino JS, Klausner RD (1989) Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell* 56: 801-813.

Liu SQ, Ito Y, Imanishi Y (1993) Cell growth on immobilized cell growth factor. IX. Covalent immobilization of insulin, transferrin, and collagen to enhance growth of bovine endothelial cells. *J Biomed Mater Res* 27: 909-915.

Lynch SE, Colvin RB, Antoniades HN (1989) Growth factors in wound healing: single and synergistic effects on partial thickness porcine skin wounds. *J Clin Invest* 84: 640-646.

Lynch SE, Buser D, Hernandez RA, Weber HP, Stich H, Fox CH, Williams RC (1991) Effects of the platelet-derived growth factor/insulin-like growth factor combination on bone regeneration around titanium dental implants. Results of a pilot study in beagle dogs. *J Periodontol* 62: 710-716.

Manolagas SC, Jilka R (1995) Bone marrow, cytokines, and bone remodeling emerging insights into the pathophysiology of osteoporosis. *N Engl J Med* 232: 305-311.

Marinkovich MP, Keene DR, Rimberg CS, Burgeson RE (1993) Cellular origin of the dermal-epidermal basement membrane. *Dev Dyn* 197: 255-267.

Marks SC and Hermey DC (1996) The structure and development of bone, in: Bilezikian JP, Raisz LG, Rodan GA (eds): *Principles of Bone Biology*, Academic Press 1: 3-14.

Massague J (1992) Receptors for the TGF-β family. *Cell* 69: 1067-1070.

Massie B, Couture F, Lamoureux L, Mosser DD, Guilbault C, Jolicoeur P, Belanger F, Langelier Y (1998) Inducible overexpression of a toxic protein by an adenovirus vector with a tetracycline-regulatable expression cassette. *J Virol* 72: 2289-2296.

Matsuda N, Lin WL, Kumar NM, Cho MI, Genco RJ (1992) Mitogenic, chemotactic, and synthetic responses of rat periodontal ligament fibroblastic cells to polypeptide growth factors in vitro. *J Periodontol* 63: 515-525.

McKee MD, Warshawsky H (1984) In vivo experimentation on rat incisor enamel organs through a surgical window. *Anat Rec* 210: 693-705.

Meyer J-M, Ruch J-V, Kubler MD, Kupferle C, Lesot H (1995) Cultured incisors display major modifications in basal lamina deposition without further effect on odontoblast differentiation. *Cell Tissue Res* 279: 135-147.

Miake Y, Yanagisawa T, Takuma S (1982) Electron microscopic study on the effects of vinblastine on young odontoblasts in rat incisor. *J Biol Buccale* 10: 319-330.

Michaels JE (1980) Alterations in the distribution of glycoproteins in epithelial cells of murine colon after injection of tunicamycin. *Calcif Tiss Res* 210: 121-132.

Midura RJ, Hascall VC (1996) Bone sialoprotein - A mucin in disguise? *Glycobiology* 6:677-681.

Mohan S (1993) IGF binding proteins in bone cell regulation. *Growth Regul* 3: 67-70.

Mohan S, Baylink DJ (1991) Bone growth factors. *Clin Orthop* 263: 30-48.

Moss ML (1972) Twenty years of functional cranial analysis. *Am J Orthod* 61: 479-485.

Nagai H, Tsukuda R, Mayahara H (1995) Effects of basic fibroblast growth factor on bone formation in growing rats. *Bone* 16: 367-373.

Nakamura K, Kurokawa T, Aoyama I, Hanada K, Tamura M, Kawaguchi H (1998) Stimulation of bone formation by intraosseous injection of basic fibroblast growth factor in ovariectomised rats. *Int Orthop* 22: 49-54.

Nakashima M (1994) Induction of dentin formation on canine amputated pulp by recombinant human bone morphogenetic proteins (BMP)-2 and -4. *J Dent Res* 73: 1515-1522.

Nanci A, Uchida T, Warshawsky H (1987) The effects of vinblastine on the secretory ameloblasts: An ultrastructural, cytochemical, and immunocytochemical study in the rat incisor. *Anat Rec* 219: 113-126.

Nanci A, Ahluwalia JP, Pompura JR, Smith CE (1989a) Biosynthesis and secretion of enamel proteins in the rat incisor. *Anat Rec* 224: 277-291.

Nanci A, Ahluwalia JP, Zalzal S, Smith CE (1989b) Cytochemical and biochemical characterization of glycoproteins in forming and maturing enamel of the rat incisor. *J Histochem Cytochem* 37: 1619-1633.

Nanci A, Smith CE (1992) Development and calcification of enamel. In: *Calcification in Biological Systems* (Bonucci E, ed),. Boca Raton: CRC Press, Inc, 313-343.

Nanci A, Zalzal S, Kogaya Y (1993) Cytochemical characterization of basement membranes in the enamel organ of the rat incisor. *Histochemistry* 99: 321-331.

Nanci A, Fortin M, Ghitescu L (1996a) Endocytotic functions of ameloblasts and odontoblasts: Immunocytochemical and tracer studies on the uptake of plasma proteins. *Anat Rec* 245: 219-234.

Nanci A, Hashimoto J, Zalzal S, Smith CE (1996b) Transient accumulation of proteins at interrod and rod enamel growth sites. *Adv Dent Res* 10: 135-149.

Nanci A, Zalzal S, Lavoie P, Kunikata M, Chen W-Y, Krebsbach PH, Yamada Y, Hammarström L, Simmer JP, Fincham AG, Snead ML, Smith CE (1998) Comparative immunochemical analyses of the developmental expression and distribution of ameloblastin and amelogenin in rat incisors. *J Histochem Cytochem* 46: 911-934.

Nanci A, Smith CE (2000) Matrix-mediated mineralization in enamel and the collagen-based hard tissues. In: *Chemistry and Biology of Mineralized Tissues* (Goldberg M, Boskey A, Robinson C, eds), Rosemont: American Academy of Orthopaedic Surgeons, 217-224.

Nanci A, Mocetti P, Sakamoto Y, Kunikata M, Lozupone E, Bonucci E (2000a) Morphological and immunocytochemical analyses on the effects of diet-induced hypocalcemia on enamel maturation in the rat incisor. *J Histochem Cytochem* 48:1043-1058.

Nanci A, Zalzal S, Fortin M, Mangano C, Goldberg HA (2000b) Incorporation of circulating bone matrix proteins by implanted hydroxyapatite and at bone surfaces: implications for cement line formation and structuring of biomaterials, in Davies JE (ed): *Proceedings of the International Workshop on Bone Engineering* (In press).

Niyibizi C, Baltzer A, Lattermann C, Oyama M, Whalen JD, Robbins PD, Evans CH (1998) Fracture healing enhancement. Potential role for gene therapy in the enhancement of fracture healing. *Clin Orthop Rel Res* 355S: S148-S153.

O'Connell BC, Lillibridge CD, Ambudkar I, Kruse D (1998) Somatic gene transfer to salivary glands. *Ann N Y Acad Sci* 15: 171-80.

Olden K, Pratt RM, Yamada KM (1978) Role of carbohydrates in protein secretion and turnover: Effects of tunicamycin on the major cell surface glycoprotein of chick embryo fibroblasts. *Cell* 13: 461-473.

Orsini G, Nanci A (2000) I fattori di crescita e il loro ruolo nella riparazione ossea, in: De Franco M., Mangano C., Fornara R., Piattelli A.,(Eds) *Fattori di crescita e biomateriali. Rigenerazione ossea in odontostomatologia*. Masson, Milano, Italia, 47-80.

Otani Y, Quinones S, Saus J, Kurkinen M, Harris EDJr (1990) Cycloheximide induces stromelysin mRNA in cultured human fibroblasts. *Eur J Biochem* 192: 75-79.

Overall CM, Limeback H (1988) Identification and characterization of enamel proteinases isolated from developing enamel. Amelogeninolytic serine proteinases are associated with enamel maturation in pig. *Biochem Pharmacol* 256: 965-972.

Parfitt AM (1987) Bone and plasma calcium homeostasis. *Bone* 8: 51-58.

Park JB, Matsuura M, Han KY, Norderyd O, Lin WL, Genco RJ, Cho MI (1995) Periodontal regeneration in class III furcation defects of beagle dogs using guided tissue regenerative therapy with platelet-derived growth factor. *J Periodontol* 66: 462-477.

Paulsson M (1992) Basement membrane proteins: Structure, assembly, and cellular interactions. *CRC Crit Rev Biochem Mol Biol* 27: 93-127.

Plank VJ, Rychlo A (1952) Eine schnellentkalkungsmethode. *Zentralbl Pathol* 89: 252-254.

Pow DV, Morris JF (1992) Tunicamycin, puromycin and brefeldin A influence the subcellular distribution of neuropeptides in hypothalamic magnocellular neurones of rat. *Cell Tissue Res* 269: 547-560.

Prince MH (1998) Gene transfer: a review of methods and application. *Pathology* 30: 335-347.

Radomsky ML, Thompson AY, Spiro RC, Poser JW (1998) Potential role of fibroblast growth factor in enhancement of fracture healing. *Clinic Orthop and Relat Res* 355S: S283-S293.

Reddi AH (1998) Role of morphogenetic proteins in skeletal tissue engineering and regeneration. *Nature Biotechnol* 16: 247-252.

Reddi AH, Cunningham NS (1993) Initiation and promotion of bone differentiation by bone morphogenetic proteins. *J Bone Miner Res* 8 (Suppl. 2): S499-S502.

Redondo LM, Cantera JMG, Hernandez AV, Puerta CV (1995) Effect of particulate porous hydroxyapatite on osteoinduction of demineralized bone autografts in experimental reconstruction of the rat mandible. *Int J Oral Maxillofac* Surg 24: 445-448.

Relan NK, Schuger L (1999) Basement membranes in development. *Pediatr Dev Pathol* 2: 103-118.

Reynolds PN, Feng M, Curiel DT. (1999) Chimeric viral vectors--the best of both worlds? *Mol Med Today* 5: 25-31.

Riew KD, Wright NM, Cheng SL, Avioli LV, Lou J (1998) Induction of bone formation using a recombinant adenoviral vector carrying the human BMP-2 gene in a rabbit spinal fusion model. *Calcif Tissue Int* 63: 357-360.

Ripamonti U (1996) The generation of bone in primates by bone morphogenetic proteins, in Lindholm TS (ed): *Bone Morphogenetic Proteins: Biology, Biochemistry and Reconstructive Surgery*. Austin, R.G. Landes Company (Academic Press), 131-145.

Ripamonti U, Heliotis M, Rueger DC, Sampath TK (1996) Induction of cementogenesis by recombinant human osteogenic protein-1 (hOP-1/BMP-7) in the baboon (*Papio ursinus*). *Arch Oral Biol* 41:121-126.

Ripamonti U, Reddi AH (1997) Tissue engineering and morphogenesis of periodontal tissues by bone morphogenetic proteins. *Crit Rev Oral Biol Med* 8: 154-163.

Ripamonti U, Duneas N, Van den Heever B, Bosch C, Crooks J (1997) Recombinant transforming growth factor-β1 induces endochondral bone in the baboon and synergizes with recombinant osteogenic protein-1 (bone morphogenetic protein-7) to initiate rapid bone formation. *J Bone Miner Res* 12: 1584-1595.

Ripamonti U, Crooks J, Kikbride AN (1999) Sintered porous hydroxyapatites with intrinsic osteoinductive activity: geometric induction of bone formation. *S Afr J Sci* 95: 335-343.

Robinson C, Brookes SJ, Shore RC, Kirkham J (1998) The developing enamel matrix: nature and function. Eur J Oral Sci 106: 282-291.

Roodman GD (1998) Osteoclast differentiation and activity. *Biochem Soc Trans* 26: 7-13.

Ruch J-V, Lesot H, Karcher-Djuricic V, Meyer JM, Mark M (1983) Epithelial-mesenchymal interactions in tooth germs: Mechanisms of differentiation. *J Biol Buccale* 11: 173-193.

Rutherford RB, Ryan ME, Kennedy JE, Tucker MM, Charette MF (1993) Platelet-derived growth factor and dexamethasone combined with a collagen matrix induce regeneration of the periodontium in monkeys. *J Clin Periodontol* 20: 537-544.

Rutheford RB, Charette M, Rueger D (1994a) Role of osteogenic (bone morphogenetic) protein and platelet-derived growth factor in periodontal wound healing, in: Genco RJ, Hamada S, Lehner T, Mc Gee J, Mergenhagen S (eds). *Molecular Pathogenesis of Periodontal Disease.* Washington DC: American Society for Microbiology Press, 247-437.

Rutheford RB, Spangberg L, Tucker M, Rueger D, Charette M (1994b) The time-course of the induction of reparative dentine formation human osteogenic protein-1. *Archs Oral Biol* 39: 833-838.

Sahlberg C, Reponen P, Tryggvason K, Thesleff I (1999) *Timp-1*, *-2* and - *3* show coexpression with *gelatinases A* and *B* during mouse tooth morphogenesis. Eur J Oral Sci 107: 121-130.

Sasaki T, Higashi S, Tachikawa T, Yoshiki S (1983) Thin-section, tracer, and freeze-fracture study of the smooth-ended maturation ameloblasts in rat incisors. *Acta Anat* 117: 303-313.

Sasaki T, Debari K, Garant PR (1987) Ameloblast modulation and changes in the Ca, P, and S content of developing enamel matrix as revealed by SEM-EDX. *J Dent Res* 66: 778-786.

Sasaki T, Goldberg M, Takuma S, Garant PR (1990) Cell biology of tooth enamel formation. In: *Monographs in oral science* (Myers HM, ed). Basel: Karger, 1-204.

Sawada T, Yanagisawa T, Takuma S, Hasegawa H, Watanabe K (1992) Electron-immunocytochemical localization of laminin and type-IV collagen in the enamel organ of the rat incisor. *Acta Histochem Cytochem* 25: 395-403.

Sawada T, Nanci A (1995) Spatial distribution of enamel proteins and fibronectin at early stages of rat incisor tooth formation. *Arch Oral Biol* 40: 1029-1038.

Schuger L (1997) Laminins in lung development. *Exp Lung Res* 23: 119-129.

Shillitoe EJ (1998) Gene therapy for oral cancer: recent progress in research. *Oral Oncol* 34: 157-160.

Sigurdsson TJ, Tatakis DN, Lee MB, Wikesjo UME (1995) Periodontal regenerative potential of space-providing expanded polytetrafluoroethylene membranes and recombinant human bone morphogenetic proteins. *J Periodontol* 66: 511-521.

Simmer JP, Fincham AG (1995) Molecular mechanisms of dental enamel formation. *Crit Rev Oral Biol Med* 6: 84-108.

Simmer JP, Fukae M, Tanabe T, Yamakoshi Y, Uchida T, Xue J, Margolis HC, Shimizu M, DeHart BC, Hu CC, Bartlett JD (1998) Purification, characterization, and cloning of enamel matrix serine proteinase 1. *J Dent Res* 77: 377-386.

Slavkin HC (1990) Molecular determinants of tooth development: A review. *Crit Rev Oral Biol Med* 1: 1-16.

Smith CE, Warshawsky H (1975) Histological and three dimensional organization of the odontogenic organ in the lower incisor of 100 gram rats. *Am J Anat* 142: 403-430.

Smith CE, McKee MD, Nanci A (1987) Cyclic induction and rapid movement of sequential waves of new smooth-ended ameloblast modulation bands in rat incisors as visualized by polychrome fluorescent labeling and GBHA-staining of maturing enamel. *Adv Dent Res* 1: 162-175.

Smith CE, Nanci A (1989) A method for sampling the stages of amelogenesis on mandibular rat incisors using the molars as a reference for dissection. *Anat Rec* 225: 257-266.

Smith CE, Borenstein S, Fazel A, Nanci A (1989a) *In vitro* studies of the proteinases which degrade amelogenins in developing rat incisor enamel. In: *Tooth enamel V* (Fearnhead RW, ed), Yokohama: Florence Publishers, 286-290.

Smith CE, Pompura JR, Borenstein S, Fazel A, Nanci A (1989b) Degradation and loss of matrix proteins from developing enamel. *Anat Rec* 224: 292-316.

Smith CE, Nanci A (1995) Overview of morphological changes in enamel organ cells associated with major events in amelogenesis. *Int J Dev Biol* 39: 153-161.

Smith CE (1998) Cellular and chemical events during enamel maturation. *Crit Rev Oral Biol Med* 9: 128-161.

St.Amand TR, Zhang Y, Semina EV, Zhao X, Hu YP, Nguyen L, Murray JC, Chen YP (2000) Antagonist signals between BMP4 and FGF8 define the expression of Pitx1 and Pitx2 in mouse tooth-forming anlage. *Develop Biol* 217: 323-332.

Taichman LB (1998) Gene therapy. *Periodontal Clin Investig* 20: 7-9.

Takahashi K, Yamane A, Bringas P, Caton J, Slavkin HC, Zeichner-David M (1998) Induction of amelogenin and ameloblastin by insulin and insulin-like growth factors

(IGF-I and IGF-II) during embryonic mouse tooth development in vitro. *Connect Tiss Res* 38:269-278.

Takano Y (1979) Cytochemical studies of ameloblasts and the surface layer of enamel of the rat incisor at the maturation stage. *Arch Histol Jpn* 42: 11-32.

Takano Y, Ozawa H (1980) Ultrastructural and cytochemical observations on the alternating morphologic changes of the ameloblasts at the stage of enamel maturation. *Arch Histol Jpn* 43: 385-399.

Takatsuki A, Arima K, Tamura G (1971) Tunicamycin, a new antibiotic. I. Isolation and characterization of tunicamycin. *J Antibiot* 24: 215-223.

Takatsuki A, Kohno K, Tamura G (1975) Inhibition of biosynthesis of polyisoprenyl sugars in chick embryo microsomes by tunicamycin. *Agric Biol Chem* 39:2089-2091.

Takatsuki A, Tamura G (1982) Inhibition of glucoconjugate biosynthesis by tunicamycin, in *Tunicamycin*, Tamura G, Ed, Japan Scientific Societies Press, Tokyo, 35-49.

Tanaka T (1980) The origin and localization of dentinal fluid in developing rat molar teeth studied with lanthanum as a tracer. *Arch Oral Biol* 25: 153-162.

Thesleff I, Pratt RM (1980a) Tunicamycin inhibits mouse tooth morphogenesis and odontoblast differentiation in vitro. *J Embryol Exp Morphol* 58: 195-208.

Thesleff I, Pratt RM (1980b) Tunicamycin-induced alterations in basement membrane formation during odontoblast differentiation. *Dev Biol* 80: 175-185.

Thesleff I, Partanen A-M, Vainio S (1991) Epithelial-mesenchymal interactions in tooth morphogenesis: The roles of extracellular matrix, growth factors, and cell surface receptors. *J Craniofac Genet Dev Biol* 11: 229-237.

Thesleff I, Åberg T (2000) Molecular regulation of tooth development. *Bone* 25:123-125.

Timpl R, Brown JC (1994) The laminins. *Matrix* 14: 275-281.

Timpl R (1996) Macromolecular organization of basement membranes. *Current Opinion in Cell Biology* 8: 618-624.

Tomita T, Hashimoto H, Tomita N, Morishita R, Bak Lee S, Nakamura N, Yonenobu K, Kaneda Y, Ochi T (1997) In vivo direct gene transfer into articular cartilagene by intraarticular injection mediated by HVJ (Sendai Virus) and liposomes. *Arthritis & Rheumatism* 40: 901-906.

Toyono T, Nakashima M, Kuhara S, Akamine A (1997) Expression of TGF-β superfamily receptors in dental pulp. *J Dent Res* 76: 1555-1560.

Urist MR (1994) The search for and discovery of bone morphogenetic protein, in Urist MR, O'Conner BT, Burwell RG (eds): *Bone grafts, derivates and substitutes.* Butterworth, London, 315-362.

Vaahtokari A, Aberg T, Jernvall J, Keranen S, Thesleff I (1996) The enamel knot as a signaling center in the developing mouse tooth. *Mech Dev* 54: 39-43.

Vu D-D, Daniel NG, Nanci A (1999) In vivo model for the experimental manipulation of calcified tissues: A surgical approach for accessing the odontogenic organ and associated tissues in the rat incisor. *J Histochem Cytochem* 47: 1-14.

Wang Q, McEwen DG, Ornitz DM (2000) Subcellular and developmental expression of alternatively spliced forms of fibroblast growth factor 14. *Mech Dev* 90: 283-287.

Warshawsky H, Smith CE (1974) Morphological classification of rat incisor ameloblasts. *Anat Rec* 179: 423-446.

Warshawsky H, Josephsen K, Thylstrup A, Fejerskov O (1981) The development of enamel structure in rat incisors as compared to the teeth of monkey and man. *Anat Rec* 200: 371-399.

Warshawsky H (1985) Ultrastructural studies on amelogenesis. In: *The chemistry and biology of mineralized tissues* (Butler WT, ed), Birmingham: Ebsco Media, Inc, 33-44.

Webster EH, Hilfer SR, Searls RL, Kornilow J (1993) Effect of tunicamycin on maturation of fetal mouse lung. *Am J Physiol Lung Cell Mol Physiol* 265: L250-L259.

Winn SR, Uludag H, Hollinger JO (1998) Sustained release emphasizing recombinant human bone morphogenetic protein-2. *Adv Drug Del Rev* 31: 303-318.

Wozney JM (1992). The bone morphogenetic protein family and osteogenesis. *Mol Reprod Dev* 32: 160-167.

Wozney JM (1995) The potential role of bone morphogenetic proteins in periodontal reconstruction. *J Periodontol* 66: 506-510.

Yang J-JW, Hilfer SR (1982) The effect of inhibitors of glycoconjugate synthesis on optic cup formation in the chick embryo. *Develop Biol* 92:41-53.

Yoshida K, Bessho K, Fujimura K, et al. (1999) Enhancement by recombinant human bone morphogenetic protein-2 of bone formation by means of porous hydroxyapatite in mandibular bone defects. *J Dent Res* 78: 1505-1510.

Xu Z, Parker SB, Minkoff R (1990) Influence of epithelial-mesenchymal interaction on the viability of facial mesenchyme II: Synthesis of basement-membrane components during tissue recombination. Anat Rec 228: 58-68.

Zheng J, Ito Y, Imanishi Y (1994) Cell growth on immobilized cell-growth factor. 10. Insulin and polyallylamine co-immobilized materials. *Biomaterials* 15: 963-968.