Clinical and Molecular Studies on Impacted Canines and the Regulatory Functions and Differentiation Potential of the Dental Follicle

Pamela Uribe-Trespalacios

Department of Orthodontics
Institute of Odontology
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2017

Cover illustration: *"Grounds of the research project"* by Pamela Uribe
Illustrating the main components of the studies: The human dental follicle, radiographic localisation of a permanent impacted maxillary canine, and CX43-immunofluorescent staining in human dental follicular tissue.

To my family,

Gonzalo, Adriana and Melissa

TABLE OF CONTENTS

ABSTRACT

Background: Impaction of the permanent maxillary canines, which is a common problem in dentistry, may require surgery and long-term orthodontic treatment. Until now, impaction has mostly been linked to physical obstructions and the direction of movement of the tooth. However, the molecular co-ordination of bone formation and bone resorption necessary for the eruption process, which is suggested to be regulated by the dental follicle, needs to be investigated further.

Aims: The overall objectives of this thesis were to determine which clinical factors are related to impacted canines, and to investigate the regulatory functions and differentiation potential of the dental follicle.

Patients and methods: The positions of impacted and normally erupting canines (orthopantograms), the skeletal variables (profile radiographs), and dento-alveolar traits (casts) were evaluated as potential predictive factors for impaction using a multivariate data analysis (N=90 patients). The gene expression profiles of bone-regulatory markers were determined by RT-qPCR and immunofluorescence staining of human dental follicles. Whole dental follicles (N= 11) obtained from impacted canines, with or without signs of root resorption, and from control teeth (normal erupting teeth and mesiodens), together with the apical (N= 15) and coronal (N= 15) segments (processed independently), were analysed. *In vitro* osteogenic differentiation of human dental follicle cells (hDFC) was followed by the quantification of gene expression of osteoblast-phenotypic markers and alizarin red staining. Quantifications of the molecular permeability of gap junctional intercellular communication and of CX43 expression were performed with the dye parachute technique and flow cytometry, respectively. Next-generation sequencing and bioinformatics processing were used for the identification of differentially regulated genes and pathways involved in the differentiation of hDFC.

Results: Clinical variables related to the spatial location of the un-erupted tooth exert the strongest influences on impaction. However, they cannot be attributed to the cause of impaction, and they cannot be used as predictors. The RT-qPCR analyses revealed that the transcript levels for osteoclast-related markers (*M-CSF, MCP-1, RANKL*) were minimally expressed compared to those for osteoblastic markers (*RUNX2, COL-1, OSX, ALP, OCN*). No differential patterns of expression were identified between the impacted canines, with or without clinical signs of root resorption, or compared to the follicles from mesiodens or the normally erupting teeth. When the apical and coronal sections were analysed independently, significant differential expression was detected for the *RANKL* gene in the coronal part of the dental follicles, as compared with their corresponding apical parts. The induced expression levels of *RANKL* and *OPG* in cultured hDFC obtained from different patients were also significantly different. *CX43* was observed to be highly expressed in the follicular tissues, and its expression was increased when the cells were cultured in osteogenic medium, and even further enhanced when the cells were exposed to silica (Si). We found that multipotent stem cells residing in the dental follicle could be induced to differentiate towards an osteoblastic lineage under favourable *in vitro* conditions, resulting in regulation of the osteoblastic phenotypic markers (*RUNX2, OSX, BMP2, ALP*, and *OCN, BSP)* and active deposition of a mineralised matrix. In addition, Si enhanced osteogenic differentiation in combination with osteogenic induction medium, as revealed by increases in the expression of *CX43* and gap junction communication activity in the hDFC.

Conclusions: The results presented in the thesis reveal that clinical variables are influential, but not determinants, for tooth impaction. The dental follicle in the late pre-eruptive stage mainly expresses osteoblast-regulatory markers, whereas the levels of osteoclast-related markers are very low. Significant expression of CX43 and gap junction communication activity were detected, indicating an important role for these factors in the functional processes in the dental follicle. The significant upregulation of *RANKL* expression in the coronal part of the dental follicles suggests the importance of recruiting and activating osteoclasts, so as to form the eruption path through the alveolar bone. Moreover, the differential expression of induced *RANKL* in cultured hDFC may explain the diversity of events noted in the clinical setting during tooth eruption. Mesenchymal cells located in the dental follicle provide the optimal precursors, which can be cultured under *in vitro* conditions and further triggered with Si to differentiate towards an osteoblastic lineage.

SAMMANFATTNING PÅ SVENSKA

Bakgrund: Retinerade hörntänder i överkäken är ett relativt vanligt kliniskt problem som ofta kräver en lång behandling innefattande både kirurgi och tandreglering. Orsak till hörntänders retention är inte klarlagd men har relaterats till fysiskt hinder eller felaktig riktning på tandens eruption. En annan regleringsmekanism som behöver studeras ytterligare är den molekylära koordinationen av benbildning och benresorption som krävs för eruptionsprocessen, och som troligen regleras av tandfollikeln.

Mål: De övergripande målen med avhandlingen var att dels analysera om kliniska faktorer är relaterade till retinerade hörntänder och dels att studera tandfollikelns reglerande funktioner och differentieringspotential.

Material och methoder: Läget på retinerade och normalt erumperande hörntänder (orthopantogram), skelettala variabler (profil röntgen), dentoalveolära variabler (studiemodeller) utvärderades som potentiella prediktiva faktorer för retention av hörntänder med hjälp av multivariat dataanalys (N= 90). Genuttrycksprofil för benreglerande markörer i humana tandfolliklar analyserades med RT-qPCR och immunofluorescence färgning. Tandfolliklar (N= 11) erhållna från operation av retinerade hörntänder, med eller utan tecken på rotresorption, och från kontroll tänder (normalt erupterande tänder och mesiodens), samt apikala (N= 15) och koronala (N= 15) segment från folliklar analyserades. Osteogen differentiering hos odlade humana tandfollikelceller (hDFC) analyserades med kvantifiering av genuttryck för osteoblastfenotypiska markörer samt infärgning av mineraliserade områden med Alizarin Red. Kvantifiering av gap junctionkommunikation och CX43-uttryck utfördes med flödescytometri och dye transfer parachute teknik. Next generation sequencing (NGS) och bioinformatik analys användes för att identifiera genreglering och signalvägar under differentieringsprocessen av hDFC.

Resultat: Multivariat analys påvisade att de kliniska variablerna relaterade till lokalisationen av den retinerade tanden var de mest inflytelserika faktorerna avseende retention. Dessa faktorer kan emellertid inte hänföras som orsak till retention eller användas som prediktorer. RT-qPCR analys visade att transkriptionsnivåerna av osteoklastrelaterade markörer (*M-CSF, MCP-1, RANKL*) uttrycktes minimalt jämfört med de osteoblastiska markörerna (*RUNX2, COL-1, OSX, ALP, OCN*). Inga tydliga mönster av genuttryck identifierades hos retinerade hörntänder, med eller utan kliniska tecken på rotresorption, eller jämfört med folliklarna från mesiodens eller de normalt erupterade tänderna. När de apikala och koronala sektionerna analyserades påvisades ett signifikant ökat genuttryck för *RANKL* i de koronala delarna av tandfolliklarna, jämfört med motsvarande apikala delar. Det inducerade uttrycket av *RANKL* och *OPG* i odlade hDFC från olika patienter visade sig också vara signifikant olika. CX43 påvisades vara starkt uttryckt i follikelvävnader och uttrycket förhöjdes genom odling av cellerna i osteogent medium och förstärktes ytterligare när follikelcellerna exponerades för kiseldioxid (Si). Resultaten visade att multipotenta stamceller i tandfollikeln kan differentiera mot osteoblaster under gynnsamma *in vitro*-betingelser, vilket resulterar i reglering av osteoblastiska fenotypiska markörer (*RUNX2, OSX, BMP2, ALP, OCN och BSP*) och en aktiv bildning av mineraliserad matrix. Vidare påvisades att Si potentierar osteogen differentiering i kombination med ett osteogent induktionsmedium och stimulerar *CX43*-uttryck och gap junctionkommunikation mellan hDFC.

Slutsats: Sammanfattningsvis visar resultaten i avhandlingen att kliniska variabler är inflytelserika, men inte prediktorer för retention av hörntänder. Tandfolliklar erhållna från tänder under sent pre-eruptivt stadium uttrycker främst osteoblastregulatoriska markörer, medan de osteoklastrelaterade markörerna har mycket lågt uttryck. En signifikant *CX43*-uttryck och gap junctionkommunikation visades i tandfolliklarna vilket indikerar att signaler mellan celler via gap junctions spelar en viktig roll för olika funktionella processer i tandfollikeln. Den signifikanta uppreglering av RANKL-uttryck i den koronala delen av tandfolliklarna indikerar betydelsen av osteoklastrekrytering och aktivering för bildandet av tandens eruptionsväg genom det alveolära benet. Vidare kan skillnaderna i det inducerade RANKL svaret i odlade hDFC från olika patienter eventuellt förklara en del av de olikheter i tanderuption som kan ses kliniskt. Slutligen visar resultaten att de mesenkymala celler som finns i tandfollikeln är optimala prekursorer som kan odlas under *in vitro*-betingelser och induceras mot osteoblaster, vilket ytterligare kan förstärkas med Si.

PREFACE

This thesis is based on the following studies, which are referred to in the text by their Roman numerals.

I.   **Uribe P,** Ransjö M, and Westerlund A. Clinical predictors of maxillary canine impaction: a novel approach using multivariate analysis. European Journal of Orthodontics, 2017, 153–160. doi: 10.1093/ejo/cjw042.

II.   **Uribe P,** Larsson L, Westerlund A, and Ransjö M. Gene expression profiles in dental follicles from patients with impacted canines. *Submitted for publication*

III.   **Uribe P,** Plakwicz P, Larsson L, Czochrowska E, Westerlund A, and Ransjö M. Local patterns of regulatory factors expressed in human dental follicles. *Submitted for publication*

IV.   **Uribe P,** Johansson A, Westerlund A, Larsson L, Magnusson C, and Ransjö M. Effect of soluble Silica on Cx43 gap junction communication and osteogenic differentiation in human dental follicle cells. *In manuscript*

ABBREVIATIONS IN BRIEF

ALP	Alkaline phosphatase
ARS	Alizarin Red staining
ATP	Adenosine triphosphate
BMP	Bone morphogenetic protein
BSP	Bone sialoprotein
Ca^{2+}	Calcium, ionised
cAMP	Cyclic adenosine monophosphate
CTSK	Cathepsin K
CBCT	Cone beam computed tomography
CBX	Carbenoxolone
CCD	Cleidocranial dysplasia
cDNA	Complementary deoxyribonucleic acid
COL-1	Type I collagen
CT	Calcitonin
CTR	Calcitonin receptor
CX43	Connexin 43
DFC	Dental follicle cells
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FN	Fibronectin
FSK	Forskolin
GJC	Gap junction communication
HA	Hydroxyapatite
HCl	Hydrochloric acid
hDFC	Human dental follicle cells
IL-1	Interleukin-1
IL-6	Interleukin-6
LCM	Laser capture microdissection
LRP5	LDL receptor related protein 5
M-CSF	Macrophage colony stimulating factor
MIQE	Minimum information for publication of quantitative Real-Time PCR
MMP9	Matrix metalloproteinase 9
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cells
MVDA	Multivariate data analysis

OCN	Osteocalcin
OIM	Osteogenic induction medium
OPG	Osteoprotegerin
Opg	Panoramic radiography
OPLS-DA	Orthogonal projections to latent structures - discriminant analysis
OPN	Osteopontin
OSX	Osterix
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PDL	Periodontal ligament
PFE	Primary failure of eruption
PGE	Prostaglandin
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
qPCR	Quantitative polymerase chain reaction
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RGD	Arginyl-glycyl-aspartic acid
RNA	Ribonucleic acid
RT-qPCR	Real-Time quantitative polymerase chain reaction
RUNX2	Runt-related transcription factor 2
SFRP-1	Secreted frizzled related protein 1
Si	Soluble silica
SOST	Sclerostin
TNFα	Tumour necrosis factor alpha
TRAP	Tartrate-resistant acid phosphatase
VEGF	Vascular endothelial growth factor
VitD	Vitamin D
α-MEM	Minimum essential medium - alpha modification

DEFINITIONS IN BRIEF

Thesis Frame

Osteogenesis | The generation and development of bone tissue as a result of osteoblast differentiation.

Osteoclastogenesis | The development of osteoclasts through different stages, including commitment, differentiation, fusion, and activation of hematopoietic precursors.

Osteopaenia | Condition characterised by low bone mineral density and deterioration of trabecular bone, leading to osteoporotic fractures.

Osteoporosis | Systemic bone disease resulting from loss of bone mass and destruction of the bone microstructure, characterised by enhanced bone fragility and increased fracture risk.

Osteopetrosis | Rare genetic disorder caused by osteoclast failure, and characterised by increased bone mass and severe bone fragility.

Study I

Primary outcome variable | Dependent variable that is of the greatest importance in relation to the study's primary objective (also known as the "end-point").

Possible predictor variables | Other variables in the study that affect the primary outcome and that can be set or measured by the experimenter. They are sometimes referred to as independent variables when they are manipulated rather than just measured.

Malocclusion | Condition in which the teeth are not in a normal position in relation to the adjacent teeth in the same jaw and/or the opposing teeth when the jaws are closed.

Agenesis | Defective development or congenital absence of teeth. Tooth absence can result from a failure to initiate tooth formation (aplasia), reduced odontogenic potential of the dental lamina, or arrested development during the early stage. Agenesis is classified according to the number of missing teeth as hypodontia, oligodontia or anodontia.

Study II

PAXgene	Formalin-free fixation method, consisting of dual-cavity containers that are prefilled, which preserves the tissue morphology and biomolecules. Fixation is comparable to formalin fixation, except that it avoids destructive nucleic acid and protein crosslinking and degradation.
Reference genes	Internal control method for normalising mRNA data. Reference gene mRNAs should be stably expressed, and their abundances should show a strong correlation with the total amount of mRNA present in the sample.
$\triangle\triangle$Cq	Method to determine differences in concentrations between samples based on normalisation with a single reference gene. The difference in Cq values ($\triangle$Cq) between the target and the reference gene is calculated, and the $\triangle$Cq values of the different samples are compared directly.
Up-regulation and down-regulation	Refer to an increase or decrease, respectively, in the mRNA expression levels of a certain gene in relation to the selected reference genes.

Study III

Basal/Apical	Relating to, or situated towards the apex of the tooth.
Coronal	Direction towards the crown of a tooth, as opposed to apical.

Study IV

Osteoinduction	The process by which osteogenesis is induced, involving the recruitment of immature cells and the stimulation of these cells to develop into osteoblasts.
Nodule	Formation of mineralised matrix deposits in cell culture, which provides a means to assess osteoblastic lineage progression in osteogenic cell cultures.

INTRODUCTION

Tooth eruption is defined as the axial movement of a tooth from its developmental site within the alveolar bone to its functional position in the dental arch (Massler 1941). It is a localised, bilateral, symmetric, and precisely timed developmental process. Eruption begins only after mineralisation of the crown is completed, and it requires resorption of the alveolar bone and, in the case of the permanent dentition, resorption of the roots of the preceding deciduous tooth (Carlson 1944). Several mechanisms control the eruption process, taking into account the predetermined location, the onset at a specific age, and synchronisation with its contralateral tooth. For descriptive purposes, the eruption process can be divided in two parts: intra-osseous and supra-osseous (Weinmann 1944). Intra-osseous events involve bone resorption and translocation of the developing tooth within the bone. Supra-osseous events include the movement of the tooth once a section of the crown has surpassed the alveolar crest.

Animal experimental models have been used to study tooth eruption, with the most frequently used models being rodents with continuously erupting teeth and dogs with non-continuous tooth eruption. The evidence gained from these animal studies suggest that changes in alveolar bone metabolism during the intra-osseous stage of eruption are orchestrated by the dental follicle (Cahill and Marks 1980; Marks and Cahill 1987). The technical and ethical difficulties associated with the isolation of dental structures from animals and humans have hindered the elucidation of the responsible mechanisms. As a result, the tissue components, cell types, and signalling pathways involved in the eruption process in the human dentition remain largely unknown.

Tooth eruption theories

Numerous factors have been implicated in the control of the eruption process, including root elongation, the periodontal ligament, pulpal pressure, vascularity, and degree of innervation.

Root elongation: It is unlikely that root elongation moves the tooth in a three-dimensional manner in the space. In fact, root formation *per se* is not required for this process, as root-less teeth do erupt (Cahill and Marks 1980; Gowgiel 1967; Shields et al. 1973). It may, however, accelerate the eruption process. It has been shown that the immobilisation of erupting hamster's molars prevents root growth but not alveolar bone formation, and when released, these molars resume normal root growth and erupt rapidly (Gregg and Avery 1964). These findings are interpreted as meaning that root elongation is a consequence rather than a cause of eruption, and that alveolar bone formation is an important factor in eruption.

Periodontal ligament (PDL): This theory suggests that contraction of the oblique inclined periodontal ligament generates contractile or motive forces that initiate the eruption (Ten Cate 1985). In cases of osteopetrotic mutations, the PDL is present but the teeth do not

erupt (Marks 1989). In the case of root-less teeth, in particular those observed in dentinal dysplasia type I (which by definition lack a PDL), eruption occurs (Cahill and Marks 1980; Gowgiel 1967; Shields et al. 1973). This suggests that the PDL is not definitely involved in the eruption process.

Pulpal pressure: The pressure level in the pulp of the erupting teeth of dogs has been demonstrated to be higher than in the tissue above the erupting teeth (Van Hassel and McMinn 1972). This theory relies on the notion that a pressure gradient produces an extrusive force (Sicher 1942). However, the chronology of these pressure events has not been correlated with the onset of eruption.

Vascular theory: It has been suggested that the blood pressure level in the periodontal ligament generates an eruptive force (Massler 1941). It has been demonstrated that injection of a vasoconstrictor close to the root apex decreases tooth eruption, whereas the administration of a vasodilator increases eruption. This hypothesis has only been considered in the context of the pre-functional eruptive spurt stage (Cheek et al. 2002).

Innervation theory: Lack of eruption of permanent teeth has been described in dogs from whom the inferior alveolar nerve was removed. As a result, it was hypothesised that the nervous system exerted an influence on tooth eruption (Harputluoglu 1990). More recently, a new concept has been introduced that may explain the factors that influence the eruption process. The theory designates as essential for tooth eruption the following three components: the space in the eruption path; a lifting force mediated by the pressure from below: and the adaptability of the periodontal membrane (Kjaer 2014). This lifting, which results from innervation-induced pressure on the apical part of the tooth being transferred to the periodontal membrane, triggers the crown follicle to initiate resorption of the surrounding tissue. This pressure is considered to be the force that drives the teeth in the direction of the eruption.

The intra-osseous eruption stage enables accommodation of the root growth and tooth drift. The required bone remodelling events are likely to be co-ordinated by the dental follicle through local signals to the adjacent tissues (Marks and Cahill 1984). The supra-osseous phase is initiated after the mucosa overlying the alveolar crest is pierced. A major consequence of mucosal penetration is the formation of the junctional epithelium on the tooth surface (Schroeder and Listgarten 1971). The oral epithelium and the dental follicle covering the tooth are fused as the crown pushes its way into the oral cavity.

The role of the dental follicle in tooth eruption

Teeth develop from invagination of the oral ectoderm, which invades the mesenchyme of the jaws. Synergistic interactions between the oral epithelium cells and the mesenchymal cells derived from the neural crest (ectomesenchyme) initiate the morphogenesis and give rise to most of the dental tissues and the periodontium (Miletich and Sharpe 2004). The peripheral part of the condensed dental mesenchyme forms the dental follicle, which surrounds the tooth germ and eventually contributes in the development of the periodontal tissues.

The experimental studies performed by Marks and Cahill (1986) provide the most convincing explanation of the characteristic changes that occur in the alveolar bone around the erupting tooth. The bone surfaces surrounding the erupting teeth were observed with photomicrographs and show evidence of scalloped bone indicative of bone resorption in the direction of eruption, trabeculae and newly bone formation in the opposite direction, and smooth surfaces in between, undergoing neither resorption nor formation (Cahill 1974; Marks and Cahill 1986; Marks et al. 1983). Temporary impaction of erupting teeth by a trans-mandibular wire prevents tooth movement, although it does not affect the bone resorption that creates the eruption pathway and leads to timely exfoliation of the deciduous predecessor (Cahill 1969). Resorption of this bone is mediated by osteoclasts, and it proceeds at the same rate as that of the corresponding teeth on the contralateral unobstructed side (Cahill 1974). Once the tooth is released, the eruption process terminates. Moreover, studies conducted in osteopetrotic rats have revealed insufficient tooth eruption and limited bone resorption (Marks 1973; 1981). Taken together, these observations suggest that alveolar bone resorption is not dependent upon tooth eruption, whereas movement of the tooth crown to the oral cavity is dependent upon bone resorption.

The dental follicle, which is soft tissue located between the bony crypt and the un-erupted tooth crown, is crucial for tooth eruption. The dental follicle plays an essential role in the local control of alveolar bone polarisation, i.e. bone formation and resorption, such that removal of the dental follicle restricts eruption (Cahill and Marks 1980; Marks and Cahill 1984). This idea has been reinforced by studies showing that tooth eruption continues even after ablation of the gubernaculum dentis, destruction of one or both of the roots, or surgical removal of the tooth crown of the third permanent pre-molars in dogs (Cahill and Marks 1980). The fact that tooth eruption proceeds in the absence of root formation, indicates that a periodontal ligament is not required for eruption. In contrast, removal of the dental follicle prevents eruption, with no radiographical evidence of bone resorption or the formation of an eruption path. To confirm the indispensable role of the dental follicle, dental crowns were surgically removed and metal beads were substituted for dental follicles just prior to scheduled eruption; the replacements erupted in a timely fashion after formation of the usual eruption paths and trabecular bone from the base of the bony crypt (Marks and Cahill 1984). Removal of either the apical half or coronal half of the follicle prevented eruption. Bone resorption and the formation of an eruption path do not occur after the removal of the coronal part, and bone formation does not occur after removal of the apical part of the follicle (Marks and Cahill 1987).

Taken together, these observations imply that impaction is also related to the biological factors and signal transduction pathways that are involved in the bone remodelling needed for tooth eruption and that are suggested to be regulated by the dental follicle (Wise et al. 2011; Wise et al. 1985). Consequently, osteoclasts and osteoblasts are activated on the dental bone surfaces just prior to the onset of eruption (Marks et al. 1983; Wise and Fan 1989; Wise et al. 1985).

That active eruption begins only after crown formation is completed suggests a role for the enamel organ in the early signalling of eruption (Carlson, 1944). It is important to consider the proximity and the tight adherence of the reduced enamel epithelium and the true dental follicle. To elucidate the ability of the enamel organ to cause eruption, Larson et al. (1994) removed the external layer (the true dental follicle) just prior to eruption. None of the teeth that lacked dental follicles erupted, whereas the teeth from which the follicles were separated

and then replaced did erupt. These experiments suggest that the enamel organ alone is unable to account for the radiographical or histological indicators of tooth eruption. Eruption of the teeth relies on the true dental follicle alone or in combination with the enamel organ, but not on the enamel organ itself. In this case, tooth eruption may be considered as an example of a collaborative epithelial-mesenchymal interaction during development (Gorski and Marks 1992).

As reviewed above, the intra-osseous events of tooth eruption are attributed to the dental follicle. While part of the follicle is lost after mucosal penetration, as the tooth erupts, the follicle undergoes changes that result in the development of the suspensory mechanism for the tooth (i.e. the periodontal ligament, cementum, and alveolar bone). Subsequent events can be controlled by these dental follicle derivatives and other involved tissues.

Post-emergent eruption

Post-emergent eruption occurs in four stages (Proffit and Frazier-Bowers 2009). In the first stage, the *pre-functional burst,* the tooth moves from the site of initial emergence into the mouth. Pre-occlusal eruption from gingival emergence to the occlusal plane is thought to be mediated by forces that are generated through bone apposition at the base of the crypt (Schroeder et al. 1992; Wise et al. 2011). The second and third stages are paralleled by the vertical growth of the face. These stages are the *juvenile equilibrium*, during which both jaw growth and eruption proceed quite slowly, followed by the *adolescent eruptive spurt* as growth accelerates and the teeth have to move from their original eruptive position to remain in occlusion. Once a tooth has reached the occlusal contact, collagen fibres in the periodontal ligament become oriented to support the tooth so as to counteract the forces of occlusion. Concomitantly, the arrangements of the alveolar crest-, horizontal-, oblique- and apical-fibres of the PDL are established. As the collagen matures, it cross-links and shortens, and it provides the potential propulsive mechanism for eruption, which is postulated to occur only after the number and orientation of the fibres have changed in response to exposure to oral forces (Moxham and Berkovitz 1984). The fourth stage entails the *adult equilibrium*. However, eruption continues throughout life to compensate for occlusal wearing of the teeth and to allow growth of the jaws (Thilander 2009). Eruption can be reactivated if the contact with the antagonist is ever lost.

Eruption problems

Abnormal eruption can be caused by a lack of functionality of the tissue layers that are important for the eruption process. The aetiology is related to either systemic or local regulation of the genes involved in the bone remodelling process. In patients with certain developmental syndromes, e.g. cleidocranial dysplasia (CCD), hyper-IgE syndrome, and osteopetrosis, multiple teeth are usually affected. In patients with a local eruption disturbance, only one or few of the permanent teeth are involved.

To identify the cause of disturbed eruption, the first step is to examine the implicated teeth with respect to timing of eruption and positioning (Table 1). Arrested eruption may occur during any of the eruption stages. The main causes of eruption disturbances are: ectopic position of the tooth germ; obstacles in the eruption path; and failure of the eruption mechanism (Andresen 1997).

Table 1. Pathological eruption of teeth in humans.

PROBLEM	DIAGNOSIS	DESCRIPTION	CAUSE
Abnormal timing	Premature	Before the expected time of eruption	*Syndromes and vascular and endocrine disorders*
	Delayed	Past the expected time of eruption	
Abnormal positioning	Ectopic/Displaced	Eruption in the wrong direction or location	*Inappropriate location of tooth buds, blockage of the eruption path, lack of space*
	Transposition	Positional interchange with another tooth	
Lack of eruption	Absence	No eruption is present	*Syndromes and dysplasia*
Arrested eruption	Impaction	Retained and embedded in the alveolar bone	*Blockage Genetic theory Guidance theory*
	Primary retention	Before emergence (no other recognisable disorder and no mechanical interferences)	*Failure of the eruption mechanism (Dental follicle: "PTHrP")*
	Secondary retention	After emergence	*Failure of the eruption mechanism (PDL: Trauma, ankylosis, hypercementosis)*

Primary retention

Primary failure of eruption (PFE) is defined as a non-syndromic eruption failure before gingival emergence in the absence of a recognisable physical barrier in the eruption path (Proffit and Vig 1981). The major features of PFE are: infra-occlusion of the affected teeth; significant posterior open-bite malocclusion accompanying normal vertical facial growth; and inability to move the involved teeth with orthodontic forces. When the eruption of a permanent tooth is at least 2 years behind schedule, PFE should be suspected (Raghoebar et al. 1991). The affected teeth are not ankylosed but they fail to follow the eruption path that has been cleared for them, i.e. the osteoclastic activity that resorbs the apical alveolar bone. The PDL exhibits abnormal features, such that not only does the affected tooth fail to erupt, it does not respond to orthodontic forces. Typically, the posterior but not the anterior teeth are affected. The first permanent molars are most likely to be involved, although pre-molars or canines can also be affected. The PFE phenomenon is considered to be due to a primary defect in the intra-osseous eruptive process. Genetic and clinical findings have further confirmed that PFE is an inherited disorder (Frazier-Bowers et al. 2007). Recent studies have proposed the gene for parathyroid hormone-related protein (*PTHrP*) as a candidate for causing eruption failure (Frazier-Bowers et al. 2010b). The *PTHrP* gene acts in the bone homeostatic pathway. PTHrP can disrupt the balance between bone resorption, which is necessary to establish the path for an erupting tooth, and bone formation, which is

necessary to rebuild the bone through which the tooth has transited (Frazier-Bowers et al. 2010a).

Secondary retention

Secondary retention is defined as an arrest of eruption after tooth emergence (Raghoebar et al. 1989). Infra-occlusion of a molar at an age when the tooth would normally be in occlusion is the most common clinical characteristic. The term "secondary" is used to indicate that the retention occurs subsequent to gingival penetration or at a later stage of the eruptive process. Until the phase in which secondary retention occurs, the eruptive process appears normal (Raghoebar et al. 1991). The aetiology of secondary retention may be linked to not only the oral epithelial lining, but also to the cell layers in the perio-root sheet. It is plausible that in traumatic cases, radicular bleeding could result in a resorption process that is later repaired by bone (Kjaer 2017). The tooth is then compromised due to ankyloses or hypercementosis. These conditions result from the inability of the periodontal tissue to reorganise and adapt to eruptive movements. Other related terms used in the literature include submerged, half-retention, re-impaction, re-inclusion, and ankyloses.

Impaction

According to a recent systematic review (Naoumova et al. 2011), there is still no consensus as to an exact definition or classification in the literature for tooth impaction. There are many descriptions and interpretations of an impacted tooth, most of which are related to putative aetiological factors. Impaction is defined as the cessation of eruption of a tooth due to a clinically or radiographically detectable physical barrier in the eruption path, or due to abnormal positioning of the tooth, and for which there is clinical and radiographic evidence that further eruption may not occur within the normal period of growth (Thilander and Jakobsson 1968). Impaction should be considered when there is an un-erupted tooth after complete root development, or when the contralateral tooth has been erupted for at least 6 months with complete root formation (Lindauer et al. 1992). In cases of impaction, early removal of the physical barrier increases the likelihood of spontaneous eruption of the tooth. If the impaction is due to space loss, extraction or space augmentation can be performed. If it is due to ectopic eruption of tooth germ, then the treatment options are surgical exposure, surgical repositioning, auto-transplantation, or removal of the affected tooth. However, these definitions only consider physical obstructions and the mechanical movement of the tooth, thereby neglecting all the biological and molecular co-ordination events involved in the eruption process. In this context, impaction may also be related to the biological factors and signal transduction pathways involved in the bone remodelling that is needed for tooth eruption and that is suggested to be regulated by the dental follicle.

Impacted permanent maxillary canines

Permanent maxillary canines play a fundamental role in facial appearance, dental aesthetics, arch development, and functional occlusion. The process of eruption of the permanent canines leading to their final positioning in the oral cavity is complex and the longest of all the permanent teeth (Lappin 1951). The canine moves a distance of around 22 mm between

the ages of 5 and 15 years (Coulter and Richardson 1997). Its eruption path comprises a series of events, including movements in three directions: posterior, vertical, and lateral. The germ of the canine is situated high in the maxilla as it begins to develop with the crown mesially and palatally directed. The calcification process starts at the age of 1 year, and at around 6 years of age, the calcification of the enamel is completed (Dewel 1949). When the canine migrates down and forward toward the occlusal plane, the tooth gradually becomes more upright until it reaches the distal aspect of the lateral incisors root and the mesial aspect of the root apex of the deciduous canine. Almost three-quarters of the root is formed before the canine erupts, and the root formation is completed around 2 years after eruption (Nanda, 1983). The permanent canine is among the last teeth to erupt in the maxilla, and the mean age of emergence varies depending on the studied population. In American children, the mean age of eruption was found to be 12.3 years for girls and 13.1 years for boys (Hurme, 1949). In a Swedish population, the mean eruption time was 10.8 years in girls and 11.6 years in boys (Hagg and Taranger 1986).

If the tooth does not follow this type of trajectory, the canine tends to become impacted. Impacted permanent maxillary canines are a common problem in dentistry, often requiring surgery and long-term orthodontic treatment. The occurrence of impacted maxillary canines may affect the neighbouring structures, and its causative factors and preventative approaches remain matters of debate.

Incidence

Permanent maxillary canines are the second most frequently impacted teeth after the third molars (Bishara 1992). Maxillary canine impaction occurs in approximate 2%–3% of the population (Peck et al. 1994; Thilander and Jakobsson 1968). The ratio of palatal to buccal impaction is 8:1; moreover, it is twice as common in female patients as in male patients (Cooke and Wang 2006; Ericson and Kurol 1987b). Impaction in the maxilla is more than ten-fold more frequent than impaction in the mandible. Of all the patients who have impacted maxillary canines, 8% present with bilateral impaction (Ericson and Kurol 1988; Thilander and Jakobsson 1968). In a Caucasian population, maxillary canine displacement was found to be five-times more common than in an Asian population, with the majority of the canines in the Caucasians showing palatal impactions, while buccal displacements were more common among Asians (Oliver et al. 1989).

Aetiology

The aetiology of impacted canines is obscure and most probably multifactorial (Becker et al. 1999; Peck et al. 1994; Sajnani and King 2012c). It has been proposed that buccal and palatal impacted canines are two different entities characterised by different aethiopathogeneses. Buccal canine impaction is thought to be a form of crowding (Bishara 1992; Jacoby 1983; Peck et al. 2002; Thilander and Jakobsson 1968). In contrast, some authors believe that the presence of excess space in the upper arch can lead to palatal canine impaction by allowing the canine to cross back to the palatal side (Mercuri et al. 2013). Others have linked the congenital absence or presence of small lateral incisors and late-developing teeth to palatal canine impaction (Baccetti 1998a; Becker et al. 1999; Brin et al. 1986; Leifert and Jonas 2003). The aetiology of palatal impaction has also been related to a presumably defective eruption mechanism (Kjaer 2017). Other factors described in the literature that are thought to cause canine impaction include: obstruction; abnormal position of the tooth bud; tooth

morphology; dental crowding; a long and complicated path of eruption; early loss of the deciduous canine; prolonged retention of the deciduous teeth; lack of vertical movement; and systemic diseases (Bishara 1992; Brin et al. 1993; Ericson and Kurol 1986b; Jacoby 1983; Sajnani and King 2012c; Sorensen et al. 2009).

Several studies have attempted to identify predictive factors for the displacement and impaction of canines, to facilitate early identification and enable an interceptive treatment aimed at spontaneous correction and eruption. However, the use of a considerable variety of diagnostic tools, study designs, and research approaches has generated results that are conflicting and far from conclusive. The sector location of the un-erupted permanent canine (Lindauer et al. 1992; Olive 2005; Sajnani and King 2012c; Warford et al. 2003) and dentoalveolar features, including delayed dental development in relation to age (Becker and Chaushu 2000; Rozylo-Kalinowska et al. 2011; Sajnani and King 2012a), have been proposed as indicators of eventual impaction. Other dental anomalies, such as aplasia, peg-shaped laterals, and agenesis of adjacent teeth, have been associated with impacted canines, suggesting a genetic aetiology (Baccetti 1998a; Brin et al. 1986; Leifert and Jonas 2003; Peck et al. 2002; Sacerdoti and Baccetti 2004). Moreover, skeletal features, e.g. class II division 2 malocclusion, a deep overbite, a hypodivergent profile, and abnormal maxillary width, have also been linked to impaction (Al-Nimri and Gharaibeh 2005; Anic-Milosevic et al. 2009; Basdra et al. 2000; Harzer et al. 1994; Langberg and Peck 2000; Leifert and Jonas 2003; Ludicke et al. 2008; McConnell et al. 1996).

Two major theories associated with palatally displaced maxillary canines are found in the literature and may explain the main contributing factors.

The **guidance theory** proposes that the canine erupts along the root of the lateral incisor, which serves as a guide, such that if the root of the lateral incisor is absent or malformed, the canine will not erupt (Becker et al. 1999; Brin et al. 1986). These authors have suggested that the presence of a lateral incisor root is an important variable in directing the erupting canine in a favourable direction. In line with this rationale, others have suggested that this anomaly is due to local predisposing factors, such as congenitally missing lateral incisors, supernumerary teeth, odontomas, tooth transposition, and other mechanical determinants, all of which interfere with the eruption path of the canine (Becker et al. 1999; Brin et al. 1986; Hitchin 1951; Thilander and Myrberg 1973). Becker and colleagues have reported a 2.4-fold increase in the incidence of palatally impacted canines adjacent to the sites of missing lateral incisors, as compared with palatally impacted canines, in the general population (Becker et al. 1999).

The **genetic theory** is based on the observation that palatally displaced canines rarely occur as an isolated sign, but instead are generally accompanied by genetically determined tooth anomalies, such as agenesis or peg-shaped laterals incisors and aplasia of other teeth. It has been postulated that some genetically and hereditary modulated mechanism underlies the linkage between coincidental dental abnormalities, as evidenced by their frequency of association (Peck et al. 1994; 2002). Lending further support to this theory, Baccetti (1998b) has reported that palatally impacted maxillary canines are genetically reciprocally associated with anomalies such as enamel hypoplasia, infra-occlusion of primary molars, aplasia of second premolars, and small maxillary lateral incisors. Peck and co-workers have noted the high probability of additional dental abnormalities occurring in combination with a palatally displaced canine, such as congenital tooth absence and delayed eruption (Peck et al. 1994).

Recently, a concept has emerged that integrates both of the above-mentioned theories into a single theory. The **sequential theory** suggests that both buccal and palatal impactions have similar aetiological factors. Genetic mechanisms may strongly influence the localisation and direction of the developing tooth, while the guidance from the lateral incisor and the stage of development play crucial roles in determining the final position (palatal or buccal) of the impacted canine (Sajnani and King 2012c).

A major limitation of the studies listed in the literature is that only radiographical and clinical findings have been studied and contemplated over the years. Additional parameters associated with the aetiology of impacted canines, such as biological factors and signal transduction pathways, have been disregarded and warrant further investigation. Therefore, it is not clear as to whether there are biological mechanisms involved in the impaction of canines related to the bone remodelling process needed for eruption to occur, and it is not known if these mechanisms are regulated by the dental follicle. A better understanding of these regulatory pathways would provide insights into the factors responsible for tooth impaction.

Diagnosis

Diagnostic methods allow the early detection and prevention of canine impaction. There is a sequential routine method used for the localisation and supervision of an impacted maxillary canine. Once the diagnosis is acquired, there is no need to further advance to the next step.

1. Inspection and palpation of the canine bulge
2. Radiographic assessment of the un-erupted canine localisation
3. Extraction of the deciduous canine
4. Radiographic follow up and clinical supervision every 6 months
5. Surgical exposure of impacted maxillary canine

Clinical:

- **Inspection:** Various clinical signs of impaction have been documented in the literature, including delayed eruption of the permanent canine, asymmetry in the exfoliation and eruption between the right side and left side of the maxilla, over-retention of the deciduous canines, absence of the labial bulge and/or presence of a palatal bulge, and distal crown tipping of the lateral incisor (Bishara 1998; Shapira and Kuftinec 1998). According to Ericson and Kurol (1986a), three specific clinical signs are to be regarded as indications for further radiographical control: 1) a pronounced difference in eruption of the canines between the left side and the right side; 2) absence of the bulge in the normal position when the occlusal development is advanced; and 3) lateral incisors appearing late during eruption or showing a pronounced buccal displacement or proclination.

- **Palpation:** A labial bulge is normally palpable about 1 year before the emergence of the permanent canines. Therefore, in the absence of the labial bulge between the ages

of 9 and 11 years, ectopic eruption should be suspected and routine radiographical control of the position of the canine is recommended until the canine can be palpated buccally (Ericson and Kurol 1986a). Manipulation of the deciduous canine to evaluate mobility may also be carried out. Non-significant mobility of the deciduous canine after the age of 13 years strongly indicates displacement of the permanent canine (Bedoya and Park 2009; Bishara 1998; Jacobs 1999).

Radiographic:

Radiographic localisation is crucial for predicting the success of interceptive treatments, early detection of root resorptions of the adjacent teeth, and planning the means of surgical access and the direction of orthodontic traction. In routine orthodontic practice, panoramic radiograph is commonly used. Recently, computerised tomography (CBCT) has been proposed as a novel alternative for accurately defining the positions of the canines. Despite the more detailed information yielded by this technique, the higher dosage of irradiation and higher costs outweigh its relative advantages (Schmuth et al. 1992). Accurate localisation in the three planes of space requires more than one image and relies on a combination of clinical and radiographical findings. The positions of the canines, tooth development, possible overlaps with the adjacent incisors, and the linear and angular measurements are frequently used as variables in the radiological assessment.

Ericson and Kurol (1988) have used linear, angular, and sector measurements to estimate the *effectiveness of interceptive extraction of the deciduous canine* in panoramic radiographs (Fig. 1). Powers and Short (1993) have also looked at angulation as a predictor, finding that if the tooth is angled at more than 31° to the mid-line, its chances of eruption after extraction of the deciduous canine are decreased (Fig. 2).
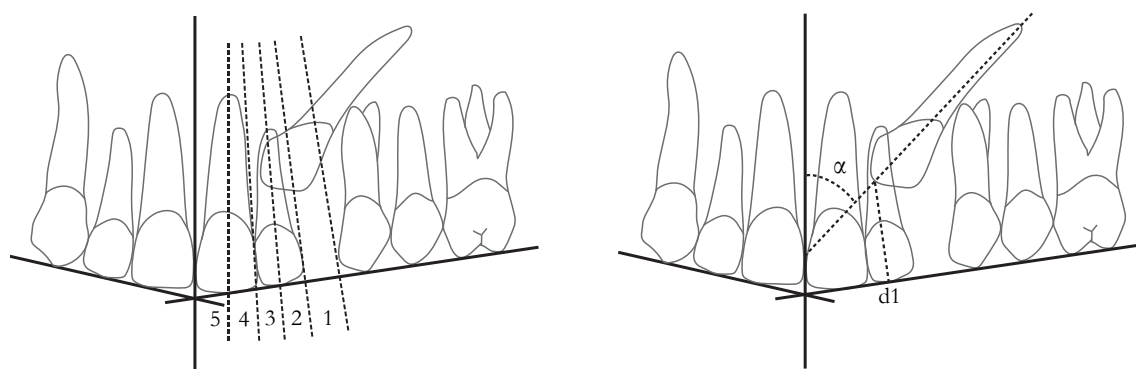

Figure 1. a) Distribution of the permanent maxillary canines according to the medial position of the canine crown in sectors 1–5. b) Mesial inclination (α) to the mid-line and distance (d1) to the occlusal plane of the permanent canine in the frontal plane, according to Ericson and Kurol (1988).
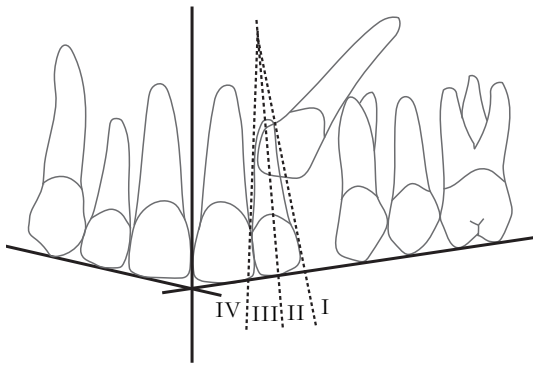
Figure 2. Sector distribution by Lindauer et al. (1992). Sector I is located distal to a tangent to the distal crown and root of the lateral incisor. Sector II includes the area from the tangent of the distal surface to a mid-line bisector of the lateral incisor tooth. Sector III includes the area extending from the mid-line bisector to a tangent to the mesial surface of the lateral incisor crown and root. Sector IV includes all areas mesial to Sector III.
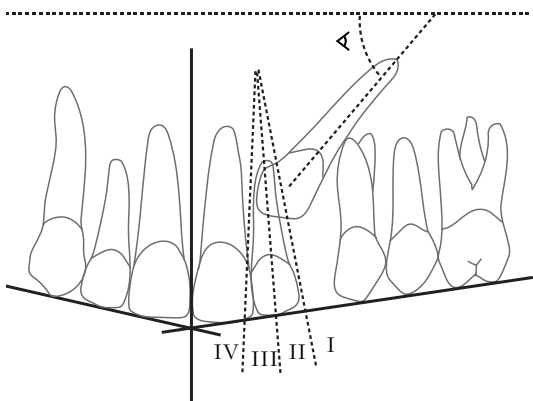
Figure 3. Sector and angular measurements used by Wardford et al. (2003). Sector delineation is taken from the description by Lindauer et al. (1992). Angulation is determined by the measurement of the mesial angle formed by the long axis of the un-erupted tooth with a bicondylar line drawn from the most-superior points of the condyles.

These criteria were further employed as a *method to predict the risks of impaction of the canine* with minor modifications (Lindauer et al. 1992) (Fig. 2). Using this method, the authors found that 78% of the canines that were destined to become impacted could be identified, all of which had cusp tips located in Sectors II–IV. Warford et al. (2003) used the method described by Lindauer et al., adding angulation of the axis of the impacted canine, and confirmed that 82% of the impacted canines were located in Sectors II–IV (Fig. 3). Sajnani and King (2012b) added to the evaluated parameters the distance from the cusp tip to the occlusal plane (d1) and root development, in order to identify the vertical level of the impacted canine.

Sequelae

Impacted canines are usually asymptomatic. However, impaction may cause severe complications, such as malocclusions and pathological conditions, e.g. dentigerous cysts. The most common irreversible and adverse effect of maxillary canine impaction is root resorption (Guler et al. 2012; Nagpal et al. 2005; Thilander and Jakobsson 1968). This loss of tooth cementum and/or dentin is due to the activities of resorbing cells (Tronstad 1988). The degree of resorption is suggested to depend on the nature and strength of the pressure produced by the impacted canine (Fuss et al. 2003). The way in which activation of the osteoclasts is triggered and which local factors are released from the follicle remain undetermined. The only major observation is that, radiographically, the resorption area is located adjacent to the impacted tooth.

Introduction

Root resorption is reported to occur in 12% of the incisors that lie close to ectopic maxillary canines (Thilander and Jakobsson 1968), although it is seen in up to 67% of the incisors when investigated with CBCT (Walker et al. 2005). The risk for root resorption occurrence increases when the ectopically erupting canine has a completely developed root and presents a medial inclination that overlaps more than 50% with the adjacent lateral incisors on radiographs (Ericson and Kurol 1987a). The most common area for resorption of the lateral incisors is the middle third of the root (82%), followed by the apical third (13%) (Ericson and Kurol 1987a). Extensive root resorption may result in removal of the damaged tooth and therefore, it is a serious complication.

Radiographical studies conducted by Ericson and Kurol (1987a; 1987b) suggest that there is no association between enlarged follicles and root resorption. They compared a resorption group with a control group that presented ectopically positioned canines that did not develop resorption in the adjacent teeth. They found that the incidence of follicular enlargement in the control group did not differ significantly from that of the resorption group, and concluded that follicular enlargement was not a risk factor. Morphological and histological studies indicate that the dental follicle of the canine will often expose the root of the adjacent incisor during eruption, without resorbing any of the hard tissues of the root (Ericson et al. 2002). However, considering the proposed regulatory functions of the follicle during the eruption process, the resorption of neighbouring teeth during maxillary canine impaction could be related to the follicle being in close physical contact with the root. The mechanisms are not clear but they may involve follicle-derived factors that regulate bone cellular activities, resulting in the recruitment and activation of osteoclasts that resorb the root cementum and dentin.

Treatment

Early diagnosis and prompt intervention represent the most desirable approach in managing impacted canines. The extraction of the deciduous canine, in the late mixed dentition stage (10–13 years of age), to prevent permanent canine impaction, is based on the assumption that the persistence of the primary tooth represents a mechanical obstacle for the emergence of the permanent tooth (Jacobs 1998).

Ericson and Kurol (1988) have used linear, angular, and sector measurements to assess the effectiveness of interceptive extraction of the deciduous canine (Fig. 1). If the crown of the permanent canine lay distal to the mid-line of the lateral incisor root, extraction of the deciduous canine normalised the erupting direction of the permanent canine in 91% of cases. In contrast, the success rate decreased to 64% if the permanent canine crown lay mesial to the mid-line of the lateral incisor root. If no improvement was seen at 12 months post-extraction, then none was expected. Other factors that contribute to a successful outcome were investigated by Power and Short (1993), who found that horizontal overlap of the lateral incisor was the most significant factor. If the impacted tooth exceeded half the width of the adjacent tooth, a successful outcome was deemed unlikely. For ethical reasons, Ericson and Kurol did not design a study that included an untreated control group. However, a recent study (Naoumova et al. 2015) included a control group and reported that 69% of the permanent canines erupted spontaneously after deciduous canine extraction, as compared to 39% in the control group. More significant angular and distance changes occurred in the extraction group than in the control group. All the previous authors have recommended

clinical and radiographical re-evaluation every 6 months, although if the patient is more than 13 years old, alternative treatment modalities should be considered.

In cases when the diagnosis has been made late or the outcome of the interceptive treatment is unsuccessful, uncovering of the impacted tooth is part of the definitive solution. The most common procedure is surgical exposure, followed by orthodontic treatment to bring the canine into the dental arch (Fig. 4) (Sampaziotis et al. 2017). Occasionally, the impacted canine is considered for extraction due to ankylosis, extensive root resorption, and problematic location of the tooth and/or extreme dilacerated roots. In other cases, the patients are not interested in treatment. In these circumstances, if there is no evidence of resorption of the adjacent teeth and if the deciduous canine has a good aesthetic and prognosis, it may be better not to give any active treatment but instead regularly monitor the tooth by radiography (Bishara 1998).
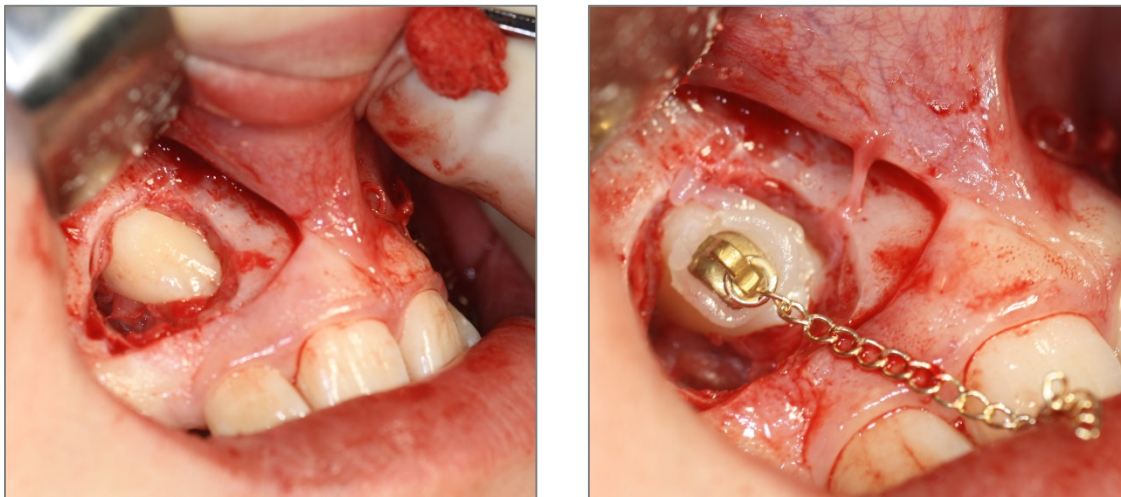

Figure 4. Closed surgical exposure is performed to uncover and identify the location of the buried tooth. The crown of an impacted canine is exposed using a wide flap, together with removal of the superficial bone and underlying follicular tissue. An attachment is then bonded and a chain is connected for vertical orthodontic traction [Use of images authorised by the patient].

Bone tissue, bone cells, and bone remodelling

Sequential remodelling of the jawbone surrounding the tooth is a prerequisite for normal tooth eruption. The remodelling process is dependent upon the chronologically regulated activities of different bone cells.

Bone tissue

The skeletal system, with over 200 named bones, has many mechanical and metabolic functions that are important for the human body (Karsenty 2003). It provides the rigid framework that supports the body and protects the inner organs, and it allows all the body

movements. Most importantly, the skeleton, as an organ system, is crucial for the endocrine signalling that regulates energy metabolism, and it is the site of haematopoiesis. The main component of bone is a mineralised extracellular matrix (EMC), which is composed of inorganic and organic phases. The principal inorganic component, accounting for approximately 65% of the dry weight, is hydroxyapatite (HA) $[Ca_{10}(PO_4)_6(OH)_2]$, which is a mineral composed of calcium (Ca^{2+}) and phosphate. The organic phase, which accounts for the remaining 35% of the dry weight, is composed of collagen fibres (90%), mainly type I collagen (COL-1), as well as non-collagenous proteins (10%), such as fibronectin (FN), osteocalcin (OCN), osteonectin, and glycosaminoglycans (Young 2003).

Two different forms of osteogenesis exist: endochondral ossification, and intra-membranous ossification. During endochondral ossification of long bones, mesenchymal stem cells (MSC), which have differentiated into chondrocytes, create the cartilaginous patterns that are subsequently mineralised and transformed into bone. In contrast to endochondral ossification, during intra-membranous ossification, the MSC differentiate directly into osteoblasts rather than with a cartilage template. The differentiated cells produce immature non-mineralised bone matrix (termed *osteoid*) that mineralises over time (Teti 2011). Bones that are formed without previously being modelled in cartilage are the flat bones of the skull and the face, the maxilla and mandible, and the clavicle.

In general, bone has an outer layer of compact bone, also known as cortical bone, which surrounds a more porous centre, the trabecular bone. Bone marrow is found inside the highly vascularised trabecular bone, and also in the larger cavities of long bones.

Bone cells

Bone contains four different types of cells: osteoblasts, osteoclasts, osteocytes, and lining cells. Bone formation and maintenance are mediated by the coupled activities of osteoblasts and osteoclasts.

Osteoblasts

Osteoblasts play a central role in bone formation, a process in which the cells initially synthesise osteoid, and then promote its mineralisation. Active osteoblasts are mononucleated cells, derived from undifferentiated MSC, generally rounded or cuboidal in shape, and they line up on bone surfaces. Osteoblasts represent only 4%–6% of the total resident cells in the bone tissue (Aubin et al. 1995). Osteoblast differentiation starts with the commitment of osteoprogenitor cells from MSC, which thereafter differentiate into immature and more mature osteoblasts that express osteoblast-phenotypic genes. With time, the mature osteoblasts become osteocytes or bone-lining cells, or they undergo apoptosis. Osteoblast commitment, differentiation, and functions are all governed by several transcription factors, resulting in the expression of phenotypic genes and acquisition of the osteoblast phenotype (Marie 2008).

Runt-related transcription factor 2 (RUNX2), formerly known as cbfa1, is a master regulator of osteoblast differentiation during the early development of committed osteoprogenitors. That RUNX2 plays an essential role is demonstrated by the findings that RUNX2-deficient

mice have a cartilaginous skeleton and complete absence of ossification, while RUNX2-over-expressing mice exhibit osteopaenia (Komori et al. 1997; Liu et al. 2001). RUNX2 insufficiency in humans results in cleidocranial dysostosis (CCD), a disease characterised by increased bone density of the jaw bones and multiple supernumerary teeth that do not erupt. The RUNX pathway is linked to the promoter that controls the expression of all the major osteoblast genes, including COL-1, osteopontin (OPN), bone sialoprotein (BSP), and OCN, resulting in the establishment of an osteoblast phenotype. Nonetheless, after the cells commit to the osteoblastic lineage, over-expression of RUNX2 negatively regulates osteoblast function and matrix production, as evidenced by examining transgenic mice (Liu et al. 2001).

In contrast, the Osterix (OSX) protein is vital for promoting the earlier stages of osteogenesis; once osteoprogenitors express *OSX*, they are committed to an osteoblastic fate. OSX-deficient mice lack osteoblasts and have defective bone formation (Nakashima et al. 2002). Even though the regulation of OSX is not fully understood, *OSX* transcription appears downstream of RUNX2 and acts to direct pre-osteoblasts to mature osteoblasts (Marie 2008).

While there are several bone morphogenetic proteins (BMP), the most potent and influential in osteogenesis differentiation are BMP2, 4, 5, 6, and 7. Signalling of the BMP2 pathway is initiated by the binding of one of the BMP proteins to the receptor complex. Autocrine BMP production is necessary for the RUNX2 transcription factor to be activated. Furthermore, BMPs and RUNX2 co-operatively interact to stimulate osteoblast gene expression (Chen et al. 2012; Lai and Cheng 2002). Loss of BMP2 and BMP4 results in severe impairment of osteogenesis (Bandyopadhyay et al. 2006).

The Wnt proteins are a family of secreted glycoproteins that are critical regulators of osteoblast differentiation and activity (Long 2011). The best-characterised member is the canonical Wnt pathway. Interaction of some Wnt proteins with Frizzled and LRP5/6 receptors leads to β-catenin phosphorylation. This results in the activation and translocation of β-catenin into the nucleus, where it binds to transcription factors and activates downstream genes that act at multiple stages of osteoblast differentiation. In the absence of canonical Wnt signalling, the MSC adopt chondrogenic and adipogenic fates rather than the osteoblastic fate (Day et al. 2005). One of the targets of Wnt signalling is RUNX2, given that β-catenin/TCF1 promotes *RUNX2* expression and activity (Gaur et al. 2005). Inactivation of the *β-catenin* pathway impedes osteoblast differentiation from mesenchymal progenitors (Glass and Karsenty 2006). The original discovery arose from genetic studies in humans where inactivating mutations in the Wnt co-receptor LRP5 led to osteoporosis, while gain-of-function mutations caused osteosclerosis (Cui et al. 2011). In mature osteoblasts, osteoprotegerin (OPG) is known to be a direct target of β-catenin (Glass and Karsenty 2006). In addition, osteocytes express inhibitors of the Wnt pathway, such as sclerostin (SOST). Although the mechanisms that induce termination of the bone formation phase remain unclear, it seems likely that *SOST*, which is an LRP5 ligand, prevents activation of the Wnt signal, thereby blocking an important osteoblast inducer (Cui et al. 2011; Prideaux et al. 2016).
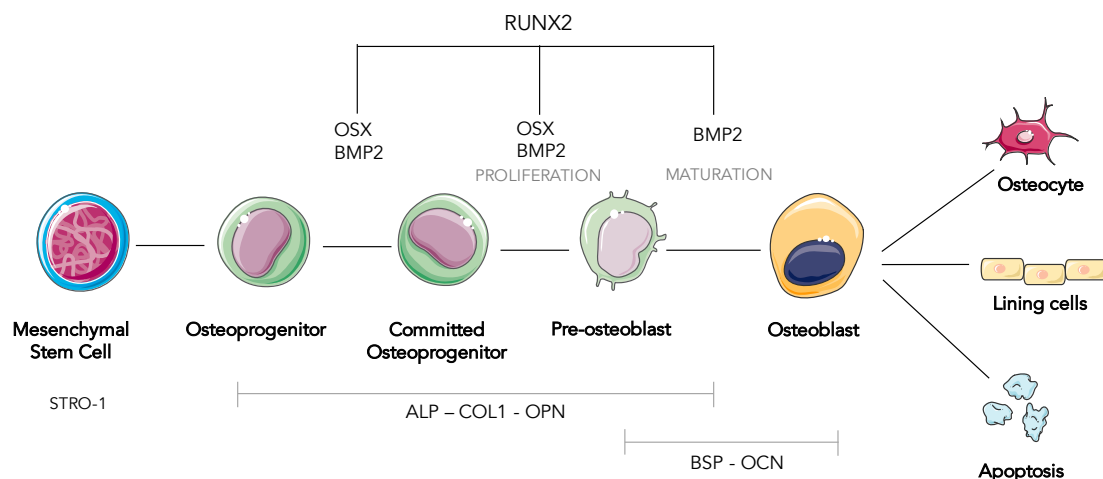
Figure 5. Schematic of osteoblast differentiation from the MSC. Transcription factors and signalling are involved, with RUNX2 functioning up-stream of OSX, which is required for osteoblastic differentiation. ALP, COL-1, OPN, BSP and OCN are the phenotypic markers in the progressive stages of differentiation [Adapted from Soltanoff et al. (2009)].

Mature and active osteoblasts express alkaline phosphatase (ALP), OPN, OCN, and BSP, and lie adjacent to the newly synthesised osteoid (Capulli et al. 2014). This stage, which involves the laying down of bone, has limited replicative potential. ALP is a key enzyme in the process of matrix mineralisation. OCN and BSP are two of the most abundant non-collagenous proteins in bone, and BSP serves as a nucleating site for HA crystal formation (Florencio-Silva et al. 2015). Together, these proteins represent both early and late markers of osteogenic differentiation, and they are all crucial for the osteogenic phenotype (Soltanoff et al. 2009). The mineralisation process is then completed with the formed HA crystals being deposited between the organised collagen fibres.

Osteoblasts carry out another vital function in bone metabolism in controlling the differentiation and activities of other cells, such as MSC and osteoclasts. A subset of destined osteoblasts will become osteocytes and become embedded within the bone matrix. The remainder of the osteoblasts are thought to undergo apoptosis or to become inactive, bone-lining cells (Long 2011).

Osteoclasts

Osteoclasts are highly specialised cells that are capable of resorbing mineralised tissues, such as bone, cementum, and the dentin of the tooth root. They are motile, large, multinucleated cells with a short life-span. Osteoclasts are derived from haematopoietic mononuclear cells of the monocyte/macrophage lineage. The precursors proliferate and are stimulated to form multinucleated osteoclasts through fusion of the precursors. The recruitment and activation of osteoclasts are crucially dependent upon the macrophage colony stimulating factor (M-CSF), receptor activator of nuclear factor kappa-B ligand (RANKL), and OPG produced by stromal cells, including osteoblasts. M-CSF induces receptor activator of nuclear factor kappa-B (RANK) expression in the committed precursors (Arai et al. 1999). RANKL binds

to the RANK receptor on the mononuclear cells and stimulates them to fuse together to form active multinucleated osteoclasts (Boyle et al. 2003). Both M-CSF and RANKL are required to induce the differentiation process and the expression of genes that typify the osteoclast lineage, including those encoding tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK), calcitonin receptor (CTR), and the integrin-$\alpha_V\beta_3$, leading to the development of mature osteoclasts.

OPG is a decoy receptor that binds to the RANK receptor on osteoclast precursors and mature osteoclasts, thereby controlling the amount of RANKL that can stimulate osteoclast recruitment and bone resorption (Kular et al. 2012). The expression of RANKL and OPG is therefore co-ordinated to regulate bone resorption and bone density both positively and negatively by controlling the activation state of RANK on osteoclasts. RANKL is indispensable for tooth eruption, since in RANKL-null mice, the teeth do not erupt (Kong et al. 1999).
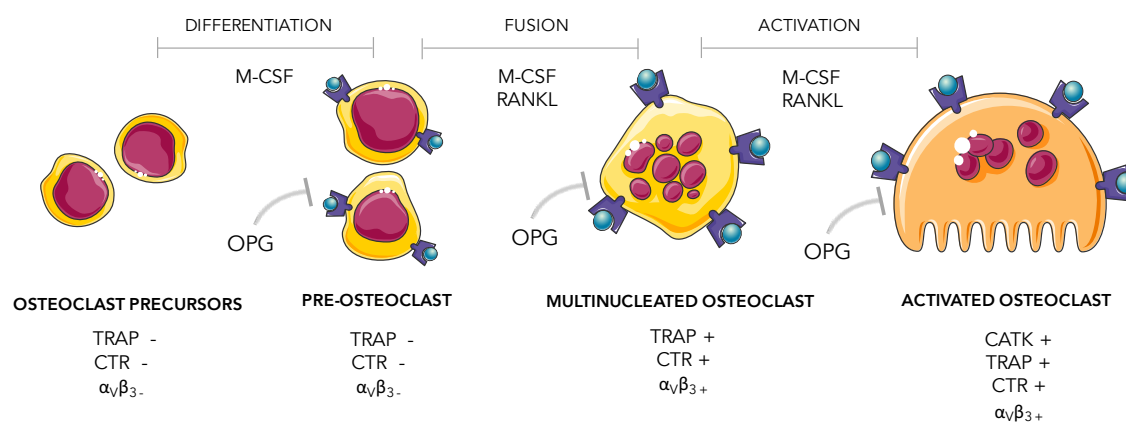

DIFFERENTIATION	FUSION	ACTIVATION
M-CSF	M-CSF RANKL	M-CSF RANKL
OPG	OPG	OPG
OSTEOCLAST PRECURSORS	PRE-OSTEOCLAST	MULTINUCLEATED OSTEOCLAST	ACTIVATED OSTEOCLAST

OSTEOCLAST PRECURSORS
TRAP -
CTR -
$\alpha_V\beta_3$-

PRE-OSTEOCLAST
TRAP -
CTR -
$\alpha_V\beta_3$-

MULTINUCLEATED OSTEOCLAST
TRAP +
CTR +
$\alpha_V\beta_3$+

ACTIVATED OSTEOCLAST
CATK +
TRAP +
CTR +
$\alpha_V\beta_3$+

Figure 6. Schematic of osteoclastogenesis from haematopoietic precursors and differentiation into mature osteoclasts. M-CSF and RANKL are essential for differentiation. OPG can regulate negatively osteoclastogenesis and the activation of mature osteoclasts. RANK receptors are indicated in purple and RANKL is indicated in blue. The phenotypic markers for the different stages of differentiation are shown in the lower part of the figure [Adapted from Boyle et al. (2003)].

Osteoclastic bone resorption involves several stages: the proliferation of osteoclast precursors; differentiation into mononuclear pre-fusion osteoclasts; fusion into multinucleated osteoclasts; attachment of mature osteoclasts to calcified tissues; polarisation, i.e. the development of a ruffled border and clear zone (actin ring), followed by the secretion of hydrogen ions and proteolytic enzymes into the space beneath the ruffled border; and finally, apoptosis. (Lerner 2000; Vaananen and Laitala-Leinonen 2008). Any disequilibrium in the regulation of the essential factors that control the differentiation process will lead to an abnormal increase in osteoclast formation and activity, e.g. osteoporosis, where resorption exceeds formation resulting in decreased bone formation. In contrast, osteopetrosis and genetic mutations that affect osteoclastogenesis lead to decreased bone resorption, which results in aberrant accumulation of bone mass (Florencio-Silva et al. 2015).
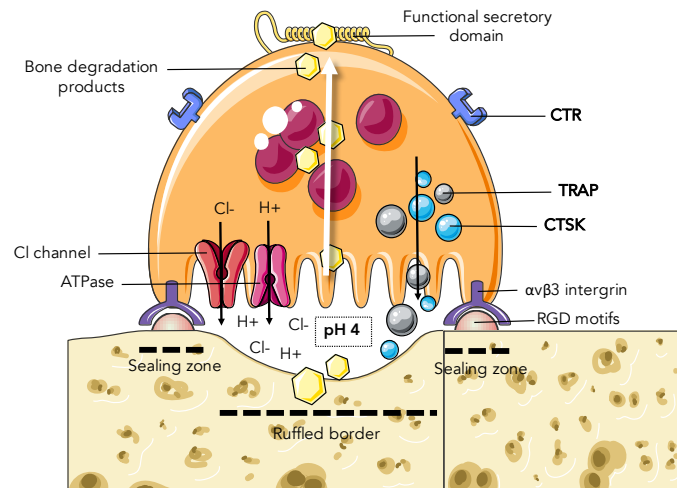
Figure 7. Schematic representation of a resorbing osteoclast. The active osteoclast binds tightly to the mineralised extracellular matrix to initiate the resorption process. The sealing zone creates an isolated extracellular microenvironment that is enclosed by the cell and the bone surface, wherein bone resorption takes place. This binding is mediated by integrin-$\alpha_V\beta_3$ interactions with matrix proteins (i.e. BSP, OPN, vitronectin) that contain the Arg-Gly-Asp (RGD) motifs. The osteoclasts are then polarised, with the ruffled border membrane exporting intracellular acidic vesicles targeted to the sealed zone. Acidification is accomplished by the interplay of enzymes and by the targeted secretion of hydrochloric acid (HCl) into the resorption *via* a proton pump that is mediated by the vacuolar H+-ATPase (v-H+-ATPase). HCl-mediated demineralisation exposes the organic phase of the bone matrix, which is made up of approximately 95% COL-1. The degradation of the remaining bone matrix proteins is carried out by secreted lysosomal enzymes, mainly CATK, TRAP, and matrix metalloproteinase 9 (MMP9). Both the organic and inorganic degradation products from bone are endocytosed by the ruffled membrane and subsequently released from the functional secretory domain at the plasma membrane into the bloodstream [Adapted from Vaananen and Laitala-Leinonen (2008)].

For tooth eruption to occur, co-ordinated resorption of the overlying bone is required for the formation of the eruption path. Examination of the alveolar bone surfaces surrounding the eruptive tooth revealed an infiltration of bone cells with the morphologic characteristics of multinucleated osteoclasts prior to the onset of eruption (Marks 1981). The presence of a persistent un-erupted dentition is observed in cases with osteopetrotic mutations, where bone formation is almost normal and bone resorption is greatly reduced (Marks and Cahill 1987; Marks et al. 1983). These observations have been interpreted as an indication that tooth eruption is an osteoclast-dependent event in which bone resorption is essential for the teeth to erupt.

Osteocytes

Osteocytes, which comprise approximately 90% of the total bone cells, are considered to be terminally differentiated cells of the osteoblast lineage. As osteoblasts mature, around 20% of them become surrounded by their secreted extracellular matrix (Prideaux et al.

2016). Once the mature osteocyte is totally entrapped within a mineralised bone matrix, several of the previously expressed osteoblast markers, such as *OCN, BSP, COL-1*, and *ALP* are down-regulated (Florencio-Silva et al. 2015). This process is accompanied by morphological and ultrastructural changes, including a reduction in the size and growth of the cell processes. These cytoplasmic processes are connected to other neighbouring osteocyte processes *via* gap junctions, as well as to the cytoplasmic processes of osteoblasts and bone-lining cells on the bone surface. The concept of the osteocyte acting as a "mechanosensor" is now widely accepted. These cells within the bone respond to mechanical loading by transmitting signals to the osteoblasts and osteoclasts on the bone surface, thereby modulating their activities. Recent studies have led to many of these signalling factors being identified, e.g. RANKL, OPG, and sclerostin (Nakashima et al. 2011; Simonet et al. 1997). In this way, the osteocytes seem to act as orchestrators of bone remodelling, through the regulation of osteoblast and osteoclast activities (Prideaux et al. 2016).

Bone remodelling

Systemic regulation

The systemic regulation of bone and bone cells function is governed primarily by four hormones: parathyroid hormone (PTH), calcitonin (CT), vitamin D3 (VitD), and oestrogen, which modulate bone remodelling through paracrine signalling. PTH is one of the most important regulators of Ca^{2+} homeostasis. It is involved in the regulation of both bone formation (through its effects on osteoblast differentiation and survival) and bone resorption (indirectly through stimulating the expression by osteoblasts of M-CSF and RANK-L) (Teti 2011). The role of PTH is to maintain the serum Ca^{2+} equilibrium. Low levels of Ca^{2+} trigger PTH synthesis. PTH signalling exerts its actions depending on the dose and duration of secretion. Continuous PTH secretion promotes bone resorption, whereas intermittent and low-dosage secretions favour bone formation. In contrast, CT is a potent osteoclast-inhibitory peptide that is produced by the thyroid gland. It acts to reduce the blood Ca^{2+} levels, counteracting the effects of PTH. The secretion of CT is regulated by the serum Ca^{2+} concentration. CT inhibits bone resorption by affecting the integrity of the ruffled borders of osteoclasts, which leads to decreased ECM breakdown (Lerner 2000).

Oestrogens affect both osteoblasts and osteoclasts, and therefore they have a crucial role in bone homeostasis. Oestrogen depletion decreases osteoblast survival and activity (e.g. in osteoporosis during menopause). Osteoblasts increase M-CSF and RANKL expression in the absence of oestrogen, whereas stimulation with oestrogen decreases the differentiation of osteoblasts, inhibiting their bone-resorbing activity (Oury 2012). VitD deficiency severely affects bone mineralisation, producing low bone mineral density. VitD is produced by cholesterol in the skin upon exposure to sunlight and it is activated in the kidney in the form of 1,25(OH)2-vitamin D. It regulates intestinal calcium uptake, participating in Ca^{2+} and phosphate homeostasis, thereby preventing bone resorption. (Florencio-Silva et al. 2015) Mechanical loading also influences bone physiology. Loading stimulates and maintains bone mass, whilst immobilisation favours bone loss. Upon mechanical stimulation, osteocytes produce several secondary messengers, such as ATP, Ca^{2+}, and prostaglandins (PGE) which influence bone structure (Prideaux et al. 2016).

Local factors

The dynamically regulated bone tissue is dependent upon local cross-talk between cells to co-ordinate the coupling of bone formation and resorption. As such, the bone cells require an extensive inter-connected network to co-ordinate the remodelling process. This co-ordination of cells during growth and tissue development is often mediated by paracrine signalling (Fig. 8) or *via* intercellular gap junction communication between adjacent cells.
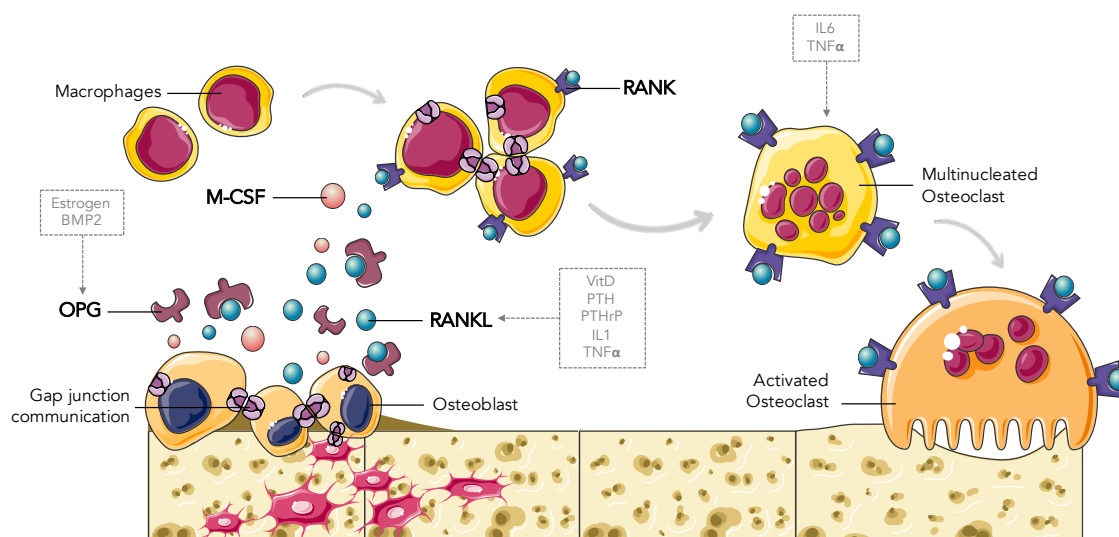

Figure 8. Schematic of the intercellular communication between bone cells. Osteoclast activation occurs after the binding of M-CSF and RANKL, which are produced by osteoblasts/stromal cells, to its membrane receptors. Osteoblasts also produce OPG, which decreases or increases osteoclastogenesis upon binding to the RANK receptor. In addition to the factors produced by osteoblasts to regulate osteoclastogenesis, several cytokines, such as tumour necrosis factor alpha (TNF-α), interleukin 1 (IL-1), and interleukin 6 (IL-6), are involved in modulating the bone remodelling process. These cytokines stimulate the production of M-CSF and RANKL. Gap junction communication is indicated between the osteoblasts, osteocytes, and osteoclast precursor cells as an alternative communication pathway.

Gap junction communication

Morphological studies conducted in rodents have shown that gap junction communication (GJC) exists between osteocytic processes, between osteocytes and osteoblasts on the bone surface, and among osteoblasts (Doty 1981). Gap junction coupling seems to be required for osteoblast differentiation and the formation of bone matrix and mineralisation (Watkins et al. 2011).

Gap junctions are trans-membrane channels termed connexons, which are composed of protein subunits called connexins. There are 21 known connexins in humans. Connexin 43 (CX43) is the most abundant connexin expressed in bone (Civitelli 2008). When two neighbouring cells dock the hemi-channels (gap junction channels) are formed, providing

direct cell-to-cell communication (Fig. 9). These trans-membrane channels facilitate the exchange between cells of small ions, molecules, and second messengers (e.g. cAMP) that have molecular masses of less than ~1.2 kDa.
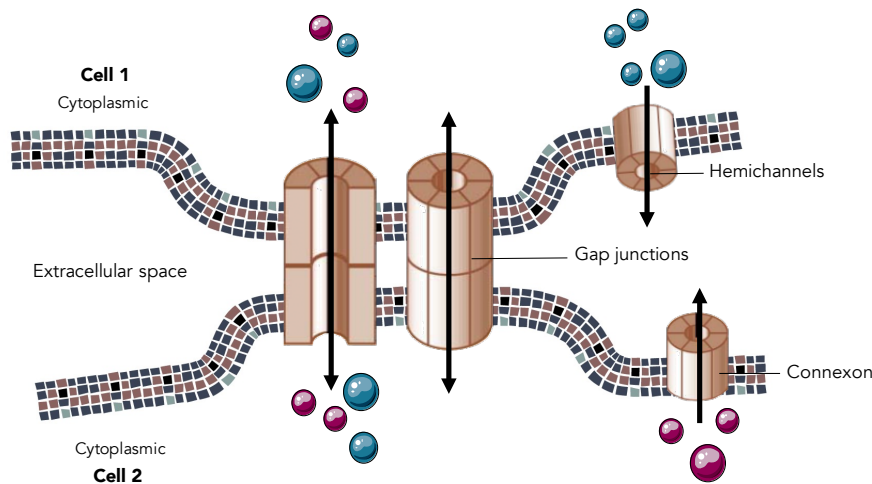

Figure 9. Schematic of the connexin hemi-channels and gap junctions in the plasma membranes of adjacent cells. Six connexin proteins form the hemi-channel or connexon with a central pore. Intercellular gap junctions form when hemi-channels from adjacent cells dock onto one another. Hemi-channels from neighbouring cells align to form gap junction channels, which allow intercellular communication.

Several studies in mice have shown that the gap junction interactions between bone cells play a crucial role in bone development. Accumulated data suggest that CX43-GJC may be important for the signals in the early phases of osteogenesis, perhaps at the time of osteogenic commitment from undifferentiated precursors (Watkins et al. 2011). CX43-null mice exhibit delayed skeletal mineralisation, craniofacial abnormalities, and osteoblast dysfunction (Lecanda et al. 2000). Gap junction intercellular communication is also suggested to be involved in the regulation of osteoclast function. Even though the mechanism is not yet fully understood, it has been reported that blockage of GJC significantly inhibits osteoclastogenesis *in vitro* (Matemba et al. 2006; Ransjo et al. 2003). These findings confirm that CX43-GJC is an important regulator of osteoblastogenesis and osteoclastogenesis.

Bone regulatory factors expressed in the dental follicle

The use of experimental set-ups in rodents with arrested tooth eruption was the first approach to seeking regulatory factors active in the eruption process. Injections of different molecules elucidated epidermal growth factor (EGF), TNF-α, and M-CSF as accelerators of the eruption process. Subsequently, with the establishment of *in vitro* cultures of stellate reticulum and dental follicle cells (DFC) from rat mandibular molars (Wise et al. 1992), the possibility arose to determine the effect of a given molecule on the gene expression patterns in cells obtained from the tissues required for tooth eruption. With increasing knowledge of

bone regulatory factors, the roles of these factors also in the eruption process attracted interest. The expression profiles of *M-CSF, MCP-1, RANKL,* and *OPG* as the central regulators of osteoclastogenesis, and *BMP2*, as the principal orchestrator of bone formation, have been investigated in the dental follicles collected from rats. With newly available technologies and more precise techniques, e.g. laser capture microdissection (LCM) and qPCR, it was possible to study the molecular, chronological, and spatial regulation of gene expression in the dental follicle. Using LCM to isolate the coronal and apical halves from the dental follicles of rat first mandibular molars, it could be shown that the expression of *RANKL* was higher in the coronal half than in the apical half, whereas the expression of *BMP2* was higher in the apical half than in the coronal half. Thus, the spatial effects on bone resorption and formation in the adjacent alveolar bone are most likely the result of regional differences in gene expression within the dental follicle (Wise and Yao 2006). These results correlate with the ultrastructural features of the alveolar bone surrounding an erupting tooth described previously (Marks and Cahill 1986). Therefore, as suggested from these experimental studies in rats, the spatial localisation of different levels of gene expression appears to be the mechanism through which the dental follicle controls both the alveolar bone resorption and formation needed for eruption.

Targeted RT-qPCR studies were implemented to study in detail the sequential gene expression in the rat dental follicle for different stages of the tooth eruption process. To study these biological events in the eruption process, the molars of rats were chosen as the model based on their limited eruption compared to the incisors of other rodents. In the rat, the first molar usually erupts around Day 18 post-natally. M-CSF and MCP-1 are reported to be maximally expressed in the dental follicle on Day 3, which coincides with the maximal influx of mononuclear cells (Que and Wise 1997; Wise et al. 1995). *In vitro*, both M-CSF and MCP-1 are secreted by the DFC and are chemotactic for monocytes (Que and Wise 1997). The mononuclear cells that are recruited to the dental follicle must fuse to form osteoclasts, which drive the resorption of the alveolar bone for the eruption pathway. Although RANKL is also expressed in the dental follicle on Day 3, its expression is not upregulated at this time-point (Liu et al. 2005). However, the down-regulation of *OPG* at Day 3 would result in a ratio of RANKL to OPG that is favourable for osteoclastogenesis. The up-regulation of *M-CSF* also influences the down-regulation of *OPG* to enable osteoclast formation (Wise et al. 2005). Maximal expression of *M-CSF* at this time would also promote osteoclastogenesis, given that M-CSF upregulates the expression of *RANK* in the osteoclast precursors to enhance cell-to-cell signalling of *RANKL* and *RANK* (Arai et al. 1999). In the absence of either M-CSF or RANKL, as seen in osteopetrotic rats (Van Wesenbeeck et al. 2002), or in the absence of *RANKL*, as observed in knockout mice (Kong et al. 1999), the teeth do not erupt. Thus, the differential chronological expression of these genes in the dental follicle, as well as the spatial expression of *RANKL*, are critical for initiating and promoting the osteoclastogenesis needed for tooth eruption.

Microarray studies showed that secreted frizzled-related protein-1 (SFRP-1) is a contributory factor to the major and minor bursts of osteoclastogenesis that occur on Days 3–9. Thus, inhibition of *SFRP-1* gene expression in combination with inhibition of *OPG* gene expression, is a critical event in enabling alveolar bone resorption such that the teeth will erupt. The results of *in vitro* osteoclastogenic assays suggest that *SFRP-1* and *OPG* are inhibited by different pathways (Liu and Wise 2007).

The second and minor burst of osteoclastogenesis on Day 10 prior to eruption appears to be controlled by vascular endothelial growth factor (VEGF), which is maximally expressed in the rat dental follicle on Days 9–11 (Yao et al. 2006). *VEGF* upregulates the expression of *RANK* in osteoclast precursors, as does *M-CSF*. In conjunction with this, *TNF-α* is also maximally expressed in the rat dental follicle at Day 9, and it enhances the expression of *VEGF* in the DFC (Wise and Yao 2003). A major difference between the minor and the major burst of osteoclastogenesis is the levels of OPG and RANKL. Unlike the major burst on Day 3, the levels of *OPG* expression are high on Day 10 (Wise et al. 2000a), whereas on Day 10 the expression of *RANKL* is up-regulated such that it reaches peak expression. Thus, a favourable RANKL/OPG ratio is created that promotes osteoclast formation.

Turning to bone formation in the rat experimental tooth eruption model, the chronological gene expression of *BMP2* begins to increase at Day 3, with maximal expression seen on Day 9 post-natally (Wise et al. 2004). These expression periods correlate with the ultrastructural initiation of alveolar bone formation on Day 3 at the base of the socket, and with the rapid bone formation by Day 9, as previously reported (Wise et al. 2007). Thus, *BMP2* may be regulating the osteogenesis of the basal bone growth in the base of the crypt. It has been suggested that the peak expression of *TNF-α* at Day 9 also influences the up-regulation of *BMP2* and *BMP3*, thereby promoting osteoblastic differentiation (Yao et al. 2010).

The capacity of the dental follicle to regulate bone resorption, as well as osteogenesis, is well-documented in rodents and dogs. The use of animals to model human biological development relies on the assumption that the basic processes are sufficiently similar to allow extrapolation. However, projection of these data to humans should be approached with caution, given the known differences between these diverse species. Human dental follicles can only be collected in restricted circumstances owing to ethical considerations, and mainly from impacted canines and third molars. The signalling pathways and regional differences in the patterns of regulatory molecules in the dental follicle need to be analysed further in humans.

Tissue engineering

Various factors, such as trauma, tumours, and congenital deformities, can cause craniofacial defects. Regenerative medicine aims to repair and replace lost or damaged tissues, thereby resembling the natural regeneration process. The term *tissue engineering* was first described in 1993 by Langer and Vacanti to describe the "process of the application of biological, chemical and engineering principals towards the repair, restoration, or regeneration of living tissue using biomaterials, cells, and factors alone or in combination". In 2010, Laurencin redefined tissue engineering in terms of *regenerative engineering* as "the integration of materials, science and tissue engineering with stem, developmental cell biology and regenerative medicine toward the regeneration of complex tissues, organs, or organ systems". Since then, the use of a combination of stem or progenitor cells and different bioactive molecules has been discussed.

The concept of harvesting adult stem cells, for either testing biomaterials in an organic environment or transplantation for tissue reconstruction in patients, is commonly mentioned in the context of regenerative medicine. Stem cells are defined by functional assays as

meeting the criteria of multipotency and self-renewal (Bianco et al. 2013). The biological property that uniquely identifies MSC is the multipotent capacity to differentiate into different lineages of specialised cells. Depending on the type of stem cell and its ability and potency to differentiate into different tissues, the following categories of stem cells have been established (Huang 2013):

1. *Totipotent stem cells:* Each cell is capable of developing into an entire organism;
2. *Pluripotent stem cells:* Embryonic stem cells that when grown in the right environment *in vivo* are capable of giving rise to basically every cell type and forming all types of tissues; and
3. *Multipotent stem cells:* Post-natal stem cells (adult stem cells) that are capable of giving rise to multiple lineages of cells. These include:
  - Hematopoietic stem cells
  - Mesenchymal stem cells (MSC)
    - Bone marrow-derived MSC
    - Tissue-derived MSC

The regenerative potential of dental follicle stem cells

In humans, five different sources of dental MSC have been identified: exfoliated deciduous teeth (Miura et al. 2003); dental pulp (Gronthos et al. 2000); the periodontal ligament (Seo et al. 2004); root apical papilla (Sonoyama et al. 2008); and the dental follicle (Yao et al. 2008). The dental follicle contains multipotent ectomesenchymal cells. These neural-crest-derived precursor cells can be isolated based on their adherence to plastic and high proliferation rates (Morsczeck et al. 2005a). DFC have a fibroblastic-like morphology and express markers typical of progenitor stem cells, such as nestin, notch-1, CD44, CD105, and STRO-1 (Kemoun et al. 2007; Morsczeck et al. 2005a). The dental follicle, which contributes to regulating the formation of the tooth and its surrounding periodontium, is also demonstrated to have resident cells with differentiation capacity. Under *in vitro* conditions, DFC differentiate into different cell types, especially cells of the periodontium (Kemoun et al. 2007; Morsczeck et al. 2005a; Vollner et al. 2009). The ability of DFC to differentiate into a wide variety of cell types, together with the possibility to obtain a high number of cells, make them good candidates for the establishment of a validation model for osteo-inductive materials, as well as an alternative source of cells for the repair and regeneration of periodontal defects.

Second- and third-generation biomaterials are designed to incorporate biocompatible modulatory signals that will induce favourable cellular responses, such as cell survival, directed cell differentiation, and specific lineage commitment (Yu et al. 2015). The activation of specific genes and signalling pathways to obtain desired cellular responses is therefore of interest for the interactions between biomolecules and DFC.

Several studies have suggested that silicon (Si) is essential for the normal development of connective tissues and the skeleton (Carlisle 1986). Previous animal studies have demonstrated that Si depletion inhibits the development of the ECM and the formation of HA (Carlisle 1986; Schwarz and Milne 1972). Si has been shown to play a structural role in COL-1 synthesis and stabilisation (Schwarz 1973), as well as in stimulating osteoblast proliferation and differentiation (Reffitt et al. 2003) Bioactive glasses, which belong to the group of second-generation biomaterials, are silica-based ceramic materials that have

different bioactivities and bone-bonding properties. The first artificial bioactive material, "Bioglass", was invented by Larry Hench in 1969. It was composed of 46.1 mol% SiO_2, and was later termed 45S5 Bioglass®. Bioglass® was the first artificial osseo-integrative material that was designed to form direct chemical bonds with bone (Hench 2006). Its usage was extended to a number of dental and orthopaedic applications, with the aim of producing bioactive components that would favour biological responses in the physiological environment (Jones 2013; Tadjoedin et al. 2000). Since Si is a major component of bioglass, it has been proposed to play a crucial role in its osteogenic effect.

Regenerative engineering strategies can significantly benefit from the morphogenetic events that are crucial for the process of formation of the desired tissue. A tissue or a cell culture is required to examine the biological responses, and further on, these cells or tissues may be implemented as sources for regenerative engineering. Therefore, it is of interest to identify the interactions between potential biomaterials/biomolecules and human DFC (hDFC).

AIMS

The overall aims of this thesis were to determine whether clinical factors are related to the impaction of canines and to investigate the regulatory functions and differentiation potential of the dental follicle.

Specific aims

- To uncover whether clinical variables can be used to predict canine impaction.

- To study the gene expression patterns of bone regulatory factors in dental follicles from different patients, and to investigate if differences in expression correlate with the clinical conditions of the impacted canine.

- To examine local differences in the expression of regulatory mediators in the human dental follicles of erupting teeth.

- To study the osteogenic differentiation potential of cultured human dental follicle cells and the effects induced by soluble silica.

MATERIALS AND METHODS

This section provides a general description of the main experimental procedures and methodologies used in this thesis work. More detailed protocols can be found in the respective papers.

Studies and experimental designs

- **Paper I** is a *clinical case-control study* based on patients with impacted canines and age- and gender-matched controls.

- **Paper II** is a *descriptive study* of the gene-expression profiles of dental follicles from impacted and normally erupting teeth

- **Paper III** consisted of two parts. The first part, is a *descriptive analysis* of the gene expression levels of bone-related markers in the apical and coronal dental follicles. The second part, involves an *experimental in vitro* stimulation of hDFC to further induce expression of *RANKL*.

- **Paper IV** is an *experimental study* to examine the osteogenic differentiation potential of hDFC.

Study I   Study II   Study III   Study IV
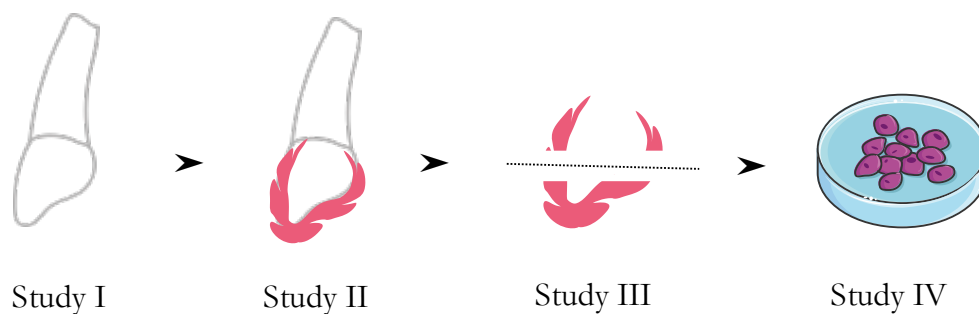
Patients

Patients

The patients included in **Study I** were referred from general practitioners, during the period of January to December of 2011, to the Clinic of Orthodontics at Mölndal Hospital, Mölndal, Sweden, for comprehensive treatment of impacted maxillary canines. A diagnosis of maxillary canine impaction was established if: (i) no spontaneous correction of a malpositioned canine occurred within at least 1 year of extraction of the deciduous canine; or (ii) eruption was considerably delayed (based on chronological age and contralateral eruption). The inclusion criterion for the study group was presentation for comprehensive treatment of impacted maxillary canines between January and December of 2011. Patients who had any craniofacial syndrome or cleft palate were excluded from the study. Forty-five subjects, with a total of 58 impacted permanent maxillary canines, were enrolled (19 females and 26 males; mean age, 14.2 years; range, 11–17 years). Age- and gender-matched orthodontic patients (N= 45) who attended the same clinic and had normally erupting permanent maxillary canines were enrolled in the control group. This control group was selected in order of consultation, and the availabilities of cephalograms and panoramic radiographs were established as inclusion criteria. The collection of data was approved by the Regional Ethics Board at the University of Gothenburg (Dnr. 898-13) and by the National Data Inspection Board.

Samples

For **Study II,** samples of the exposed dental follicles were collected during surgical exposure of the impacted teeth (Fig. 4). All operations were performed at the Clinic of Oral and Maxillofacial Surgery and the Clinic of Pedodontics at Mölndal Hospital, Mölndal, Sweden. Five females and four males (mean age, 13.2 years; range, 10–16 years) were enrolled. In total, 11 dental follicles from permanent teeth were collected: 8 from permanent maxillary impacted canines, 2 from mesiodens, and 1 from a normally erupting tooth. All teeth were intra-osseous and entirely covered by bone. All the teeth had a considerable root length. No gingival tissue was attached to the collected specimens. Patients who had craniofacial syndromes, a systemic metabolic diagnosis or any bone disease were excluded from the study. This study was approved by the Regional Ethics Board at the University of Gothenburg (Dnr. 898-13) and by the National Data Inspection Board. Informed consent was obtained from all the patients and their parents.

For **Study III,** dental follicles were collected during the surgical exposure of impacted teeth used for auto-transplantation or an orthodontic treatment that involved forced eruption. Isolation of the coronal and apical portions of the dental follicle was performed after removal of the tooth from the bone crypt. The dental follicle was divided into three fractions. The middle portion was discarded, and the coronal and apical portions were independently fixed. All operations were performed at the Department of Periodontology at the Medical University of Warsaw, Warsaw, Poland. Nine females and three males (mean age, 13.3 years; range, 10–16 years) were enrolled. In total, 12 dental follicles from permanent teeth were collected. All the teeth were intra-osseous and entirely covered by bone. All the teeth had

considerable root length. The collected specimens were free of gingival tissue. Patients with craniofacial syndromes, a systemic metabolic diagnosis or any bone disease were excluded from the study. Informed consent was obtained from all the participating juvenile patients and their parents. This study was approved by the Bioethics Committee at the Medical University of Warsaw, Warsaw, Poland (KB/124/2016).

Immediately after their removal, the dental follicles were placed in PAXgene® tissue containers (PreAnalytiX, Hombrechtikon, Switzerland). PAXgene® is a formalin-free system that is designed to improve the quality of the molecular analysis without diminishing the quality of the downstream and histopathological analyses. These are dual-cavity containers that are pre-filled with fixation and stabiliser reagents. The specimens were fixed in PAXgene Tissue FIX Solution for 2–4 h, and then transferred to the PAXgene® tissue STABILIZER solution in the same container. Specimens were stored at -80°C until used.

Cells

In **Study III** and **Study IV**, primary cultures of hDFC were established using the dental follicles obtained from four different patients who presented with impacted canines and were referred for surgical exposure to the Department of Pedodontics, at the University of Gothenburg, Gothenburg, Sweden. After rinsing the dental follicle in MEM Alpha medium 1× (α-MEM; Gibco Life Technologies, Grand Island, NY, USA), the tissues were minced using a sterilised scalpel, cultured in α-MEM that was supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco Life Technologies), 2 mM Glutamax (Gibco Life Technologies), and Antibiotic-Antimycotic reagent (Gibco Life Technologies) at 37°C in humidified air with 5% CO_2. After 48 h, non-adherent cells were removed by changing the medium. hDFC from passages 3–5 were used in the experimental set-ups. Informed consent was obtained from all the patients and their parents after they were fully informed about the aims of the studies. This procedure was approved by the Regional Ethics Board at the University of Gothenburg (Dnr. 898-13) and by the National Data Inspection Board.

Methods

Orthopantomography

Panoramic radiography (Opg), which technically involves collecting images with a rotating system, allows a wide view of the oral and maxillofacial complex, as in frontal overbite. Opg is one of the standard diagnostic tools in orthodontics. The clinician uses it to assess the tooth positioning and the tissues that surround these teeth. These assessments are either visualised or in the form of linear and angular measurements.

For **Study I**, all the panoramic radiographs were obtained with the same X-ray unit (Schick; PanElite, New York, NY, USA) and using standardised settings (64 kV, 8 mA, ~1.25 Å, constant magnification). All the Opgs were coded and the measurements were performed in

a blinded fashion. Graphimetric methods were used depending on the rigid structures of the facial skeleton. A sector location for the impacted tooth was assigned to one of five categories based on the canine crown overlap of the roots of the adjacent teeth, according to the method described by Ericson and Kurol (1988), and including the modification of Lindauer and co-workers (1992).

The angulation of the impacted maxillary canine was determined as the angle formed by the long axis of the canine with the mid-line denoted as 'α', and the angle formed by the long axis of the canine with the occlusal plane denoted as 'σ'. The mid-line was defined by the inter-maxillary suture, anterior nasal spine, nasal septum, and the inter-nasal suture. The occlusal plane was determined by drawing a horizontal line through the incisal edge of the permanent maxillary central incisor and the occlusal plane of the first permanent maxillary molar on the given side. A perpendicular line was then drawn from the cusp tip of the impacted maxillary canine to the occlusal plane. This distance was denoted as 'd1'. The lateral distance of the canine was measured using a perpendicular line drawn at the mid-line. The distance (in mm) to the crown tip was recorded as 'd2'. Sector I is located distal to a tangent to the distal crown and root of the lateral incisor, Sector II includes the area from the tangent of the distal surface to a mid-line bisector of the lateral tooth, Sector III includes the area from the mid-line bisector to a tangent to the mesial surface of the lateral incisor crown and root, Sector IV includes the area from the tangent of the distal surface to a mid-line bisector of the crown of the central incisor, and Sector V encompasses the area from the bisector of the central incisor to the mid-line.
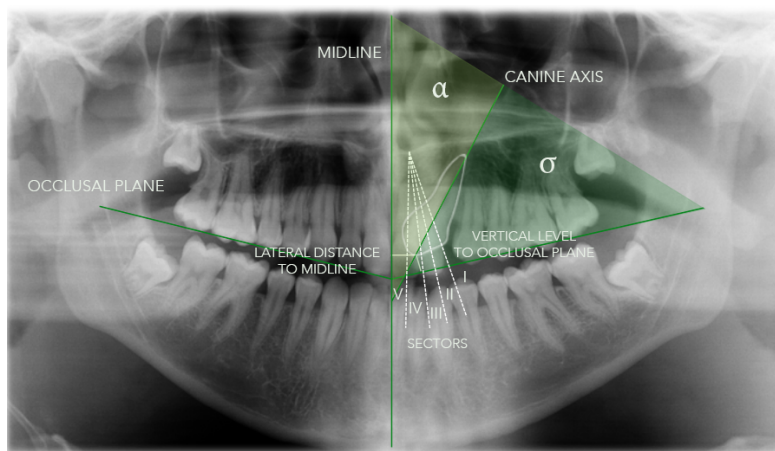

Figure 10. Panoramic radiographical evaluation.

Cephalometrics

Cephalometrics involves the study of head films and is used for the identification and description of a set of craniofacial structural relationships. This method consists of a predetermined set of measured angles and distances applied to each cephalometric tracing. Most analyses use the relatively stable elements in the cranial base as the reference points and planes to which the changing or growing structures are related. This analytical method is useful for the diagnosis and treatment planning of the individual patient when the values derived from the tracing of the patient's initial head film are compared to established norms.

If serial radiographs are evaluated, they can be used to estimate increments of growth and the eventual effects of certain treatments.

In **Study I**, all the cephalograms were taken with the same cephalostat (Schick; PanElite) using standardised settings (64 kV, 8 mA, ~1.25 Å, constant magnification). All the landmarks used for facial and cranial base-line measurements are shown in Figure 11, according to Bjork (1963). The digital lateral cephalograms were traced using the FACAD software (Ilexis AB, Linköping, Sweden) based on Bergen's cephalometric norms (Hasund 1977).
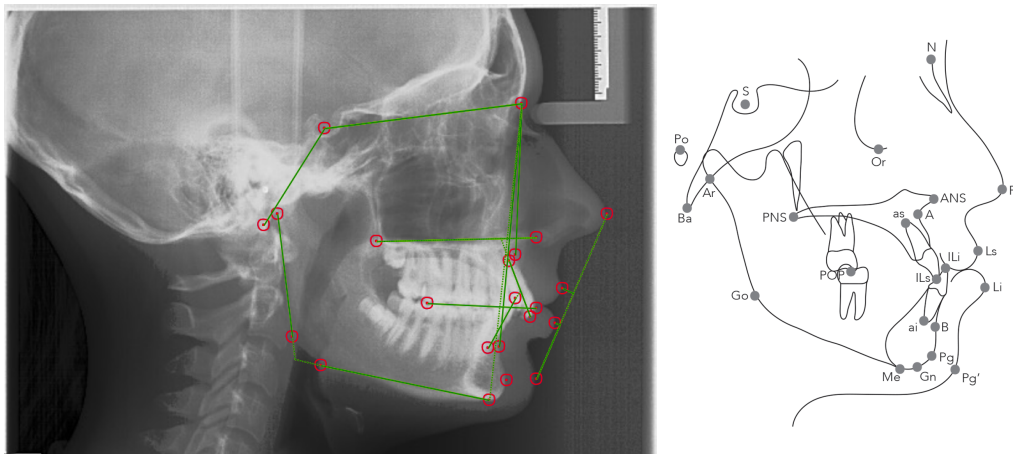
Figure 11. Profile radiograph evaluation (landmarks).

Casts measurements

The cast models used in orthodontic studies are accurate plaster reproductions of the teeth and their surrounding soft tissues. They are a valuable diagnostic aid, making it possible to study the arrangement of teeth and the occlusion. The cast model provides a three-dimensional view of the maxillary and mandibular dental arches in all three planes of space, i.e. the sagittal, vertical, and transverse planes. Model analysis allows careful scrutiny of several parameters, such as dentition and jaw relationships, and provides objective measurements for detailed evaluation and treatment planning. Traditionally, measurements on dental casts are performed with the either Vernier callipers or needle-point dividers.

In **Study I**, the lengths and widths of the arches were obtained as previously described by Thilander (2009). The reference points and lines used are illustrated in Figure 12. The arch length was obtained by measuring the arch perimeter to the first permanent molars, divided into right/left posterior and anterior segments. Briefly, the posterior length (P2–C) represents the distance between the distal surface of the second pre-molar and the mesial surface of the permanent canine on the right and left sides. The anterior length (I1–I2) represents the distance between the mesial surface of the permanent canine and the mid-line of the dental arch. The circumference of each dental arch is represented by the distance (P2–P2). When the permanent canine was absent, the mesial surface of the first pre-molar was

used. The width of each dental arch was obtained by measuring the distance between the corresponding teeth on the right and left sides at different levels. The anterior arch width was designated as the distance between the contact points of the mesial surface of the first pre-molar (P1–P1). The posterior arch width was assessed as the distance between the central fossa of the first permanent molars (M1–M1).
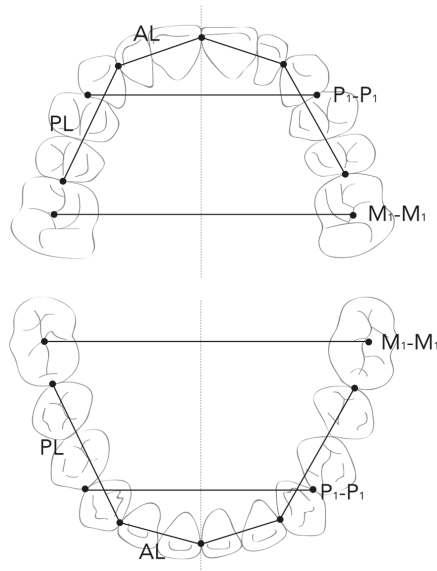
Figure 12. Dental cast evaluation.

Dental age assessment

The modified classification proposed by Demirjian and Goldstein (1976) was used to assess dental age based on the degree of calcification of the permanent teeth. The developmental stages of the seven-left mandibular permanent teeth were determined. Briefly, the degree of tooth formation was assigned to one of eight stages, from the first appearance of calcification to the closure of the apex (stages A through H). Each stage of the seven teeth was translated into a maturity score, and the sum of the scores was transformed into a dental maturity age, which differed according to the gender of the patient based on the conversion system provided by Demirjian and Goldstein.
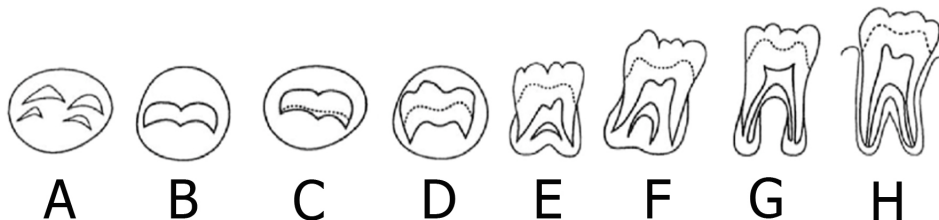
Figure 13. Demirjian's 8-stage classification (Demirjian and Goldstein 1976)

Real-Time quantitative polymerase chain reaction

Real-Time quantitative polymerase chain reaction (RT-qPCR) allows the quantitation of mRNA expression in biological samples. In combination with reverse-transcription PCR, which involves complementary DNA (cDNA) synthesis from RNA, RT-qPCR can be used to quantify changes in gene expression. RT-qPCR uses fluorescent reporter molecules to monitor the amplification of products during each cycle of the PCR reaction. In these studies, SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) that contained the double-stranded DNA-binding dye was used to track the process of DNA amplification. As the RT-qPCR proceeds, at each round of amplification, the dye binds to the double-stranded DNA, resulting in an increase in the level of fluorescence at the end of each cycle. The quantification of the double-stranded DNA product in the reaction is proportional to the amount of fluorescence.

The Minimum Information for Publication of RT-qPCR Experiments (MIQE) guidelines were followed to ensure the relevant experimental conditions and assay characteristics (Bustin et al. 2009). In **Study II** and **Study III**, RT-qPCR was used to determine the gene expression profiles of osteoblast- and osteoclast-related markers in human dental follicle tissues from impacted canines. In **Study III**, the induced expression levels of *RANKL* and *OPG* were quantified in hDFC from distinct patients. In **Study IV**, the expression of osteoblastic phenotypic markers was analysed using RT-qPCR. Before the RT-qPCR analysis, RNA extraction from the tissues or cells of interest (dental follicle tissues in **Studies II and III**; hDFC in **Studies III and IV**) and cDNA preparation were performed.

The dental follicles were homogenised in the lysis buffer using a gentle MACS dissociator (Miltenyi Biotech, Bergish Gladbach, Germany). RNA extraction was carried out using the commercially available PAXgene Tissue RNA Kit for tissues and the RNeasy Plus Mini Kit for cells (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNase I treatment was performed to remove any contaminating DNA. RNA concentrations were quantified with the Qubit™ Fluorometer (Invitrogen, Burlington, ON, Canada). All the reverse-transcription steps were performed using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories). Universal RNA Spike (TATAA Biocenter, Gothenburg, Sweden) was added to each sample to allow quality control throughout the entire RT-qPCR experimental workflow. To select the most stable reference genes for normalisation, a panel of twelve reference genes was screened in representative samples from the retrieved tissues or cells. The expression profiles of the screened reference genes were evaluated using the geNorm (Vandesompele et al. 2002) and Normfinder (Andersen et al. 2004) programmes. The analyses of the target genes and the best two selected reference genes were performed in duplicate on a CFX 96 Real-Time System (Bio-Rad Laboratories) using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories). An inter-plate calibrator (TATAA Biocenter) was added to each plate to compensate for the variation between runs. The quantities of the target genes were normalised using the geometric mean of the Cq values of the selected reference genes. The normalised relative quantities were calculated using the delta-delta Cq method and 90% PCR efficiency (Pfaffl 2001).

The primers used in the RT-qPCR were purchased from Bio-Rad Laboratories and are listed in Table 2.

Materials and Methods

Table 2. Bio-Rad SYBR® Green primers used for the RT-qPCR

Gene identification	Abbreviation	Unique Assay ID
Target gene encoding:		
Sp7 transcription factor	OSX	qHsaCED0003759
Runt-related transcription factor 2	RUNX2	qHsaCED0044067
Bone morphogenetic protein 2	BMP2	qHsaCID0015400
Alkaline phosphatase	ALP	qHsaCID0010031
Gap junction protein, alpha 1, 43 kDa	CX43	qHsaCID0012977
Bone gamma-carboxyglutamate protein (BGLAP)	OCN	qHsaCED0038437
Integrin-binding sialoprotein	BSP	qHsaCED0002933
Tumour necrosis factor receptor superfamily, memb.11b	OPG	qHsaCED0046251
Tumour necrosis factor (ligand) superfamily, memb.11	RANKL	qHsaCID0015585
Chemokine (C-C motif) ligand 2 (CCL2)	MCP-1	qHsaCID0011608
Colony-stimulating factor 1 (macrophage)	M-CSF	qHsaCID0016847
Reference gene encoding:		
Glucuronidase, beta	GUSB	qHsaCID0011706
Hypoxanthine phosphoribosyltransferase 1	HPRT1	qHsaCID0016375
Peptidylprolyl isomerase A (cyclophilin A)	PPIA	qHsaCED0038620

Immunofluorescence

Immunofluorescence is a robust tool to detect the location and expression levels of proteins of interest based on the use of fluorochromes that are bound to antibodies. It can be used on cells or tissue sections.

In **Study III**, the PAXgene-fixed and paraffin-embedded tissue sections were deparaffinised, antigen-retrieved, and stained with the following primary antibodies (Abcam, Cambridge, UK): anti-MCS-f (ab9693, diluted 1:400); anti-osteocalcin (anti-OCN, ab13420, diluted 1:350); and anti-CX43 (ab87645, diluted 1:350). After washing, the sections were incubated with the following secondary antibodies (Invitrogen, Thermo Fisher Scientific) for M-CSF (A11011, anti-rabbit Alexa 568-conjugated, diluted 1:200), OCN (A21050, anti-mouse Alexa 633-conjugated, diluted 1:50), and CX43 (A21222, anti-goat Alexa 488-conjugated, diluted 1:200). This was followed by treatment with HOECHST solution (H3570) for the visualisation of cell nuclei, before mounting in Fluoroshield™ mounting medium (Sigma-Aldrich, St. Louis, MO, USA).

Images were recorded using the LSM 710 NLO microscope from Carl Zeiss available at the Centre of Cellular Imaging at the University of Gothenburg (Gothenburg, Sweden).

Cell culture

Cell culture refers to the removal of cells from a tissue and their subsequent growth in a favourable artificial environment. Primary cells are isolated directly from human or animal tissue using enzymatic or mechanical methods, in contrast to cell lines, which have been

immortalised and have acquired the ability to proliferate indefinitely, either through genetic mutations or artificial modifications.

In **Study III** and **Study IV**, primary cultures of hDFC were established using the dental follicles isolated from patients who presented with impacted canines and were referred for surgical open exposure. The tissues were minced using a sterilised scalpel, cultured in α-MEM that was supplemented with 10% (v/v) FBS, 2 mM Glutamax, and Antibiotic-Antimycotic reagent at 37°C in humidified air with 5% CO_2. After 48 h in culture, non-adherent cells were removed by changing the medium. The hDFC from passages 3–5 were used in the experimental set-ups. In **Study III**, hDFC were cultured in α-MEM with or without osteogenic induction medium (OIM; 50 mg/ml L-ascorbic acid 2-phosphate sesqui-magnesium salt and 10 mM β-glycerophosphate disodium salt hydrate; Sigma-Aldrich). Thereafter, 10^{-6} M forskolin (FSK; Sigma-Aldrich) was added to stimulate RANKL production. In **Study IV**, hDFC were cultured in the presence of Si, with or without supplementation of osteogenic induction medium.

Neutral Red Assay

The Neutral Red uptake assay provides a quantitative estimation of the number of viable cells in a culture (Repetto et al. 2008). The principle of this assay is based on the detection of viable cells that can take up the Neutral Red dye. Viable cells take up the chromophore by non-ionic passive diffusion and concentrate it into lysosomes, whereas the dye cannot be retained by dead cells. After the cells have been allowed to incorporate the dye, they are briefly washed or fixed. Viable cells release the incorporated dye when immersed in an acidified ethanol solution, and the absorbance (OD 540 nm) of the solubilised dye is quantified using a spectrophotometer. Consequently, the amount of retained dye is proportional to the number of viable cells. The assay quantitates cell viability and can be used to measure cell replication, cytostatic effects or cell death, depending on the seeding density. In **Study IV**, this method was used to measure the effect and evaluate the dose-response when the hDFC were exposed to different concentrations of Si.

Mineralisation Assay and Alizarin Red Staining

For the induction of osteogenic differentiation, hDFC were seeded in plastic wells at a density of 10,000 cells/cm^2 in OIM (Bellows et al. 1992; Quarles et al. 1992). This medium is known to induce the osteogenic differentiation of MSC (Buttery et al. 2001). In **Study IV**, the cells were additionally exposed to 25 µg/ml of Si, to investigate the osteogenic effects.

Calcium deposits in the ECM are an indication of successful osteogenic differentiation, with bone matrix proteins producing *in vitro* bone formation. Calcium deposits can be specifically stained using the Alizarin Red stain (ARS). ARS is a dye that binds selectively to calcium-rich deposits and it is widely used for calcium mineral histochemistry. An ARS-calcium complex is formed through a chelation reaction, and the end-product is a bright-red stain. Sites that contain calcium are covered and surrounded by a dark-red precipitate, clearly distinguishable from the pale-orange background. Mineralisation is then assessed by the extraction and colorimetric quantification of ARS in a stained monolayer using cetylpyridinium chloride, in a protocol adapted from that previously described by Stanford (1995).

Cell monolayers are washed twice, fixed, and stained with ARS (Sigma-Aldrich). The monolayers are then rinsed five times to reduce non-specific ARS staining and allowed to air-dry. Stained cells are photographed and then subjected to a quantitative extraction

method. Briefly, ARS is released from the cell matrix by incubation of the monolayers with cetylpyridinium chloride in sodium phosphate. The ARS extracts are then removed and aliquots are transferred to a 96-well plate prior to reading at 562 nm absorbance in a multi-plate reader. Control values are obtained from control samples without cells.

CX43 expression and Gap junctional coupling

To quantify the expression of the CX43 protein, hDFC were prepared as a cell suspension and labelled for flow cytometry. Briefly, hDFC were cultured in the absence or presence of Si (50 µg/ml) for 48 h. The cells were detached from the wells with ice-cold EDTA using a cell scrapper. Aliquots of 10^6 cells in 100 µl α-MEM were stained and labelled with GJA1 APC-conjugated antibody (BioLegend Inc., San Diego, CA, USA) for 20 min at 4°C. Thereafter, the cells were washed twice with PBS, and finally suspended in 500 µl PBS. An isotype b with different spectrum to that of the antibody of interest was used as a control to establish the gating threshold.

The GJC capability was monitored using the transfer of fluorescent molecules *via* gap junctions with the parachute technique. This assay, in which cells labelled with a gap-junction permeable indicator are plotted against unlabelled cells, allows precise quantification of the transferred dye in recipient cells using flow cytometry. Two fluorescent dyes were applied: a lipophilic dye that labels the cell membrane (DiI); and a second fluorochrome that loads the cytosol (calcein-AM). Once inside the donor cells, the non-fluorescent calcein-AM is hydrolysed by intracellular esterases to form the fluorescent form of calcein-AM, which can only be passed on to recipient cells through the gap junction channels. If gap junctions are present, the dye diffuses from the loaded cell to the cells in the unlabelled culture. Second-, third-, and higher-order cells will acquire the dye, depending on the degree of coupling. Using a flow cytometer, the double-stained donor cells can be separated from the recipient cells (Czyz et al. 2000).

In **Study IV**, the effects of Si on GJC were studied. Briefly, hDFC were cultured in the absence or presence of Si. For the donor cells, a cell suspension from a parallel culture was prepared and loaded with a staining solution using the Calcein-AM (Molecular Probes, Eugene, OR, USA) and Dil (Molecular Probes) dyes. The cell suspension was washed and centrifuged, and the pellet was re-suspended in PBS. Two percent of the double-stained donor cells were added to the un-stained recipient cells at a donor:recipient ratio of 1:50, just above the cell layer. After different incubation times, the cultures are trypsinised, suspended in PBS, and analysed by flow cytometry. Carbenoxolone (CBX) was added as an inhibitor of GJC, and used as a negative control.
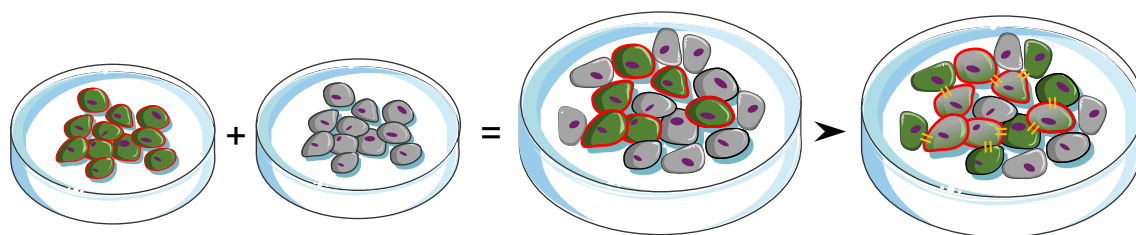

Figure 14. Protocol for the parachute technique.

Next-Generation Sequencing (RNA-Seq)

Next-generation sequencing provides a sensitive and accurate tool for measuring gene expression, so that changes that occur in response to different stimuli can be detected. Total RNA sequencing (RNA-Seq) accurately measures gene and transcript abundances and allows the detection of transcript isoforms, gene fusions, single nucleotide variants, allele-specific gene expression, and other features without any limitations with respect to prior knowledge. RNA-Seq is utilised to discover novel RNA variants and splice sites, as well as to quantify precisely mRNAs for gene expression analyses. Bioinformatics analyses are then used to converge these fragments by mapping the individual reads to the human reference genome. Each of the billion bases in the human genome is sequenced multiple times, ensuring sufficient resolution to deliver accurate data and an insight into DNA variations.

In **Study IV**, RNA-Seq was used to capture both known and novel regulatory elements in the gene expression profiles of cells that were exposed to Si. Transcriptome sequencing was performed at GATC Biotech, Germany. The RNA integrity was first analysed on an Agilent 2100 BioAnalyzer/Advanced Analytical Technologies Fragment Analyzer, to ensure a RIN/RQI value of ≥ 8. The mRNA was selected based on the poly-A tail, and a random-primed cDNA library was created for each sample. Standard Illumina adapters were used. Samples were pooled and loaded on an Illumina HiSeq and sequenced with 50-bp single reads. At least 30 million single reads were obtained from each sample.

Statistical analysis

In **Study I**, intra-examiner reliability was assessed using Dahlberg's formula (Dahlberg 1940) (method error = $\sqrt{\Sigma d2/2n}$, where d is the z difference between the two measurements of a pair, and n is the number of samples).

Multivariate data analyses
Thereafter, MVDA was used to project associations positively correlated with maxillary canine impaction. Firstly, PCA was applied to reduce the complexity of the data and to identify any intrinsic clustering within the dataset. The variables were column-scaled to unit variance and mean-centred. Moreover, prior to OPLS-DA modelling, separate PCAs were carried out to check for homogeneity and outlier identification. In a similar fashion, plots of residuals, in terms of the DmodX and DmodY control charts, were used to identify moderate outliers in the data. To support the decision-making process, some statistical reference limits were considered. For 2D score plots, Hotelling's T2 defined a 95% tolerance region, and for the DmodX bar chart, a critical distance corresponding to the 0.05 level was derived from the F-distribution. Observations outside the ellipse defined by Hotelling's T2 deviated from normality, and observations exceeding the critical distance in DmodX did not fit the model. OPLS-DA was used for pattern recognition across two different matrices; this involves reduction of data complexity and is commonly used to establish the information on X variables that relates to the variation in Y in a multivariate model. In the present case, X corresponded to the individual clinical variables, and Y denoted the specific diagnosis (impacted versus non-impacted canine). The variables were plotted along an orthogonal axis. The importance of each X-variable for the related Y-variable is presented as column bars in a corresponding column-loading plot. The quality of the multivariate analysis was assessed based on the following parameters: R2X, i.e. how well the variation of the Y-variable is

explained by the model, the goodness of fit; and Q2, i.e. how well a variable can be predicted by the model, the goodness of prediction. Variable of influence of projection (VIP) was the criterion selected to summarise the importance of the different predictors in the Y-matrix, where a VIP > 1 indicates that the X-variable is influential in explaining Y. MVDA was performed using the SIMCA-P software ver. 13 (Umetrics AB, Umeå, Sweden).

Univariate data analyses

Two statistical methods, parametric and non-parametric, were applied in the data analyses. Parametric tests assume a normal distribution of the data, whereas non-parametric tests rely on no assumptions being made as to the distribution of the data.

In **Study II and Study IV**, visual inspection of the frequency distribution (histogram) of the numerical dataset was performed to assess normal distribution prior to the analyses, and parametric statistics were used. The unpaired two-tailed *t*-test was used for the comparison of two independent groups (experimental vs. control), while one-way ANOVA followed by Holm-Sidak's multiple comparisons test was applied to evaluate differences between three or more groups. In **Study III**, the statistical analysis was conducted with no assumptions made as to the data's normal distribution, and non-parametric statistics were applied. A statistically significant difference between two paired groups was determined using the Wilcoxon matched-pairs signed-ranks test.

All the univariate analyses were performed in GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA), with p-values <0.05 being considered statistically significant.

RESULTS

Detailed descriptions of the results are found in the respective papers. Therefore, only the most important findings are presented in the following section.

Study I

For this case-control clinical study, 45 patients with a total of 58 impacted permanent maxillary canines, and 45 age- and gender-matched control subjects were selected. Radiographic, dento-alveolar, and other clinical variables were measured and recorded. MVDA were performed to identify patterns or correlations that could lead to the prediction of canine impaction.

Plots of the PCA scores are used to present the relationships among the observations and the obtained variables. A clear separation between the two groups was revealed (Fig. 15). This clear partition was explained by the variables related to the position and localisation of the un-erupted teeth, positively correlated to those patients presenting impacted canines.
The associations between each of the X-variables (clinical variables) and the Y-matrix (diagnosis) are expressed as OPLS-DA regression coefficients and coloured according to the VIP values. Warm colours have high VIP values and contribute more than the cold colours (Fig. 16). These VIP-values were used to discriminate between important and irrelevant predictors for the overall model. OPLS-DA VIP-values >1.5 were selected as the definitive predictors in the model.

The quality of the multivariate analysis was assessed based on the following parameters: R2X, i.e. how well the variation of the X-variable is explained by the model, *the goodness of fit*; and Q2, i.e. how well a variable can be predicted by the model, *the goodness of prediction*. For the applied statistical model, a predictive power >80 per cent—Q2 (0.82127) was acquired.

Upon distribution of the clinical variables, the regression model based on OPLS-DA allowed the identification of five positively correlated variables. The vertical level to the occlusal plane, lateral distance to the midline, and angulation to the mid-line exerted the strongest influences on the prediction of impaction, with canine location in the more mesial sectors being substantially associated. Neither skeletal features nor specific dento-alveolar relationships were identified as being correlated to the impacted group.

Although the variables inherent to the location of the un-erupted tooth were highlighted as possible predictors, considering that the diagnoses of these patients were already determined as "impaction", these results could only be regarded as positively associated factors. To investigate these specific variables as predictors, a prospective study design in which the patients are followed from a younger age and selected from the general population is necessary.
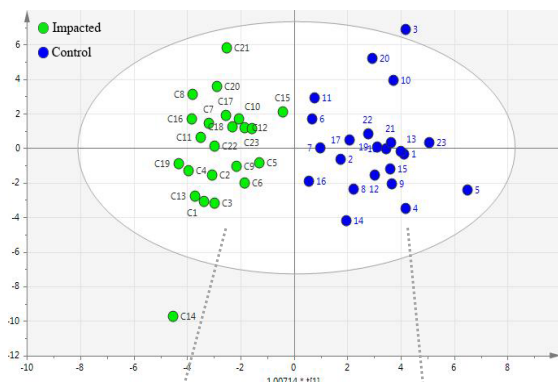
Figure 15. PCA Score Scatter Plot.
The blue dots indicate the control group and the green dots represent patients with impacted canines, with each point corresponding to one individual. The scores and loading plots show a strong association. Observations to the right have high values for variables in the same place in the loading plot. The effect is more pronounced further away from the origin.
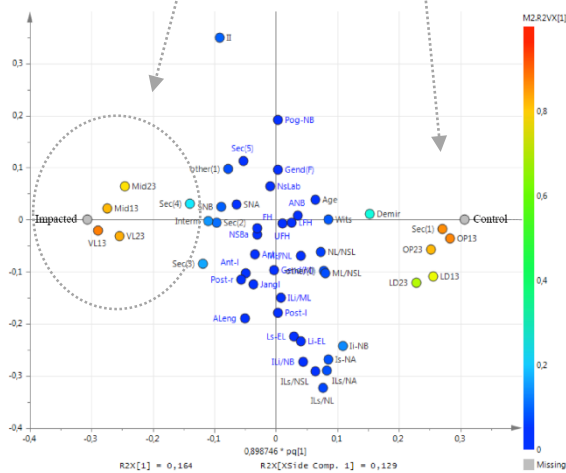

Figure 16. OPLS-DA Loading Scatter Plot.
OPLS-DA-loading graph plotted against the outcome variable (impacted or non-impacted). The separation of the data-points in the horizontal direction (between-group variation) is determined by their VIP-value colours. The separation of the data-points in the vertical direction (within-group variation) is influenced by their relevance to predictive power.

That there was no clear association to any of the skeletal growth patters or dento-alveolar characteristics led us to investigate further the local metabolism and molecular control of the dental follicle in the impacted teeth. Impaction may be related to the biological mechanisms involved in the bone remodelling that is needed for eruption and that is regulated by the dental follicle.

Study II

The gene expression profiles of markers related to bone formation and bone resorption were compared in the dental follicles from un-erupted teeth with different clinical status. Dental follicles were obtained from teeth at the time of surgical exposure. Eleven specimens from permanent teeth were collected: eight from permanent maxillary impacted canines, two from mesiodens, and one from a normally erupting pre-molar. All the teeth had considerable root length and no gingival tissue was attached to the collected samples.

Microscopic examination of histological sections revealed a relatively heterogeneous morphology, both within the histological sections from the same patient and between the different samples from individuals. The majority of the tissue sections consisted of fibrous connective tissue with dispersed fibroblast-like cells, as well as other condensed areas with numerous cells. Islands and strands of odontogenic epithelium were observed in most of the

sections. Bone tissue and diffuse calcifications were also evident. No consistent pattern was detected in the appearance of the tissue sections in relation to clinical signs of resorption in the roots of the neighbouring teeth.

RT-qPCR allowed the quantification of expression of the bone-related markers pertinent to bone formation, bone resorption, and GJC, and comparisons were made of the gene expression profiles of the different samples. Of the up-regulated genes, several were associated with osteogenesis. The highest levels of expression were observed for genes involved in bone formation, namely *RUNX2* and *CX43*. Relatively high mRNA levels were seen in all the samples for *OSX*, *ALP*, and *OCN*, whereas *BMP2* showed very low expression in all the samples. The expression levels of *RANKL* and *M-CSF*, which are markers related to osteoclast recruitment, were low in all the specimens. *MCP-1* expression was absented in every sample. In parallel, regulation of *PTHrP* was analysed in relation to a previous association with impacted teeth described in the literature (Fig. 17). Even though the expression levels were minimal in the dental follicles from the mesiodens, *PTHrP* was differentially regulated in all the samples, and no significant pattern could be ascribed to any of the defined groups: impacted, resorption or control (unpublished data). There was a relatively large variation in the expression levels of all examined genes, with the exception of the *CX43* gene, which was regulated in all the evaluated specimens relative to the expression level of the reference gene.
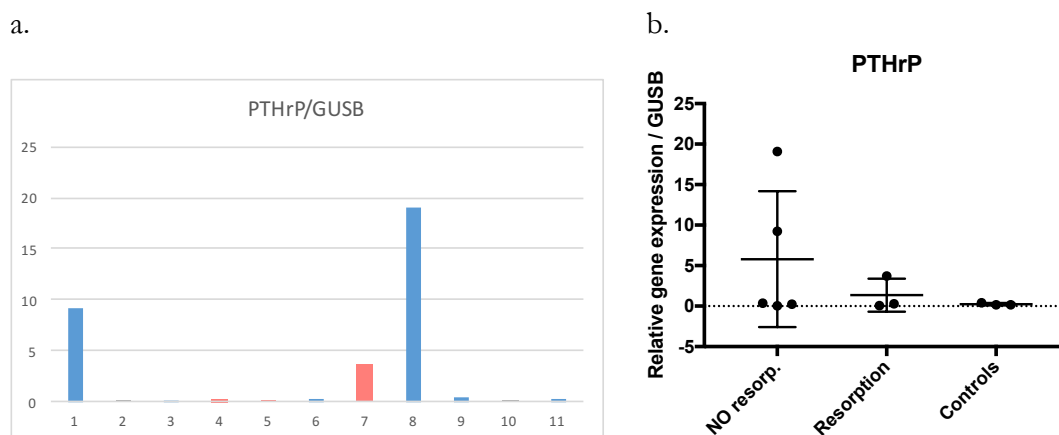
a.
b.

Figure 17. Relative expression levels of *PTHrP* in the dental follicles from un-erupted teeth with different clinical status. The data are presented as $\triangle\triangle$Cq values relative to the gene expression level of the selected reference gene (*GUSB*). a) The blue-coloured bars represent the impacted canines without signs of root resorption, the red-coloured bars illustrate the follicles of teeth presenting root resorption in the adjacent teeth, and the grey bars indicate the controls, including mesiodens and normally erupting teeth. b) ANOVA was performed to test for significant differences in gene expression between follicles obtained from patients with different clinical situations, with statistical significance being adjudged for p-values <0.05.

Overall, the genes related to bone formation showed high expression levels in all the samples, while the expression levels of markers for osteoclast recruitment and activation were negligible. No apparent patterns or significant differences in the mRNA expression of

marker genes were identified between impacted canines, with or without clinical signs of root resorption, or compared to the follicles from mesiodens or the normally erupting pre-molar.

Study III

This study was implemented in line with the theory, based on earlier animal studies, that there are regional patterns of expression of osteoblast- and osteoclast-regulatory factors, in relation to the apical and coronal parts of the dental follicle. Samples were obtained from twelve patients, and the apical and coronal portions of the dental follicle were processed independently. The highest levels of expression were detected for *COL-1* and *ALP*. The levels of expression of *OSX* and *BMP2* were relatively low in all the samples. Nonetheless, the gene that encodes the gap junction protein CX43 was strongly expressed in all the specimens relative to the reference gene. Differences in expression levels were detected both in the samples from different patients and when comparing the expression profiles of the target genes. However, there was no specific linkage between higher expression of these particular genes and the apical parts or coronal parts of the dental follicle. The relative transcription levels of the osteoclast-related markers *M-CSF*, *MCP-1*, and *OPG* were low in all the samples. No specific patterns of *M-CSF, MCP-1* or *OPG* expression were associated with either of the two sections of the dental follicle. Interestingly, significantly higher expression of *RANKL* was found in the coronal parts, as compared to the corresponding apical parts of the follicles (p=0.031).

To elucidate the spatial localisation and expression of the proteins encoded by the evaluated genes, immunofluorescence staining of M-CSF, OCN, CX43, BMP2, and RANKL was performed. Confocal microscopy images of the tissue sections revealed specific expression of M-CSF, OCN, and CX43, whereas no staining of BMP2 or RANKL could be demonstrated in any of the tissue sections. Visual discrimination of the stained specimens did not reveal a specific pattern of localisation of the selected bone-resorption or bone-formation markers to either of the follicle segments.

To characterise further the *RANKL* expression, hDFC from four patients were independently pre-cultured in osteogenic medium to initiate osteoblastic differentiation, and thereafter, cultured with FSK (10^{-6} M) to activate the PKA/cAMP signalling pathway and induce *RANKL/OPG* expression. The rationale behind this stimulation was to explore whether hDFC obtained from specific subjects would exhibit a differential capacity for regulation of the *RANKL* and *OPG* genes. Notably, FSK treatment induced substantial variation in the stimulated *RANKL* expression levels in the hDFC from different patients, implying a completely different regulatory behaviour. The *OPG* gene was highly expressed in the cultured control cells before activation with FSK. No clear time-dependent regulatory effect on *OPG* expression emerged after 24 h or 48 h in culture. Our results demonstrate that there are significant variations in the *RANKL/OPG* expression ratios between individuals in terms of the response to the same type of stimulation of cultured hDFC. This finding may have relevance for the diversity of events noted in the clinical setting during tooth eruption.

Overall, the results of this study demonstrate a consistent local differential expression of *RANKL* in human dental follicles, and diverse responses in the expression of *RANKL* in

hDFC, which in turn may explain the different eruption events observed in clinical practice. Once again, the consistent regulation of *CX43* expression validated the previous finding that GJC signals play a substantial role in regulating functions in the follicle and, eventually, in the eruption process.

Study IV

In this *in vitro* study, the osteogenic differentiation potential of the hDFC was analysed. The cells were treated with Si (50 µg/ml) during induced osteogenic differentiation, and the progression of the differentiation was evaluated after 1, 7, 14, and 21 days. Furthermore, the activity and regulation of CX43 were monitored after exposure to Si.

Cell viability analysed with the Neutral Red uptake assay showed a dose-dependent response of hDFC to Si after 48 h of culture. A significant stimulatory effect of Si on cell proliferation was seen at 25 µg/ml (p=0.001). Si concentrations in the range of 25–100 µg/ml did not significantly influence cell viability, as compared to the control. However, concentrations of Si >100 µg/ml significantly decreased the numbers of viable cells (p < 0.0001). Based on the proliferation results, the dosage of 25 µg/ml Si was selected for the subsequent experiments designed to evaluate further the effects induced by Si during osteogenic differentiation. After 21 days of culture in the presence of OIM, there was formation of mineralisation nodules in the cultured hDFC, as revealed by ARS. Significant stimulatory effects of OIM (p=0.0102) and OIM plus Si (p=0.0063) on the mineralisation process were seen after 14 and 21 days of culturing. However, no significant effect of Si alone on mineralised calcium deposition could be demonstrated.

To evaluate the lineage specificity, RT-qPCR was performed to examine the sequential expression of the osteoblast differentiation markers *OSX, RUNX2, BMP2, ALP, OCN,* and *BSP* and the gap junction protein *CX43*. All the genes were expressed in control cultures of hDFC and cells cultured in the presence of OIM with or without Si. The expression levels were low in all the cells at Day 1, except for *CX43*, which was highly expressed. The expression levels of *RUNX2, BMP2, ALP* and *BSP* progressively increased from Day 1 to Day 21 in all the groups. At Day 21, the mRNA levels of *BMP2* (p=0.0025) and *OCN* (p=0.0045) were significantly enhanced in the cells exposed to OIM plus Si and to Si alone, as compared to the control cultures. Time-dependent, significant increases in the *BSP* levels (p<0.0001) were observed in the cells exposed to OIM, OIM plus Si, and Si alone, as compared to control cultures. Interestingly, while the expression levels of *CX43* were significantly high from Day 1 in all the groups, a marked and significant up-regulation was observed after 21 days in the Si-treated group (p=0.0054).

Considering the results showing the regulation of *CX43* in response to Si, FACS analyses were conducted to quantify and measure the expression of CX43 and the functional coupling activity. Quantification of the expression of CX43 protein revealed that only a low proportion (7.6%) of the hDFC expressed CX43 on their surfaces. Most importantly, an increase in the percentage of hDFC expressing CX43 (16.2%) was observed after hDFC were exposed to the Si solution for 48 h. In addition, the GJC activity of the hDFC was analysed using the parachute dye transfer technique. The effect of Si (50 µg/ml) on the hDFC after 1, 2, and 3 h progressively increased the GJC activity, resulting in a significant increase

of stained-recipient hDFC. The transfer of dye between donor and recipient cells was completely blocked when the cells where incubated on ice, and furthermore, dye transfer was totally inhibited by treatment with the gap junction blocking compound carbenoxolone (CBX).

Taken together, these results indicate that undifferentiated MSC that are resident in the dental follicle can be driven to differentiate toward an osteoblastic lineage under favourable *in vitro* conditions, resulting in the active deposition of mineralised matrix. In particular, Si enhances osteogenic differentiation in combination with OIM. The results also suggest that Si stimulates CX43 expression and GJC activity in hDFC.

MAIN FINDINGS

- No correlation between the clinical variables and impaction was found using the comprehensive MVDA. Therefore, these variables cannot be used as predictors of canine impaction **(Study I)**.

- No apparent patterns or significant differences in gene expression profiles were found between impacted canines, with or without signs of root resorption, or when compared to control teeth **(Study II).**

- The role of the dental follicle in regulating osteoclastic activity is limited during the late pre-emergent stages of tooth eruption, irrespective of whether the tooth is normally erupting or impacted **(Studies II and III)**.

- Higher expression of *RANKL* in the coronal portion of the human dental follicle provides further support for the notion that *RANKL* is an important regulator of the osteoclastogenesis and bone resorption needed for formation of the eruption pathway **(Study III)**.

- There is a significant difference in the induced expression levels of *RANKL* and *OPG* in cultures of hDFC from different follicles and individuals. This may be linked to the differences in tooth eruption observed in the clinical setting **(Study III)**.

- The consistent regulation of *CX43* expression in the dental follicle indicates that signals related to GJC may play a fundamental role in the regulatory functions of the dental follicle during the pre-emergent stage of tooth eruption **(Studies II and III)**.

- Cultured hDFC can be induced towards osteogenic differentiation by OIM and Si. Si stimulates *CX43* expression and GJC activity in hDFC **(Study IV)**.

- Multipotent mesenchymal cells localised in the dental follicle can be cultured under *in vitro* conditions to induce osteogenic differentiation, and they are regarded as a potential source for bone regenerative therapies **(Study IV).**

GENERAL DISCUSSION

The development of advanced techniques and refined tools has given us the opportunity to investigate the complexity of human biological events. This has given rise to new avenues to delve deeper into the unresolved questions regarding specific conditions and niches in the human body, and has also facilitated a critical interpretation of the paradigms that have existed for generations. This project was designed to expand the body evidence concerning the biological setting of tooth eruption and, in particular, the clinical and molecular aspects of canine impaction.

Clinical variables and prediction of impacted maxillary canines

Permanent maxillary canine impaction has been discussed extensively over the years. The factors underlying the occurrence and management of this phenomenon remain a matter of debate. Based on the available evidence, early diagnosis and opportune interceptive treatment are the main guarantors of a successful eruption and optimal outcome. Eruption of the permanent palatally displaced canines can be facilitated by the extraction of deciduous canines (Ericson and Kurol 1988; Naoumova et al. 2015). Early interceptive treatment can decrease the degree of developing malocclusion and the risk of root resorption of the adjacent teeth, and thereby, reduce the need for future comprehensive orthodontic treatment. A recent systematic review concluded that following the extraction of the deciduous canine when the permanent canine is palatally displaced, there was a higher incidence of successful eruption (50%–69%) compared with the no-intervention control groups (36%–42%) (Almasoud 2017). To determine whether a canine is destined to become impacted, several clinical and radiographical parameters need to be considered. The position of the un-erupted tooth, the overlapping of the roots of the adjacent lateral incisors, the stage of root development, the signs of root resorption, and the occurrence of other dental anomalies, together with the linear and angular measurements from radiographs are frequently used as variables for radiological assessment, and they have been suggested as possible predictors of canine eruption. In **Study I**, all the variables listed in the literature as being related to canine impaction were measured, collated, and incorporated into the MVDA. The aim was to identify predictive variables for impacted maxillary canines. Based on our results, sector location, angulation to the mid-line, and vertical level to the occlusal plane are all indicators of impaction, yielding good predictive power.

The complex nature of most biological datasets entails technical and systematic errors, making these analyses more challenging. Large datasets, in combination with complex biological settings, have created a need for more specific and sophisticated statistical, mathematical, and high-end computational support. Multivariate techniques provide a more accurate picture by reducing the complexity of the datasets while retaining valuable information about the relationships between the observations. Although several studies have directed their efforts towards these specific clinical factors, only conventional statistical tools have been used in the evaluations. Thus, the potential interactions between and within clinical variables limit the scope of these investigations.

General Discussion

Tooth impaction should not be viewed as a black or white event. In this respect, the isolation prior to selection of independent variables is another disadvantage of univariate and bivariate approaches. Each variable is evaluated in isolation among all the concomitant variables that characterise the scenario during arrested eruption. Skeletal relationships, dento-alveolar features, and the local characteristics of the site of eruption are all part of the multifactorial event. Consequently, they may be contributing collectively and simultaneously, and should therefore be studied in a complex multivariate setting.

The MVDA revealed that the significant positively associated variables in regard to the impaction group are those that refer to the spatial location of the un-erupted tooth. If the canine is completely developed, the canine angle to the mid-line and the overlap with the lateral incisor are considered good indicators of canine impaction. Sector location and angulation of the impacted tooth have been analysed previously as predictors of eruption after extraction of the deciduous canine (Baccetti et al. 2008; Ericson and Kurol 1988; Leonardi et al. 2004; Naoumova et al. 2015; Power and Short 1993).

Study I supports the findings of Ericson and Kurol (1988) and Power and Short (1993), who reported lower probabilities of eruption when lower canine angulation to the mid-line was present. Consistent with the data presented by Wardford et al. (2003) and Alqerban et al. (2015), the degree of mesial overlap of the maxillary canine relative to the adjacent lateral incisor exerts a potent influence on the severity of impaction. Sajnani and King (2012b) found that after the age of 9 years, a statistical difference in the distance from the canine cusp tip to the occlusal maxillary plane was the most important predictor of impaction. The significance of these specific variables has also been studied using cone-beam computed tomography (CBCT), resulting in the strongest predictive capability (Alqerban et al. 2015; Naoumova et al. 2014). However, the linear measurements and their angulations used to identify the locations of the impacted canines have been found by some authors to be inadequate indicators of successful outcome in relation to the interceptive orthodontic treatment, length of treatment, and periodontal status (An et al. 2013; Crescini et al. 2007; Leonardi et al. 2004). The results of this study do not reveal any association with craniofacial skeletal relationships or malocclusion. The frequent absence of malocclusion and disperse craniofacial features in patients who present with impacted canines may explain the difficulties encountered with the early identification and delayed diagnosis of this condition, which hinder timely preventative therapies (Mercuri et al. 2013).

The term "prediction" is used to define a statement as to what will happen or might happen in the future. The issue of whether these encountered variables related to impaction can be regarded as predictors is complicated due to the fact that the study design involved patients whose canines were already regarded as *impacted*. In this situation, the variables related to the spatial location of the un-erupted tooth are interpreted as the most influential features in regard to the impaction group. To determine whether these variables are predictors, a prospective study design in which the patients are selected from the general population and followed from a younger age is imperative. Nonetheless, ethical constraints linked to needless exposure to radiation for the participating subjects may limit these types of studies.

It is also important to delineate the limitations of this study. The control group was taken from an orthodontic population. This may explain why the association with other dental anomalies was not highlighted in relation to their occurrence. As has been discussed in the literature (Baccetti 1998b; Peck et al. 1994), the existence of associations between different

tooth anomalies is not only important from an aetiological point of view, but may also have clinical relevance given that early diagnosis of one anomaly can indicate an increased risk for the later appearance of other anomalies. However, orthodontic patients may have higher incidences of malocclusions and dental anomalies compared to a randomised sample taken from the general population. Crowding was not considered in our analyses. Only arch lengths and distances were measured, although tooth length and the availability of space in the dental arches should be considered in future studies.

Palatal and buccal canines have been regarded as two completely different entities. Considering the results in **Study I**, there were no clear associations or patterns identified between the studied variables and either of the anatomical localisations (palatally and buccally). The two major theories (the guidance theory, which proposes that local conditions are responsible for the displacement of the canine; and the genetic theory, which considers genetic factors and concomitant occurrence of other dental anomalies) are, in our view, analogous and complementary. It remains uncertain as to whether an anomalous lateral incisor is a local causal factor for palatally displaced canines, or whether the displaced canines are the result of an associated genetic developmental effect. The sequential theory, which is a more recent approach to these distinct characteristics of the impacted canines, disputes this mono-causal perspective for explaining the phenomenon. The sequential theory proposes that regional genetic mechanisms strongly influence the localisation and direction of the developing tooth, while the guidance conferred by the lateral incisor at a specific stage of development plays a crucial role in determining the final position (either palatal or buccal) of the canine (Sajnani and King 2012c). Despite there being evidence of increased incidences of other dental anomalies when impacted canines are in the palatal position, it should be borne in mind that anomalies in the development of a neighbouring tooth are also controlled by genetic mechanisms. Certainly, both processes are dependent upon genetic regulation and local factors that may influence a later developmental stage. Taking into account these results (**Study I**), and applying rationale that permanent canine impaction is influenced by site-specific genetic mechanisms, the question of which of the biological factors regulated in the dental follicles pertain to impacted canines raised. **Study II and Study III** were designed to characterised the gene expression profiles of the dental follicles of impacted canines.

Characterisation of the human dental follicle

Tooth eruption is a genetically programmed, localised event that is directed by the dental follicle (Cahill and Marks 1980). The regulation of osteoblastic and osteoclastic genes is of importance during this chronological and sequential phenomenon. The data gathered in **Study II** and **Study III** describe the gene expression profiles related to bone formation and bone resorption markers in human dental follicles. It was clearly shown that the mRNA levels of osteoblastic-related markers were highly expressed compared to the levels of mRNA for the bone-resorption markers.

The relatively high expression levels of *RUNX2*, *OSX*, *BMP2*, *ALP*, and *OCN* indicate the capacity of human dental follicles for osteogenic regulation. The highest levels of expression were identified for the genes involved in bone extracellular matrix regulation, namely *COL-1* and *ALP*. The *CX43* gene was strongly expressed in all the evaluated specimens relative to the expression level of the reference gene.

The mechanism or motive force that pushes the tooth through the resorbed path has not been clearly defined, although many studies have shown that bone growth at the base of the tooth crypt is required for a tooth to erupt (Wise et al. 2011; Wise et al. 2007; Yao et al. 2010). The results from **Study II** suggest that the dental follicle is actively regulating osteogenesis when the teeth are in a late pre-eruptive stage. Previous studies have demonstrated that inhibition of members of the BMP superfamily can impede alveolar bone formation and subsequently, tooth eruption, despite the fact that an eruption path is formed (Wise et al. 2011). Therefore, it appears that *RUNX2, OSX,* and *BMP2* are key molecules in promoting osteogenesis of the alveolar bone at the base of the tooth during the eruption process.

Such alveolar bone growth is likely regulated by the apical portion of the dental follicle. This belief is based on the findings from surgical studies in the dog, in which removal of the apical half of the dental follicle prevented any bone formation (Marks and Cahill 1987), and by gene expression studies in rodents that have shown that *BMP2* is chronologically regulated (Wise et al. 2004) and is expressed at a higher level in the apical half, as compared to the coronal half of the dental follicle (Wise and Yao 2006). However, **Study III** failed to identify a specific pattern for either of the halves of the follicle, even when gene expression of the bone formation markers was quantified using RT-qPCR and detected with immunofluorescence in each of the sections. The effective involvement of these transcription factors and proteins produced by the dental follicle was further confirmed when hDFC, cultured under favourable conditions, were indeed found to be regulated in the mineralisation process. In **Study IV**, hDFC were shown to possess osteogenic activity. As the dental follicle contains multipotent cells that are capable of differentiation into multiple cell lineages (including osteoblasts), these candidate molecules secreted by the follicle may stimulate osteoprogenitor cells in or around the follicle to differentiate into the osteoblasts that are needed to form the new bone growth at the base of the alveolar socket.

In contrast, the transcription levels of osteoclast-related markers were found to be negligible or very low relative to the reference genes, and when compared to the osteoblastic markers (**Study II** and **Study III**). Experimental animal studies have demonstrated the ultra-structural features of the bony crypt around an erupting tooth, revealing bone resorption in the direction of eruption and bone formation in the opposite course. The cellular events that co-ordinate this resorption activity include the recruitment of mononuclear cells to the dental follicle, followed by the fusion of precursors to form multinucleated and actively resorptive osteoclasts (Marks et al. 1983). This is the first study to evaluate the site-specific expression of regulatory factors in the human dental follicles. A significant differential spatial gene expression pattern could be demonstrated for *RANKL* in the coronal parts of the dental follicles, as compared with their corresponding apical parts (**Study III**). These results are interpreted as supporting the hypothesis developed in earlier studies with dogs and rodents, as presented by Cahill and Marks (1980) and Wise and Yao (2006).

The role of the dental follicle during impaction

The spatiotemporal patterns and relative abundances of *M-CSF, RANKL* and *OPG* transcripts and proteins during tooth eruption in mice have been identified as key determinants of site-specific osteoclastic activity in the bone surrounding the tooth (Heinrich et al. 2005). MCP-1 (Que and Wise 1997) and M-CSF (Wise et al. 1995; Wise et al. 2005) are

synthesised by DFC. *MCS-F* is also expressed in the osteoblasts and stromal cells surrounding the eruptive tooth, and it promotes the survival and proliferation of osteoclast precursors by up-regulating *RANK* and reducing the expression of *OPG* (Wise et al. 2005). These co-ordinated events lead to enhanced intercellular signalling for RANK-RANKL, favouring osteoclast formation. The biological effect of RANKL is inhibited by OPG. OPG, which is constitutively produced by the dental follicle (Wise et al. 2002), is down-regulated when the maximum number of osteoclasts is reached on the surrounding alveolar bone (Wise et al. 2003; Wise et al. 2002) and when there is maximal expression of *CSF-1* (Wise et al. 1995). When RANKL production is higher than OPG production in osteoblasts, osteoclast formation is supported, whereas higher relative levels of OPG block this effect. The lack of either M-CSF or RANKL would culminate in arrested tooth eruption (Harris et al. 2012).

The low levels of osteoclast-activating signals in the dental follicles from impacted teeth and mesiodens may be attributed to their impaction condition. However, the expression of osteoclast-regulating factors was not higher in the dental follicles from normally erupting teeth or impacted canines with clinical and radiographical signs of root resorption in adjacent teeth. The timing of the sampling process could have had an influence on the obtained results. At the time of surgery, the examined specimens were from teeth that were in a late developmental stage and that probably had undergone cessation of the intra-osseous resorption spurt. Considering the results obtained in **Study II** and **Study III,** it can be postulated that the dental follicle does not regulate bone resorption in the final pre-emergent stages of eruption. It could also be argued that since the surgical procedure in **Study II** did not provide the complete follicular tissue, there may be regional differences, in terms of the regulatory capacity of the dental follicle, which could not be examined. As part of the treatment protocol for the auto-transplantation of vital teeth, whole dental follicles can easily be obtained for further analysis in the laboratory. Thus, the whole follicular tissue and the selection of the specific regions of interest was made possible in **Study III**. However, when studying the follicle as a whole tissue, the mRNA levels of osteoclastic markers were still found to be expressed lesser when compared to the expression levels of the osteoblast-related markers.

It is important to note that the main goal was to quantify the regulatory control exerted by the dental follicle on chemotactic and biological molecules that would promote osteoclastogenesis. The reason for this is that recruited and activated osteoclasts are only found close to bone surfaces (or root surfaces), and not in the follicle tissue *per se.* Another factor that should be considered when interpreting these findings is that the stimulation of bone resorption, as well as the osteoclast activity and life-span, are shorter and more limited compared to osteogenesis. Osteoclastic activity does not persist for very long, perhaps only days. Osteoclasts are activated and exert short-term effects on osteoblasts and stromal inter-cellular signalling when they are needed in the surrounding environment. Therefore, the limited expression of osteoclast-related markers can be attributed to the absence or extinction of the osteoclastic resorptive burst.

Achieving a better understanding of the regulatory mechanisms and validating the putative molecules are essential steps in elucidating dental eruption disorders. The demonstration of *RANKL* expression in the coronal portion of the dental follicles in **Study III** provides further support for the idea of *RANKL* as an important regulator of osteoclastogenesis. Moreover, the *in vitro*-induced expression of *RANKL* (**Study III**) demonstrates that there are significant differences between individuals in relation to: the levels of *RANKL*

expression; the responses to external stimuli; and the control of the *OPG/RANKL* ratio (with respect to favouring or limiting osteoclastic activity). It has previously been reported that RANKL expression can be induced in cultured hDFC by adding extracellular factors, such as PTHrP (Sun et al. 2015). Activation of cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) is one of the major down-stream signalling pathways for the expression of *RANKL* (Kondo et al. 2002). FSK is a potent adenylyl cyclase activator that increases the level of the second messenger cAMP in bone cells (Lerner et al. 1986). FSK was used as an experimental tool to investigate whether stimulated expression of *RANKL* in hDFC differed among the patients. Our results demonstrate that there are significant differences between individuals in terms of the levels of FSK-induced *RANKL* in their cultured hDFC. The results imply that the stimulus from the local environment at the onset of eruption elicits a different response in each individual. As a consequence, different clinical pictures and distinct eruption features are encountered even when the teeth of the different patients are found to have the same locations and characteristics.

The differential response of the hDFC that express *RANKL* can also be considered in the context of root resorption. When we compared teeth with different clinical statuses, including canines involved in resorption of neighbouring roots, no clear pattern could be identified in relation to the selected markers. Previous studies have suggested that enlarged dental follicles increase the risk for root resorption (Chaushu et al. 2015), while other studies have claimed that the existing contact of the impacted maxillary canine with the lateral incisor root is critical (Ericson and Kurol 2000). While bone-resorbing osteoclasts are required for tooth eruption, they are also involved in the root resorption process, which is often regarded as a complication associated with impacted and displaced maxillary canines. Root resorption is hypothesised to be caused by continuous forces in a specific location and possibly related to susceptible root areas. However, the reason why the roots are resorbed in one patient and remain intact in another patient, in whom the canine is identically situated, remains elusive. Nevertheless, since resorption is dependent upon local signals for the recruitment and activation of osteoclasts, it is reasonable to assume that the dental follicle is involved. In order for resorption to occur, the osteoclast precursors need to be activated *via* RANKL/M-CSF, to fuse and form multinucleated cells that will be activated once they find an exposed substrate to which they can attach and develop the acidified lacunae, which will subsequently dissolve the minerals in the ossified tissues. There is no difference in the scenario for root resorption to happen. A source, in this case the dental follicle, can respond differentially to the local environment by expressing RANKL when stimulated. It is of importance to consider that for resorption to proceed, an uncovered area of cementum needs to be available for the osteoclasts to attach. These areas can appear as a consequence of earlier trauma, such as damage to the cementocytes or cementoid layer caused by mechanical forces, or idiopathy.

The gene expression profiles of the dental follicles derived from impacted canines have not been analysed previously for the clinical signs of root resorption. In **Study II**, no apparent patterns or significant differences for the mRNA expression of osteoclast- and osteoblast-marker genes were observed between the impacted canines, with or without clinical signs of root resorption, or compared to the follicles from mesiodens, or to the normally erupting pre-molar. However, the differential response of stimulated hDFC suggests that the root resorption event is the result of unfortunate coincidental local factors that provide a convenient setting for stimulated osteoclasts to attach to an uncovered area on the root. An event that occur as a consequence of responsive dental follicles in the permanent maxillary impacted canines being stimulated by the close proximity of an adjacent root. Although this

is a limited number of samples to generate conclusions, our findings may have relevance for the diversity of features noted in the clinical setting for cases of tooth eruption.

The concept of a genetic molecular cause for tooth eruption failures is not new (Frazier-Bowers et al. 2010a). The eruption failure observed in cases of CCD has been related to the altered expression of RANKL/OPG in the dental follicle (Dorotheou et al. 2013). Furthermore, familial cases of PFE have been linked to a defect in the *PTHrP* gene (Frazier-Bowers et al. 2010a; Risom et al. 2013). Only one previous study has analysed the expression of genes in the dental follicles of human subjects in the context of eruption. In patients with CCD, the levels of *RANKL* were found to be lower in those patients who carried a *RUNX2* mutation, such that the *RANKL/OPG* ratio was not favourable for osteoclast formation to start the resorption pathway needed for eruption (Dorotheou et al. 2013). An accumulating body of evidence indicates that *PTHrP* is likely to induce osteoclastogenesis, which is necessary for the normal eruption course. *PTHrP* is known to stimulate *RANKL* expression in osteoblasts (Huang et al. 2004). In addition, *PTHrP* expression appears to be localised to the stellate reticulum of the mouse, with its receptors being present in both alveolar bone and the dental follicles (Philbrick et al. 1998). Their proximity would enable paracrine control of *MCP-1, MCS-F* and *OPG* expression, thereby participating in the recruitment of mononuclear cells to the follicle, as has been demonstrated *in vitro* using DFC (Wise et al. 2000b; Yao et al. 2007). Analysis of the samples in **Study II** failed to demonstrate any correlation between impacted canines and *PTHrP* expression. However, it is important to note that the *PTHrP* mRNA levels were minimal in both of the mesiodens.

The model of tooth eruption remains a theory that is based on experimental animal studies. Most of the earlier data on the molecular basis of tooth eruption were obtained from studies that were conducted with rodents and dogs. Animal models provide the opportunity to study the time-course of the eruption process through the acquisition of time- and area-specific samples with fewer ethical issues. Samples collected from animals have the advantages of selectivity and repeatability. The location of the sampling, the size of the specimen, the time of biopsy, and the ethical considerations are challenges that need to be deliberated upon when investigating human samples. Extrapolation of the animal data to humans should be approached with caution given the known differences between these diverse species. Tooth eruption in humans is difficult to study, primarily because it occurs so slowly and the teeth are relatively inaccessible until they emerge into the mouth. As a result, neither the eruption mechanism nor the controlling factors in human tooth eruption are completely understood (Proffit and Frazier-Bowers 2009). Since tooth eruption relies on the dental follicle, studies of the gene expression of impacted teeth may shed some light on the mechanism underlying aberrant eruption. While the results obtained in **Study II** and **Study III** cannot prove the actual cause of impaction, they still provide insights into the relatively unexplored field of tooth eruption in humans.

Mineralisation capacity and regeneration potential of hDFC

It has been shown that the dental follicle produces molecules at critical times to regulate the osteoclastogenesis and osteogenesis needed for eruption onset (Wise and Yao 2006). Multipotent stem cells are demonstrated to be present in the dental follicles of different species, and most importantly in the human dental follicle (Honda et al. 2011; Morsczeck et al. 2005a). The hDFC have excellent proliferation rates, and under specific culture

conditions, they are reported to have the capacity to differentiate towards osteoblasts, cementoblasts, fibroblasts, neurons, and adipocytes (Kanao et al. 2017; Tamaki et al. 2013; Yao et al. 2008). Animal experiments have determined that the bone formation at the base of the crypt likely serves as the eruptive force to push the tooth out of the bony crypt (Wise et al. 2011; Wise et al. 2007). Given this fact, DFC are implicated in the orchestration of osteogenesis, and it is therefore of interest to study further their osteogenic potential.

The results from **Study IV** demonstrate the capacity of hDFC to differentiate towards osteoblast-like cells under *in vitro* conditions in the presence of OIM, which contains ascorbic acid and glycerophosphate. OIM promotes an osteoblastic phenotype for the hDFC, as evidenced by the regulated expression of early and late osteoblastic markers and increased extracellular matrix formation (based on Alizarin Red staining), which is a characteristic functional feature of mature osteoblasts.

hDFC are a promising source of valuable cells that could be used to establish validation models for osteo-inductive materials, and furthermore, as an alternative reservoir of MSC for regeneration approaches and tissue engineering applications. The following concepts provide fundamental evidence that the hDFC are a reliable source of osteogenic precursors (all of which are described in **Study IV**). The hDFC:

- Are characterised by a fibroblast-like morphology, having the ability to adhere to plastic and the possibility to propagate under *in vitro* conditions while remaining stable in terms of proliferation for up to nine passages (Morsczeck et al. 2005a; Yao et al. 2004).
- Demonstrate the ability to proliferate *in vitro* under suitable conditions, and are able to form cell colonies, which is one of the properties of stem cells (Morsczeck et al. 2005a).
- Show a higher proliferation rate than multipotent cells derived from the dental pulp (Shoi et al. 2014).
- Can be successfully cryopreserved after isolation from fresh tissues, retaining the hDFC phenotype, without affecting their biological properties (Yang et al. 2017).
- Have osteogenic capabilities *in vivo* and *in vitro*. These cells have the competence to differentiate when triggered correctly and produce calcified nodules, which are areas with high levels of calcium (Honda et al. 2011; Kang et al. 2015; Morsczeck et al. 2005b; Park et al. 2012).
- Have the added advantage of being easily harvested from exposed or extracted impacted teeth.

The ability of hDFC to differentiate into osteoblasts seems likely, given the chronological and sequential expression of osteoblast-related markers and incremental matrix deposition over time. Usually, osteoblasts form bone-like mineralised nodules in culture through three stages: proliferation of osteoblast precursors; differentiation and extracellular matrix synthesis; and maturation and mineralisation of the matrix (Huang et al. 2007). In Figure 18, the successive stages of the differentiation process observed in hDFC derived from impacted canines are described for cells cultured under osteogenesis-favourable conditions (**Study IV**).

While markers typical for osteogenic differentiation were expressed, *BMP2*, *OCN* and *BSP* were clearly up-regulated after differentiation. The down-regulation of expression of the

transcription factors *OSX* and *RUNX2* during long-term culturing was also observed. Therefore, we propose that the down-regulation of these factors supports the molecular processes of osteogenic differentiation.

Exposure to an osteogenic differentiation environment, e.g. soluble factors (ascorbic acid, β-glycerophosphate, and dexamethasone) and growth factors (BMP-2 and TGFβ1), has been shown in previous studies to induce the osteogenic differentiation of DFC (Morsczeck et al. 2009; Saugspier et al. 2010). During bone formation, growth factors of the BMP family induce differentiation by up-regulating various transcription factors, including *RUNX2* and *OSX*. This in turn leads to the up-regulation of *ALP*, *OCN*, and *BSP*, and eventually results in down-stream gene transcription that directs cell differentiation and promotes the synthesis and secretion of bone extracellular matrix (Roca et al. 2005; Zhao et al. 2002). In the osteogenic differentiation process in hDFC, the *RUNX2* mRNA levels were increased on Day 7 in all the groups, with no significant difference in the levels. Although *RUNX2* is essential for osteoblast differentiation, *RUNX2*-independent osteogenic differentiation mechanisms have been proposed to operate in hDFC induced with dexamethasone (Felthaus et al. 2014; Morsczeck 2006; Saugspier et al. 2010). This mechanism depends on the expression of *ZBTB16* (Felthaus et al. 2014). The mature osteoblast (Day 21+) expresses *ALP*, *OPN*, *BSP*, and *OCN*. This stage, which is responsible for the laying down of bone, has limited replicative potential (Huang et al. 2007). *BSP* is involved in the mineralisation process during the third and final phases of osteoblast development (Choi et al. 1996), with the results implying that the precursor cells in the hDFC are transformed and differentiate towards the osteoblastic phenotype.
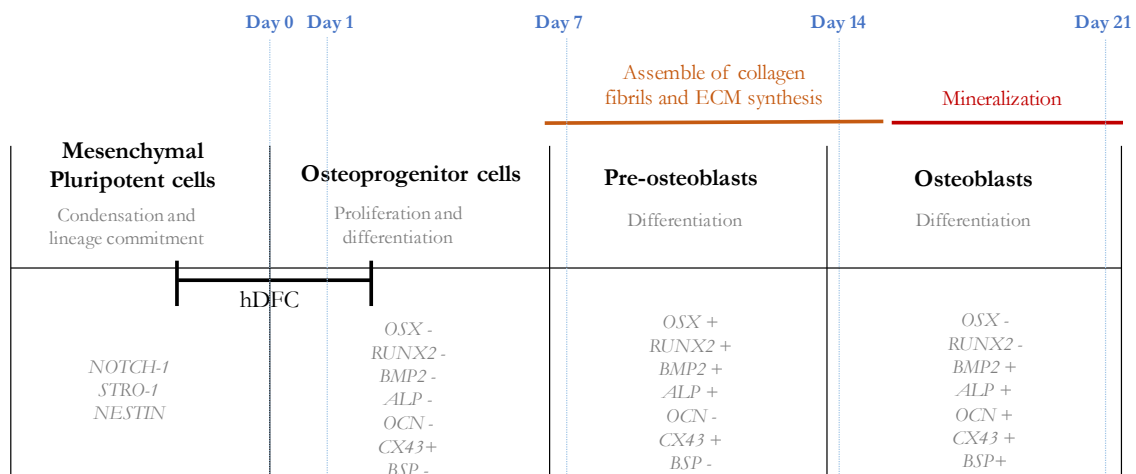

Figure 18. Time-line and gene expression profiles of hDFC during osteogenic differentiation. As the hDFC proliferate and differentiate, they synthesise the ECM that eventually mineralises. In blue, at the top, the days of culture are presented and correlated with the stage of differentiation. The regulation of the gene expression of osteoblastic markers is presented for each time-point in relation to the reference genes. The hDFC are a heterogeneous population, containing both MSC and osteoprogenitors from the start-point.

The differentiation of hDFC is not completed after 4 weeks. However, the time-points for evaluation of the molecular mechanism during osteogenic differentiation were selected based on the literature, such that the expected up-regulation of primary genes involved in the

molecular processes of differentiation would be active. hDFC from impacted canines showed continuous expression of osteoblast-specific transcription factors and strong mineralisation, as well as increased expression of osteogenic markers during osteogenic differentiation. These observations are in line with those from previous studies (Morsczeck et al. 2009; Vollkommer et al. 2015), establishing the strong osteogenic potential of hDFC.

Silica as an "osteo-inductive" factor for hDFC

Several studies have suggested that Si is influential in the normal development of connective tissue and the skeleton (Carlisle 1986). In addition to the positive effects on bone health of dietary Si, it has also been demonstrated that biomaterials that contain Si have positive effects on bone regeneration and the repair of bone defects. One interesting osteo-inductive Si-containing biomaterial is Bioglass 45S5, which was originally developed by Larry Hench in 1969 (Hench 2006; Jones 2013; Tadjoedin et al. 2000).

This is the first study to report the effect of Si on hDFC (**Study IV**). Significantly enhanced proliferation of hDFC was observed when they were cultured in the presence of Si at 25 µg/ml. For this reason, this concentration of Si was used for the posterior mineralisation assay. The dose-response results revealed that Si at concentrations >100 µg/ml exhibited an inhibitory effect on cell proliferation, which may indicate a toxic effect. The argument underlying this is that Si at high concentrations (50–60 µg/ml) may lead to polymerisation of silica (Iler 1979), as it reaches the solubility limit. Thus, our higher concentrations of Si could have predominant species such as in a polymeric form, which may have negative effects on cell biocompatibility.

The mineralisation assay demonstrated that hDFC cultured with OIM showed significantly stimulated formation of calcium deposits. In contrast, this mineralisation effect was decreased when Si was added and when the cells were cultured with Si only, as compared to the controls. These observations are discrepant with the results obtained with the murine osteoblast precursor cell line MC3T3-E1, where lower doses of Si (1–7 µg/ml) increased mineralised nodule formation, as evidenced by increased ARS (Kim et al. 2013). This discrepancy may be explained by the fact that a different concentration of Si was used, following on the finding that lower concentrations are more effective for mineral matrix deposition.

Most importantly, Si enhances osteoblast differentiation in hDFC by regulating the gene expression of osteogenesis markers. These positive changes were associated with *BMP2*, *OCN* and *CX43* up-regulation in long-term cultures of hDFC in the presence of simple Si medium. Furthermore, Si in combination with OIM maintained a significantly higher level of expression of *ALP* until the late stages of differentiation, as compared to the basic medium. The finding that Si influences *ALP* activity also appears to be in agreement with the findings from Reffitt and co-workers (2003). They reported that 1 µg/ml orthosilicic acid significantly increased the differentiation of MG-63 (human osteosarcoma-derived) cells *in vitro*, as evidenced by a 50% increase in ALP activity and a 75% rise in type I collagen synthesis (Reffitt et al. 2003). The *BSP* levels decreased towards Day 21 in the simple Si group. This can be explained by the absence of OIM, which strongly promotes phenotypic differentiation, since the signals for matrix deposition and maturation were lacking. Our results demonstrate that the expression of *BMP2* is only increased in OIM containing Si (25

µg/ml). Similar results are observed for bone MSC exposed to 0.5 µg/ml of Si, with marked increases in the expression of *BMP2*, *RUNX2*, and *COL-1* (Dong et al. 2016). These findings support the concept of Si stimulating osteogenic differentiation in hDFC, since *BMP2* plays an important role in the regulation of osteogenic genes and matrix mineralisation (Viale-Bouroncle et al. 2013).

The results presented in this thesis prove that there is an osteogenic effect of Si on undifferentiated hDFC cells, in agreement with findings previously reported by Costa-Rodrigues et al. (2016), who showed that MSC cultured with physiological levels of Si favoured osteogenic differentiation. Si stimulates the expression of factors that are essential for osteoblastic differentiation, although compared to the effect of the osteogenic medium *per se*, the influence exerted by Si in itself is not sufficient to trigger the mineralisation process. Our findings suggest that Si promotes osteoblastic differentiation and maturation, although the precise mechanism is still unknown. It has earlier been proposed that Si is a cofactor for prolyl hydroxylase, which is a catalyst in the formation of hydroxyproline in the collagen chain (Reffitt et al. 2003). Currently, it is unclear whether Si is transported into the cells or exerts its effects through extracellular mechanisms. However, in the present study and based on our earlier published results on the effects of Si on osteoclast formation *in vitro* (Mladenovic et al. 2014), we propose that Si interacts directly with the regulatory functions in the bone cells.

Short-term application of Si promoted further the CX43-mediated GJC, which is identified as a major prerequisite for osteogenic differentiation of mesenchymal stromal cells and osteoprogenitor cells. These results also support previous findings regarding dental pulp multipotent cells during differentiation and mineralisation (Syed-Picard et al. 2013). Inhibition of CX43-based cellular communication was associated with reduced differentiation potential and decreased mineralisation (Lecanda et al. 2000).

Our results demonstrate that mesenchymal cells located in the dental follicle provide the optimal precursors for the culturing of bone cells under *in vitro* conditions, and the triggering of such cells to differentiate toward the osteoblastic lineage. The present investigations reveal the gene expression profiles of transcription factors and osteoblast-phenotypic markers in the hDFC from impacted canines during osteogenic differentiation. The data presented in **Study IV** support the idea of a novel source of materials for cell-based therapies and candidates that can be used to establish a validation model for osteo-inductive materials, as well as an alternative source of cells and cellular components for the repair and regeneration of periodontal defects, with the added advantage that these cells and factors can easily be harvested from extracted impacted canines. In addition to the cellular source, the inductive micro-environment is an important component in the mineralisation process. Si could be a candidate molecule for external stimulation to enhance further the osteoblastic differentiation and activity involved in bone matrix synthesis. Understanding the underlying mechanism behind the osteo-inductivity of Si would greatly advance the design of future biomaterials.

A novel finding: CX43 regulation in the dental follicle

One of the ways in which cells co-ordinate their actions is by intercellular communication through gap junctions. Gap junctions form channels that allow the direct exchange between

cells of small ions, molecules, and second messengers. This allows efficient cell-to-cell communication and functional co-ordination through an inter-connected cellular network. It has long been recognized that extensive cellular interactions are important in embryogenesis and for the regulatory signals involved in growth control, pattern formation, and the differentiation of tissues (Lecanda et al. 2000). The dental follicle is no stranger to the involvement of developmental structures. During different developmental stages, the dental follicle plays a significant role in tooth morphogenesis, the eruption process, and the differentiation of the periodontium complex.

A major finding of this thesis is the up-regulation of *CX43* in the dental follicles from un-erupted teeth. The levels of *CX43* were observed to be highly expressed in both the coronal and apical sections of the follicles, irrespective of clinical condition, i.e. impacted, signs of root resorption or normally erupting (**Studies II** and **III**). This regulatory effect was increased by culturing the hDFC in osteogenic medium, and even further enhanced when the hDFC were exposed to Si (**Study IV**). Immunofluorescence staining revealed expression of the CX43 protein in areas with condensed cell organisation (**Study III**), and gene expression analyses revealed high-level, continuous expression of the gene encoding CX43.

The high-level expression of *CX43* in the dental follicle has not been reported previously. The overall evidence from previous experimental studies suggests that GJC is important during osteoblast proliferation, maturation, and activation (Lecanda et al. 2000). In addition, osteoblasts and osteoclasts precursors are reported to express the *CX43* protein and functional gap junction channels necessary for their development and bone-resorbing activities (Matemba et al. 2006; Ransjo et al. 2003; Zappitelli and Aubin 2014). However, considering the low-level expression for osteoclast markers and activity in the present samples, it seems likely that the high-level expression of *CX43* reflects an active osteoblast differentiation process that involves GJC.

To investigate further the role of CX43 in hDFC, **Study IV** aimed to quantify the expression and activity of CX43 in cells cultured with OIM. The pattern of *CX43* expression revealed up-regulation during the differentiation process toward an osteoblastic lineage. In the control cells after 1 day of culture, the high level of expression of CX43 was already obvious, implying activity of the protein. Seven days after culturing under favourable conditions, the up-regulation of mRNA levels for CX43 was significantly enhanced. During Days 7–10 of differentiation induction, committed osteoprogenitor cells have the features of pre-osteoblasts, a stage that is crucially dependent upon intercellular signalling. Previous studies that have established the role of *CX43* have shown that osteoblasts require *CX43* throughout their differentiation programme, particularly as they progress through the *RUNX2* to *OSX* transition (Moorer and Stains 2017). The *CX43*-dependent effect in pre-osteoblasts, which involves various signalling pathways, such as ERK and PKCδ promotion of the transcriptional activity of *RUNX2* and *SP7/OSX* driving the expression of several osteoblastic genes (Lima et al. 2009). Loss of *CX43* in any of these contexts can affect cell survival and/or the efficiency of the cellular response to an extracellular signal (Stains et al. 2014). Considering these findings, it can be postulated that during the pre-osteoblast stage, signalling through CX43-mediated GJC is vital for the differentiation of hDFC. The up-regulation of *CX43* was intense even after 21 days of hDFC culturing in the presence of Si alone. This study shows that the differentiation process in hDFC is paralleled by the Si-induced increase in *CX43* gene expression and GJC activity. Several studies have strongly suggested that Si interacts directly with the regulatory functions of bone cells. Osteoblast

differentiation was enhanced by increasing *ALP* activity and *OCN* synthesis (Costa-Rodrigues et al. 2016; Dong et al. 2016; Reffitt et al. 2003) and stimulating *COL-1* synthesis in osteoblast-like cells (Reffitt et al. 2003). These results were interpreted as supporting the hypothesis that Si enhances osteoblast differentiation. Interestingly, in the present study, we show that the osteogenic medium has no stimulatory effect on *CX43* expression. Therefore, our results also suggest that the effect of Si on *CX43* regulation is independent of OIM, which is in agreement with Hashida and colleagues (2014), who reported that CX43-mediated GJC plays an important role in osteoblast differentiation induced by *BMP2* but not that induced by ascorbic acid.

We next performed a functional test to evaluate the activity of the GJC in hDFC (**Study IV**). The dye transfer process was significantly enhanced in the hDFC exposed to Si in a time-dependent manner. It was interesting to observe that the cultured hDFC form a heterogeneous cell population, and in terms of the reactivity of CX43, a sub-group of the cells was more responsive to Si stimulation. The GJC mechanism allows signal sharing between multiple cells in response to the stimulus, regardless of the ability of the cells to sense the signal (perhaps due to a lack of receptors). Therefore, the observed incremental spreading of the calcein-AM dye may be explained by the number of active gap junction channels and the regulation of CX43 in the recipient cells amplifying the overall response. In contrast, when CX43 was inhibited, using either treatment with CBX or incubation of the cells on ice, the response was limited in all the cells and no transfer of signals was observed.

Overall, these results provide direct evidence that *CX43* plays a crucial role in the late pre-emergent stage of tooth eruption, possibly being involved in the regulation of the bone formation that is required during this stage. In line with the high levels of CX43, our results suggest that signals that are dependent upon GJC may be essential for the regulatory functions that are active during osteoblastic differentiation of hDFC. Si, as a biocompatible molecule with osteo-inductive capabilities, can regulate CX43 expression and activities that promote the differentiation mechanism in hDFC.

CONCLUDING REMARKS

- No correlation was found between the clinical variables and canine impaction in the comprehensive MVDA. Therefore, these variables cannot be used as predictors of canine impaction.

- No significant difference or any clear pattern was observed when comparing the follicles and relating their gene expression profiles to their different clinical situations (impaction, root resorption, normal eruption).

- The significant up-regulation of *RANKL* expression identified in the coronal parts compared to the apical parts of the dental follicles underlines the importance of recruiting and activating osteoclasts to form the eruption pathway through the alveolar bone.

- Multipotent mesenchymal cells localised in the dental follicle can be cultured *in vitro* and induced towards an osteoblastic phenotype. Furthermore, Si positively stimulates osteogenic differentiation in hDFC.

ACKNOWLEDGEMENTS

"It's not where you go, it's all about who you meet along the way"

The wizard of Oz

REFERENCES

Al-Nimri K, Gharaibeh T. 2005. Space conditions and dental and occlusal features in patients with palatally impacted maxillary canines: An aetiological study. Eur J Orthod. 27(5):461-465.

Almasoud NN. 2017. Extraction of primary canines for interceptive orthodontic treatment of palatally displaced permanent canines: A systematic review. Angle Orthod.

Alqerban A, Jacobs R, Fieuws S, Willems G. 2015. Radiographic predictors for maxillary canine impaction. Am J Orthod Dentofacial Orthop. 147(3):345-354.

An S, Wang J, Li J, Cheng Q, Jiang CM, Wang YT, Huang YF, Yu WJ, Gou YC, Xiao L. 2013. Comparison of methods for localization of impacted maxillary canines by panoramic radiographs. Dentomaxillofac Radiol. 42(8):20130129.

Andersen CL, Jensen JL, Orntoft TF. 2004. Normalization of real-time quantitative reverse transcription-pcr data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64(15):5245-5250.

Andresen JO. 1997. Normal and disturbed tooth eruption in humans. In: Andreasen JOP, J. K.; Laskin, D.M. , editor. Textbook and color atlas of tooth impactions. Copenhagen. p. 49-64.

Anic-Milosevic S, Varga S, Mestrovic S, Lapter-Varga M, Slaj M. 2009. Dental and occlusal features in patients with palatally displaced maxillary canines. Eur J Orthod. 31(4):367-373.

Arai F, Miyamoto T, Ohneda O, Inada T, Sudo T, Brasel K, Miyata T, Anderson DM, Suda T. 1999. Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-fms and receptor activator of nuclear factor kappab (rank) receptors. J Exp Med. 190(12):1741-1754.

Aubin JE, Liu F, Malaval L, Gupta AK. 1995. Osteoblast and chondroblast differentiation. Bone. 17(2 Suppl):77S-83S.

Baccetti T. 1998a. A clinical and statistical study of etiologic aspects related to associated tooth anomalies in number, size, and position. Minerva Stomatol. 47(12):655-663.

Baccetti T. 1998b. A controlled study of associated dental anomalies. Angle Orthod. 68(3):267-274.

Baccetti T, Leonardi M, Armi P. 2008. A randomized clinical study of two interceptive approaches to palatally displaced canines. Eur J Orthod. 30(4):381-385.

References

Bandyopadhyay A, Tsuji K, Cox K, Harfe BD, Rosen V, Tabin CJ. 2006. Genetic analysis of the roles of bmp2, bmp4, and bmp7 in limb patterning and skeletogenesis. PLoS Genet. 2(12):e216.

Basdra EK, Kiokpasoglou M, Stellzig A. 2000. The class ii division 2 craniofacial type is associated with numerous congenital tooth anomalies. Eur J Orthod. 22(5):529-535.

Becker A, Chaushu S. 2000. Dental age in maxillary canine ectopia. Am J Orthod Dentofacial Orthop. 117(6):657-662.

Becker A, Gillis I, Shpack N. 1999. The etiology of palatal displacement of maxillary canines. Clin Orthod Res. 2(2):62-66.

Bedoya MM, Park JH. 2009. A review of the diagnosis and management of impacted maxillary canines. J Am Dent Assoc. 140(12):1485-1493.

Bellows CG, Heersche JN, Aubin JE. 1992. Inorganic phosphate added exogenously or released from beta-glycerophosphate initiates mineralization of osteoid nodules in vitro. Bone Miner. 17(1):15-29.

Bianco P, Cao X, Frenette PS, Mao JJ, Robey PG, Simmons PJ, Wang CY. 2013. The meaning, the sense and the significance: Translating the science of mesenchymal stem cells into medicine. Nat Med. 19(1):35-42.

Bishara SE. 1992. Impacted maxillary canines: A review. Am J Orthod Dentofacial Orthop. 101(2):159-171.

Bishara SE. 1998. Clinical management of impacted maxillary canines. Semin Orthod. 4(2):87-98.

Bjork A. 1963. Variations in the growth pattern of the human mandible: Longitudinal radiographic study by the implant method. J Dent Res. 42(1)Pt 2:400-411.

Boyle WJ, Simonet WS, Lacey DL. 2003. Osteoclast differentiation and activation. Nature. 423(6937):337-342.

Brin I, Becker A, Shalhav M. 1986. Position of the maxillary permanent canine in relation to anomalous or missing lateral incisors: A population study. Eur J Orthod. 8(1):12-16.

Brin I, Solomon Y, Zilberman Y. 1993. Trauma as a possible etiologic factor in maxillary canine impaction. Am J Orthod Dentofacial Orthop. 104(2):132-137.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL et al. 2009. The miqe guidelines: Minimum information for publication of quantitative real-time pcr experiments. Clin Chem. 55(4):611-622.

Buttery LD, Bourne S, Xynos JD, Wood H, Hughes FJ, Hughes SP, Episkopou V, Polak JM. 2001. Differentiation of osteoblasts and in vitro bone formation from murine embryonic stem cells. Tissue Eng. 7(1):89-99.

Cahill DR. 1969. Eruption pathway formation in the presence of experimental tooth impaction in puppies. Anat Rec. 164(1):67-77.

Cahill DR. 1974. Histological changes in the bony crypt and gubernacular canal of erupting permanent premolars during deciduous premolar exfoliation in beagles. J Dent Res. 53(4):786-791.

Cahill DR, Marks SC, Jr. 1980. Tooth eruption: Evidence for the central role of the dental follicle. J Oral Pathol. 9(4):189-200.

Capulli M, Paone R, Rucci N. 2014. Osteoblast and osteocyte: Games without frontiers. Arch Biochem Biophys. 561:3-12.

Carlisle EM. 1986. Silicon as an essential trace element in animal nutrition. Ciba Found Symp. 121:123-139.

Carlson H. 1944. Studies on the rate and amount of eruption of certain human teeth. Am J Orthod Oral Surg. 42:78-91.

Chaushu S, Kaczor-Urbanowicz K, Zadurska M, Becker A. 2015. Predisposing factors for severe incisor root resorption associated with impacted maxillary canines. Am J Orthod Dentofacial Orthop. 147(1):52-60.

Cheek CC, Paterson RL, Proffit WR. 2002. Response of erupting human second premolars to blood flow changes. Arch Oral Biol. 47(12):851-858.

Chen G, Deng C, Li YP. 2012. Tgf-beta and bmp signaling in osteoblast differentiation and bone formation. Int J Biol Sci. 8(2):272-288.

Choi JY, Lee BH, Song KB, Park RW, Kim IS, Sohn KY, Jo JS, Ryoo HM. 1996. Expression patterns of bone-related proteins during osteoblastic differentiation in mc3t3-e1 cells. J Cell Biochem. 61(4):609-618.

Civitelli R. 2008. Cell-cell communication in the osteoblast/osteocyte lineage. Arch Biochem Biophys. 473(2):188-192.

Cooke J, Wang HL. 2006. Canine impactions: Incidence and management. Int J Periodontics Restorative Dent. 26(5):483-491.

Costa-Rodrigues J, Reis S, Castro A, Fernandes MH. 2016. Bone anabolic effects of soluble si: In vitro studies with human mesenchymal stem cells and cd14+ osteoclast precursors. Stem Cells Int. 2016:5653275.

Coulter J, Richardson A. 1997. Normal eruption of the maxillary canine quantified in three dimensions. Eur J Orthod. 19(2):171-183.

Crescini A, Nieri M, Buti J, Baccetti T, Pini Prato GP. 2007. Pre-treatment radiographic features for the periodontal prognosis of treated impacted canines. J Clin Periodontol. 34(7):581-587.

References

Cui Y, Niziolek PJ, MacDonald BT, Zylstra CR, Alenina N, Robinson DR, Zhong Z, Matthes S, Jacobsen CM, Conlon RA et al. 2011. Lrp5 functions in bone to regulate bone mass. Nat Med. 17(6):684-691.

Czyz J, Irmer U, Schulz G, Mindermann A, Hulser DF. 2000. Gap-junctional coupling measured by flow cytometry. Exp Cell Res. 255(1):40-46.

Dahlberg G. 1940. Statistical methods for medical and biological students. George Allen and Unwin LTD.

Day TF, Guo X, Garrett-Beal L, Yang Y. 2005. Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev Cell. 8(5):739-750.

Demirjian A, Goldstein H. 1976. New systems for dental maturity based on seven and four teeth. Ann Hum Biol. 3(5):411-421.

Dewel BF. 1949. Clinical observations on the axial inclination of teeth. Am J Orthod. 35(2):98-115.

Dong M, Jiao G, Liu H, Wu W, Li S, Wang Q, Xu D, Li X, Liu H, Chen Y. 2016. Biological silicon stimulates collagen type 1 and osteocalcin synthesis in human osteoblast-like cells through the bmp-2/smad/runx2 signaling pathway. Biol Trace Elem Res. 173(2):306-315.

Dorotheou D, Gkantidis N, Karamolegkou M, Kalyvas D, Kiliaridis S, Kitraki E. 2013. Tooth eruption: Altered gene expression in the dental follicle of patients with cleidocranial dysplasia. Orthod Craniofac Res. 16(1):20-27.

Doty SB. 1981. Morphological evidence of gap junctions between bone cells. Calcif Tissue Int. 33(5):509-512.

Ericson S, Bjerklin K, Falahat B. 2002. Does the canine dental follicle cause resorption of permanent incisor roots? A computed tomographic study of erupting maxillary canines. Angle Orthod. 72(2):95-104.

Ericson S, Kurol J. 1986a. Longitudinal study and analysis of clinical supervision of maxillary canine eruption. Community Dent Oral Epidemiol. 14(3):172-176.

Ericson S, Kurol J. 1986b. Radiographic assessment of maxillary canine eruption in children with clinical signs of eruption disturbance. Eur J Orthod. 8(3):133-140.

Ericson S, Kurol J. 1987a. Incisor resorption caused by maxillary cuspids. A radiographic study. Angle Orthod. 57(4):332-346.

Ericson S, Kurol J. 1987b. Radiographic examination of ectopically erupting maxillary canines. Am J Orthod Dentofacial Orthop. 91(6):483-492.

Ericson S, Kurol J. 1988. Early treatment of palatally erupting maxillary canines by extraction of the primary canines. Eur J Orthod. 10(4):283-295.

Ericson S, Kurol PJ. 2000. Resorption of incisors after ectopic eruption of maxillary canines: A ct study. Angle Orthod. 70(6):415-423.

Felthaus O, Gosau M, Klein S, Prantl L, Reichert TE, Schmalz G, Morsczeck C. 2014. Dexamethasone-related osteogenic differentiation of dental follicle cells depends on zbtb16 but not runx2. Cell Tissue Res. 357(3):695-705.

Florencio-Silva R, Sasso GR, Sasso-Cerri E, Simoes MJ, Cerri PS. 2015. Biology of bone tissue: Structure, function, and factors that influence bone cells. Biomed Res Int. 2015:421746.

Frazier-Bowers SA, Koehler KE, Ackerman JL, Proffit WR. 2007. Primary failure of eruption: Further characterization of a rare eruption disorder. Am J Orthod Dentofacial Orthop. 131(5):578 e571-511.

Frazier-Bowers SA, Puranik CP, Mahaney MC. 2010a. The etiology of eruption disorders - further evidence of a 'genetic paradigm'. Semin Orthod. 16(3):180-185.

Frazier-Bowers SA, Simmons D, Wright JT, Proffit WR, Ackerman JL. 2010b. Primary failure of eruption and pth1r: The importance of a genetic diagnosis for orthodontic treatment planning. Am J Orthod Dentofacial Orthop. 137(2):160 e161-167; discussion 160-161.

Fuss Z, Tsesis I, Lin S. 2003. Root resorption--diagnosis, classification and treatment choices based on stimulation factors. Dent Traumatol. 19(4):175-182.

Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PV, Komm BS, Javed A, van Wijnen AJ, Stein JL, Stein GS et al. 2005. Canonical wnt signaling promotes osteogenesis by directly stimulating runx2 gene expression. J Biol Chem. 280(39):33132-33140.

Glass DA, 2nd, Karsenty G. 2006. Canonical wnt signaling in osteoblasts is required for osteoclast differentiation. Ann N Y Acad Sci. 1068:117-130.

Gorski JP, Marks SC, Jr. 1992. Current concepts of the biology of tooth eruption. Crit Rev Oral Biol Med. 3(3):185-206.

Gowgiel JM. 1967. Observations on the phenomena of tooth eruption. J Dent Res. 46(6):1325-1330.

Gregg JM, Avery JK. 1964. Studies of alveolar bone growth and tooth eruption using tetracycline-induced fluorescence. J Oral Ther Pharmacol. 1:268-281.

Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. 2000. Postnatal human dental pulp stem cells (dpscs) in vitro and in vivo. Proc Natl Acad Sci U S A. 97(25):13625-13630.

Guler N, Comunoglu N, Cabbar F. 2012. Ki-67 and mcm-2 in dental follicle and odontogenic cysts: The effects of inflammation on proliferative markers. ScientificWorldJournal. 2012:946060.

References

Hagg U, Taranger J. 1986. Timing of tooth emergence. A prospective longitudinal study of swedish urban children from birth to 18 years. Swed Dent J. 10(5):195-206.

Harputluoglu S. 1990. Effects of removing inferior alveolar neurovascular structures on mandibular growth and the eruption of permanent dentition in puppies. Oral Surg Oral Med Oral Pathol. 70(2):147-149.

Harris SE, MacDougall M, Horn D, Woodruff K, Zimmer SN, Rebel VI, Fajardo R, Feng JQ, Gluhak-Heinrich J, Harris MA et al. 2012. Meox2cre-mediated disruption of csf-1 leads to osteopetrosis and osteocyte defects. Bone. 50(1):42-53.

Harzer W, Seifert D, Mahdi Y. 1994. [the orthodontic classification of impacted canines with special reference to the age at treatment, the angulation and dynamic occlusion]. Fortschr Kieferorthop. 55(2):47-53.

Hashida Y, Nakahama K, Shimizu K, Akiyama M, Harada K, Morita I. 2014. Communication-dependent mineralization of osteoblasts via gap junctions. Bone. 61:19-26.

Hasund A. 1977. Clinical cephalometry for the bergen technique. [Bergen, Norway]: University of Bergen.

Heinrich J, Bsoul S, Barnes J, Woodruff K, Abboud S. 2005. Csf-1, rankl and opg regulate osteoclastogenesis during murine tooth eruption. Arch Oral Biol. 50(10):897-908.

Hench LL. 2006. The story of bioglass. J Mater Sci Mater Med. 17(11):967-978.

Hitchin AD. 1951. The impacted maxillary canine. Dent Pract Dent Rec. 2(4):100-103.

Honda MJ, Imaizumi M, Suzuki H, Ohshima S, Tsuchiya S, Satomura K. 2011. Stem cells isolated from human dental follicles have osteogenic potential. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 111(6):700-708.

Huang GTZ, X.; Yan, X.;. Hewitt, K.J.; Garlick, J.A. . 2013. Stem cells, induced pluripotent stem cells, and their differentiation to specified lineages fates. In: Huang GTT, I., editor. Stem cells in craniofacial development and regeneration. John Wiley & Sons, Inc. p. 205-222.

Huang JC, Sakata T, Pfleger LL, Bencsik M, Halloran BP, Bikle DD, Nissenson RA. 2004. Pth differentially regulates expression of rankl and opg. J Bone Miner Res. 19(2):235-244.

Huang W, Yang S, Shao J, Li YP. 2007. Signaling and transcriptional regulation in osteoblast commitment and differentiation. Front Biosci. 12:3068-3092.

Iler RK. 1979. The chemistry of silica : Solubility, polymerization, colloid and surface properties, and biochemistry. Wiley.

Jacobs SG. 1998. Reducing the incidence of unerupted palatally displaced canines by extraction of deciduous canines. The history and application of this procedure with some case reports. Aust Dent J. 43(1):20-27.

Jacobs SG. 1999. Localization of the unerupted maxillary canine: How to and when to. Am J Orthod Dentofacial Orthop. 115(3):314-322.

Jacoby H. 1983. The etiology of maxillary canine impactions. Am J Orthod. 84(2):125-132.

Jones JR. 2013. Review of bioactive glass: From hench to hybrids. Acta Biomater. 9(1):4457-4486.

Kanao S, Ogura N, Takahashi K, Ito K, Suemitsu M, Kuyama K, Kondoh T. 2017. Capacity of human dental follicle cells to differentiate into neural cells in vitro. Stem Cells Int. 2017:8371326.

Kang YH, Lee HJ, Jang SJ, Byun JH, Lee JS, Lee HC, Park WU, Lee JH, Rho GJ, Park BW. 2015. Immunomodulatory properties and in vivo osteogenesis of human dental stem cells from fresh and cryopreserved dental follicles. Differentiation. 90(1-3):48-58.

Karsenty G. 2003. The complexities of skeletal biology. Nature. 423(6937):316-318.

Kemoun P, Laurencin-Dalicieux S, Rue J, Farges JC, Gennero I, Conte-Auriol F, Briand-Mesange F, Gadelorge M, Arzate H, Narayanan AS et al. 2007. Human dental follicle cells acquire cementoblast features under stimulation by bmp-2/-7 and enamel matrix derivatives (emd) in vitro. Cell Tissue Res. 329(2):283-294.

Kim EJ, Bu SY, Sung MK, Choi MK. 2013. Effects of silicon on osteoblast activity and bone mineralization of mc3t3-e1 cells. Biol Trace Elem Res. 152(1):105-112.

Kjaer I. 2014. Mechanism of human tooth eruption: Review article including a new theory for future studies on the eruption process. Scientifica (Cairo). 2014:341905.

Kjaer I. 2017. Tooth eruption and alveolar bone formation: Abnormal patterns including syndromes. In: Kjaer I, editor. Etiology-based dental and craniofacial diagnostics. Wiley Blackwell. p. 125-148.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M et al. 1997. Targeted disruption of cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell. 89(5):755-764.

Kondo H, Guo J, Bringhurst FR. 2002. Cyclic adenosine monophosphate/protein kinase a mediates parathyroid hormone/parathyroid hormone-related protein receptor regulation of osteoclastogenesis and expression of rankl and osteoprotegerin mrnas by marrow stromal cells. J Bone Miner Res. 17(9):1667-1679.

Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A et al. 1999. Opgl is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature. 397(6717):315-323.

Kular J, Tickner J, Chim SM, Xu J. 2012. An overview of the regulation of bone remodelling at the cellular level. Clin Biochem. 45(12):863-873.

References

Lai CF, Cheng SL. 2002. Signal transductions induced by bone morphogenetic protein-2 and transforming growth factor-beta in normal human osteoblastic cells. J Biol Chem. 277(18):15514-15522.

Langberg BJ, Peck S. 2000. Adequacy of maxillary dental arch width in patients with palatally displaced canines. Am J Orthod Dentofacial Orthop. 118(2):220-223.

Langer R, Vacanti JP. 1993. Tissue engineering. Science. 260(5110):920-926.

Larson EK, Cahill DR, Gorski JP, Marks SC, Jr. 1994. The effect of removing the true dental follicle on premolar eruption in the dog. Arch Oral Biol. 39(4):271-275.

Lecanda F, Warlow PM, Sheikh S, Furlan F, Steinberg TH, Civitelli R. 2000. Connexin43 deficiency causes delayed ossification, craniofacial abnormalities, and osteoblast dysfunction. J Cell Biol. 151(4):931-944.

Leifert S, Jonas IE. 2003. Dental anomalies as a microsymptom of palatal canine displacement. J Orofac Orthop. 64(2):108-120.

Leonardi M, Armi P, Franchi L, Baccetti T. 2004. Two interceptive approaches to palatally displaced canines: A prospective longitudinal study. Angle Orthod. 74(5):581-586.

Lerner UH. 2000. Osteoclast formation and resorption. Matrix Biol. 19(2):107-120.

Lerner UH, Fredholm BB, Ransjo M. 1986. Use of forskolin to study the relationship between cyclic amp formation and bone resorption in vitro. Biochem J. 240(2):529-539.

Lima F, Niger C, Hebert C, Stains JP. 2009. Connexin43 potentiates osteoblast responsiveness to fibroblast growth factor 2 via a protein kinase c-delta/runx2-dependent mechanism. Mol Biol Cell. 20(11):2697-2708.

Lindauer SJ, Rubenstein LK, Hang WM, Andersen WC, Isaacson RJ. 1992. Canine impaction identified early with panoramic radiographs. J Am Dent Assoc. 123(3):91-92, 95-97.

Liu D, Wise GE. 2007. A DNA microarray analysis of chemokine and receptor genes in the rat dental follicle--role of secreted frizzled-related protein-1 in osteoclastogenesis. Bone. 41(2):266-272.

Liu D, Yao S, Pan F, Wise GE. 2005. Chronology and regulation of gene expression of rankl in the rat dental follicle. Eur J Oral Sci. 113(5):404-409.

Liu W, Toyosawa S, Furuichi T, Kanatani N, Yoshida C, Liu Y, Himeno M, Narai S, Yamaguchi A, Komori T. 2001. Overexpression of cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures. J Cell Biol. 155(1):157-166.

Long F. 2011. Building strong bones: Molecular regulation of the osteoblast lineage. Nat Rev Mol Cell Biol. 13(1):27-38.

Ludicke G, Harzer W, Tausche E. 2008. Incisor inclination--risk factor for palatally-impacted canines. J Orofac Orthop. 69(5):357-364.

Marie PJ. 2008. Transcription factors controlling osteoblastogenesis. Arch Biochem Biophys. 473(2):98-105.

Marks SC, Jr. 1973. Pathogenesis of osteopetrosis in the ia rat: Reduced bone resorption due to reduced osteoclast function. Am J Anat. 138(2):165-189.

Marks SC, Jr. 1981. Tooth eruption depends on bone resorption: Experimental evidence from osteopetrotic (ia) rats. Metab Bone Dis Relat Res. 3(2):107-115.

Marks SC, Jr. 1989. Osteoclast biology: Lessons from mammalian mutations. Am J Med Genet. 34(1):43-54.

Marks SC, Jr., Cahill DR. 1984. Experimental study in the dog of the non-active role of the tooth in the eruptive process. Arch Oral Biol. 29(4):311-322.

Marks SC, Jr., Cahill DR. 1986. Ultrastructure of alveolar bone during tooth eruption in the dog. Am J Anat. 177(3):427-438.

Marks SC, Jr., Cahill DR. 1987. Regional control by the dental follicle of alterations in alveolar bone metabolism during tooth eruption. J Oral Pathol. 16(4):164-169.

Marks SC, Jr., Cahill DR, Wise GE. 1983. The cytology of the dental follicle and adjacent alveolar bone during tooth eruption in the dog. Am J Anat. 168(3):277-289.

Massler MS, I. 1941. Studies in tooth development theories of eruption. Am J Orthod Oral Surg. 27:552-576.

Matemba SF, Lie A, Ransjo M. 2006. Regulation of osteoclastogenesis by gap junction communication. J Cell Biochem. 99(2):528-537.

McConnell TL, Hoffman DL, Forbes DP, Janzen EK, Weintraub NH. 1996. Maxillary canine impaction in patients with transverse maxillary deficiency. ASDC J Dent Child. 63(3):190-195.

Mercuri E, Cassetta M, Cavallini C, Vicari D, Leonardi R, Barbato E. 2013. Skeletal features in patient affected by maxillary canine impaction. Med Oral Patol Oral Cir Bucal. 18(4):e597-602.

Miletich I, Sharpe PT. 2004. Neural crest contribution to mammalian tooth formation. Birth Defects Res C Embryo Today. 72(2):200-212.

Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. 2003. Shed: Stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A. 100(10):5807-5812.

Mladenovic Z, Johansson A, Willman B, Shahabi K, Bjorn E, Ransjo M. 2014. Soluble silica inhibits osteoclast formation and bone resorption in vitro. Acta Biomater. 10(1):406-418.

References

Moorer MC, Stains JP. 2017. Connexin43 and the intercellular signaling network regulating skeletal remodeling. Curr Osteoporos Rep. 15(1):24-31.

Morsczeck C. 2006. Gene expression of runx2, osterix, c-fos, dlx-3, dlx-5, and msx-2 in dental follicle cells during osteogenic differentiation in vitro. Calcif Tissue Int. 78(2):98-102.

Morsczeck C, Gotz W, Schierholz J, Zeilhofer F, Kuhn U, Mohl C, Sippel C, Hoffmann KH. 2005a. Isolation of precursor cells (pcs) from human dental follicle of wisdom teeth. Matrix Biol. 24(2):155-165.

Morsczeck C, Moehl C, Gotz W, Heredia A, Schaffer TE, Eckstein N, Sippel C, Hoffmann KH. 2005b. In vitro differentiation of human dental follicle cells with dexamethasone and insulin. Cell Biol Int. 29(7):567-575.

Morsczeck C, Schmalz G, Reichert TE, Vollner F, Saugspier M, Viale-Bouroncle S, Driemel O. 2009. Gene expression profiles of dental follicle cells before and after osteogenic differentiation in vitro. Clin Oral Investig. 13(4):383-391.

Moxham BJ, Berkovitz BK. 1984. The mobility of the lathyritic rabbit mandibular incisor in response to axially-directed extrusive loads. Arch Oral Biol. 29(10):773-778.

Nagpal A, Sharma G, Sarkar A, Pai KM. 2005. Eruption disturbances: An aetiological-cum-management perspective. Dentomaxillofac Radiol. 34(1):59-63.

Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B. 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell. 108(1):17-29.

Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ, Bonewald LF, Kodama T, Wutz A, Wagner EF et al. 2011. Evidence for osteocyte regulation of bone homeostasis through rankl expression. Nat Med. 17(10):1231-1234.

Naoumova J, Kjellberg H, Palm R. 2014. Cone-beam computed tomography for assessment of palatal displaced canine position: A methodological study. Angle Orthod. 84(3):459-466.

Naoumova J, Kurol J, Kjellberg H. 2011. A systematic review of the interceptive treatment of palatally displaced maxillary canines. Eur J Orthod. 33(2):143-149.

Naoumova J, Kurol J, Kjellberg H. 2015. Extraction of the deciduous canine as an interceptive treatment in children with palatal displaced canines - part i: Shall we extract the deciduous canine or not? Eur J Orthod. 37(2):209-218.

Olive RJ. 2005. Factors influencing the non-surgical eruption of palatally impacted canines. Aust Orthod J. 21(2):95-101.

Oliver RG, Mannion JE, Robinson JM. 1989. Morphology of the maxillary lateral incisor in cases of unilateral impaction of the maxillary canine. Br J Orthod. 16(1):9-16.

Oury F. 2012. A crosstalk between bone and gonads. Ann N Y Acad Sci. 1260:1-7.

Park BW, Kang EJ, Byun JH, Son MG, Kim HJ, Hah YS, Kim TH, Mohana Kumar B, Ock SA, Rho GJ. 2012. In vitro and in vivo osteogenesis of human mesenchymal stem cells derived from skin, bone marrow and dental follicle tissues. Differentiation. 83(5):249-259.

Peck S, Peck L, Kataja M. 1994. The palatally displaced canine as a dental anomaly of genetic origin. Angle Orthod. 64(4):249-256.

Peck S, Peck L, Kataja M. 2002. Concomitant occurrence of canine malposition and tooth agenesis: Evidence of orofacial genetic fields. Am J Orthod Dentofacial Orthop. 122(6):657-660.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time rt-pcr. Nucleic Acids Res. 29(9):e45.

Philbrick WM, Dreyer BE, Nakchbandi IA, Karaplis AC. 1998. Parathyroid hormone-related protein is required for tooth eruption. Proc Natl Acad Sci U S A. 95(20):11846-11851.

Power SM, Short MB. 1993. An investigation into the response of palatally displaced canines to the removal of deciduous canines and an assessment of factors contributing to favourable eruption. Br J Orthod. 20(3):215-223.

Prideaux M, Findlay DM, Atkins GJ. 2016. Osteocytes: The master cells in bone remodelling. Curr Opin Pharmacol. 28:24-30.

Proffit WR, Frazier-Bowers SA. 2009. Mechanism and control of tooth eruption: Overview and clinical implications. Orthod Craniofac Res. 12(2):59-66.

Proffit WR, Vig KW. 1981. Primary failure of eruption: A possible cause of posterior open-bite. Am J Orthod. 80(2):173-190.

Quarles LD, Yohay DA, Lever LW, Caton R, Wenstrup RJ. 1992. Distinct proliferative and differentiated stages of murine mc3t3-e1 cells in culture: An in vitro model of osteoblast development. J Bone Miner Res. 7(6):683-692.

Que BG, Wise GE. 1997. Colony-stimulating factor-1 and monocyte chemotactic protein-1 chemotaxis for monocytes in the rat dental follicle. Arch Oral Biol. 42(12):855-860.

Raghoebar GM, Boering G, Jansen HW, Vissink A. 1989. Secondary retention of permanent molars: A histologic study. J Oral Pathol Med. 18(8):427-431.

Raghoebar GM, Boering G, Vissink A, Stegenga B. 1991. Eruption disturbances of permanent molars: A review. J Oral Pathol Med. 20(4):159-166.

Ransjo M, Sahli J, Lie A. 2003. Expression of connexin 43 mrna in microisolated murine osteoclasts and regulation of bone resorption in vitro by gap junction inhibitors. Biochem Biophys Res Commun. 303(4):1179-1185.

Reffitt DM, Ogston N, Jugdaohsingh R, Cheung HF, Evans BA, Thompson RP, Powell JJ, Hampson GN. 2003. Orthosilicic acid stimulates collagen type 1 synthesis and

References

osteoblastic differentiation in human osteoblast-like cells in vitro. Bone. 32(2):127-135.

Repetto G, del Peso A, Zurita JL. 2008. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Protoc. 3(7):1125-1131.

Risom L, Christoffersen L, Daugaard-Jensen J, Hove HD, Andersen HS, Andresen BS, Kreiborg S, Duno M. 2013. Identification of six novel pth1r mutations in families with a history of primary failure of tooth eruption. PLoS One. 8(9):e74601.

Roca H, Phimphilai M, Gopalakrishnan R, Xiao G, Franceschi RT. 2005. Cooperative interactions between runx2 and homeodomain protein-binding sites are critical for the osteoblast-specific expression of the bone sialoprotein gene. J Biol Chem. 280(35):30845-30855.

Rozylo-Kalinowska I, Kolasa-Raczka A, Kalinowski P. 2011. Dental age in patients with impacted maxillary canines related to the position of the impacted teeth. Eur J Orthod. 33(5):492-497.

Sacerdoti R, Baccetti T. 2004. Dentoskeletal features associated with unilateral or bilateral palatal displacement of maxillary canines. Angle Orthod. 74(6):725-732.

Sajnani A, King N. 2012a. Dental age of children and adolescents with impacted maxillary canines. J Orofac Orthop. 73(5):359-364.

Sajnani AK, King NM. 2012b. Early prediction of maxillary canine impaction from panoramic radiographs. Am J Orthod Dentofacial Orthop. 142(1):45-51.

Sajnani AK, King NM. 2012c. The sequential hypothesis of impaction of maxillary canine - a hypothesis based on clinical and radiographic findings. J Craniomaxillofac Surg. 40(8):e375-385.

Sampaziotis D, Tsolakis IA, Bitsanis E, Tsolakis AI. 2017. Open versus closed surgical exposure of palatally impacted maxillary canines: Comparison of the different treatment outcomes-a systematic review. Eur J Orthod.

Saugspier M, Felthaus O, Viale-Bouroncle S, Driemel O, Reichert TE, Schmalz G, Morsczeck C. 2010. The differentiation and gene expression profile of human dental follicle cells. Stem cells and development. 19(5):707-717.

Schmuth GP, Freisfeld M, Koster O, Schuller H. 1992. The application of computerized tomography (ct) in cases of impacted maxillary canines. Eur J Orthod. 14(4):296-301.

Schroeder HE, Listgarten MA. 1971. Fine structure of the developing epithelial attachment of human teeth. Monogr Dev Biol. 2:1-134.

Schroeder HE, Luder HU, Bosshardt DD. 1992. Morphological and labeling evidence supporting and extending a modern theory of tooth eruption. Schweiz Monatsschr Zahnmed. 102(1):20-31.

Schwarz K. 1973. A bound form of silicon in glycosaminoglycans and polyuronides. Proc Natl Acad Sci U S A. 70(5):1608-1612.

Schwarz K, Milne DB. 1972. Growth-promoting effects of silicon in rats. Nature. 239(5371):333-334.

Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, Young M, Robey PG, Wang CY, Shi S. 2004. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet. 364(9429):149-155.

Shapira Y, Kuftinec MM. 1998. Early diagnosis and interception of potential maxillary canine impaction. J Am Dent Assoc. 129(10):1450-1454.

Shields ED, Bixler D, el-Kafrawy AM. 1973. A proposed classification for heritable human dentine defects with a description of a new entity. Arch Oral Biol. 18(4):543-553.

Shoi K, Aoki K, Ohya K, Takagi Y, Shimokawa H. 2014. Characterization of pulp and follicle stem cells from impacted supernumerary maxillary incisors. Pediatr Dent. 36(3):79-84.

Sicher H. 1942. Tooth eruption: Axial movement of teeth with limited growth. J Dent Res. 21(4):395-402.

Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T et al. 1997. Osteoprotegerin: A novel secreted protein involved in the regulation of bone density. Cell. 89(2):309-319.

Soltanoff CS, Yang S, Chen W, Li YP. 2009. Signaling networks that control the lineage commitment and differentiation of bone cells. Crit Rev Eukaryot Gene Expr. 19(1):1-46.

Sonoyama W, Liu Y, Yamaza T, Tuan RS, Wang S, Shi S, Huang GT. 2008. Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: A pilot study. J Endod. 34(2):166-171.

Sorensen HB, Artmann L, Larsen HJ, Kjaer I. 2009. Radiographic assessment of dental anomalies in patients with ectopic maxillary canines. Int J Paediatr Dent. 19(2):108-114.

Stains JP, Watkins MP, Grimston SK, Hebert C, Civitelli R. 2014. Molecular mechanisms of osteoblast/osteocyte regulation by connexin43. Calcif Tissue Int. 94(1):55-67.

Stanford CM, Jacobson PA, Eanes ED, Lembke LA, Midura RJ. 1995. Rapidly forming apatitic mineral in an osteoblastic cell line (umr 106-01 bsp). J Biol Chem. 270(16):9420-9428.

Sun H, Li Q, Zhang Y, Bi Y, Li X, Shu Y, Chen X, Jin Z, Ge C. 2015. Regulation of opg and rankl expressed by human dental follicle cells in osteoclastogenesis. Cell Tissue Res. 362(2):399-405.

References

Syed-Picard FN, Jayaraman T, Lam RS, Beniash E, Sfeir C. 2013. Osteoinductivity of calcium phosphate mediated by connexin 43. Biomaterials. 34(15):3763-3774.

Tadjoedin ES, de Lange GL, Holzmann PJ, Kulper L, Burger EH. 2000. Histological observations on biopsies harvested following sinus floor elevation using a bioactive glass material of narrow size range. Clin Oral Implants Res. 11(4):334-344.

Tamaki Y, Nakahara T, Ishikawa H, Sato S. 2013. In vitro analysis of mesenchymal stem cells derived from human teeth and bone marrow. Odontology. 101(2):121-132.

Ten Cate A. 1985. Oral histology: Development, structure and function St. Louis, Toronto: Mosby.

Teti A. 2011. Bone development: Overview of bone cells and signaling. Curr Osteoporos Rep. 9(4):264-273.

Thilander B. 2009. Dentoalveolar development in subjects with normal occlusion. A longitudinal study between the ages of 5 and 31 years. Eur J Orthod. 31(2):109-120.

Thilander B, Jakobsson SO. 1968. Local factors in impaction of maxillary canines. Acta Odontol Scand. 26(2):145-168.

Thilander B, Myrberg N. 1973. The prevalence of malocclusion in swedish schoolchildren. Scand J Dent Res. 81(1):12-21.

Tronstad L. 1988. Root resorption--etiology, terminology and clinical manifestations. Endod Dent Traumatol. 4(6):241-252.

Vaananen HK, Laitala-Leinonen T. 2008. Osteoclast lineage and function. Arch Biochem Biophys. 473(2):132-138.

Van Hassel HJ, McMinn RG. 1972. Pressure differential favouring tooth eruption in the dog. Arch Oral Biol. 17(1):183-190.

Van Wesenbeeck L, Odgren PR, MacKay CA, D'Angelo M, Safadi FF, Popoff SN, Van Hul W, Marks SC, Jr. 2002. The osteopetrotic mutation toothless (tl) is a loss-of-function frameshift mutation in the rat csf1 gene: Evidence of a crucial role for csf-1 in osteoclastogenesis and endochondral ossification. Proc Natl Acad Sci U S A. 99(22):14303-14308.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative rt-pcr data by geometric averaging of multiple internal control genes. Genome Biol. 3(7):RESEARCH0034.

Viale-Bouroncle S, Felthaus O, Schmalz G, Reichert TE, Morsczeck C. 2013. Transcription factors for dental stem cell differentiation. Int J Oral Maxillofac Implants. 28(6):e478-486.

Vollkommer T, Gosau M, Felthaus O, Reichert TE, Morsczeck C, Gotz W. 2015. Genome-wide gene expression profiles of dental follicle stem cells. Acta Odontol Scand. 73(2):93-100.

Vollner F, Ernst W, Driemel O, Morsczeck C. 2009. A two-step strategy for neuronal differentiation in vitro of human dental follicle cells. Differentiation. 77(5):433-441.

Walker L, Enciso R, Mah J. 2005. Three-dimensional localization of maxillary canines with cone-beam computed tomography. Am J Orthod Dentofacial Orthop. 128(4):418-423.

Warford JH, Jr., Grandhi RK, Tira DE. 2003. Prediction of maxillary canine impaction using sectors and angular measurement. Am J Orthod Dentofacial Orthop. 124(6):651-655.

Watkins M, Grimston SK, Norris JY, Guillotin B, Shaw A, Beniash E, Civitelli R. 2011. Osteoblast connexin43 modulates skeletal architecture by regulating both arms of bone remodeling. Mol Biol Cell. 22(8):1240-1251.

Weinmann JP. 1944. Bone changes related to eruption of the teeth. The Angle Orthodontist. 11(2):83-99.

Wise GE, Ding D, Yao S. 2004. Regulation of secretion of osteoprotegerin in rat dental follicle cells. Eur J Oral Sci. 112(5):439-444.

Wise GE, Fan W. 1989. Changes in the tartrate-resistant acid phosphatase cell population in dental follicles and bony crypts of rat molars during tooth eruption. J Dent Res. 68(2):150-156.

Wise GE, He H, Gutierrez DL, Ring S, Yao S. 2011. Requirement of alveolar bone formation for eruption of rat molars. Eur J Oral Sci. 119(5):333-338.

Wise GE, Lin F, Fan W. 1992. Culture and characterization of dental follicle cells from rat molars. Cell Tissue Res. 267(3):483-492.

Wise GE, Lin F, Zhao L. 1995. Transcription and translation of csf-1 in the dental follicle. J Dent Res. 74(9):1551-1557.

Wise GE, Lumpkin SJ, Huang H, Zhang Q. 2000a. Osteoprotegerin and osteoclast differentiation factor in tooth eruption. J Dent Res. 79(12):1937-1942.

Wise GE, Marks SC, Jr., Cahill DR. 1985. Ultrastructural features of the dental follicle associated with formation of the tooth eruption pathway in the dog. J Oral Pathol. 14(1):15-26.

Wise GE, Que BG, Huang H, Lumpkin SJ. 2000b. Enhancement of gene expression in rat dental follicle cells by parathyroid hormone-related protein. Arch Oral Biol. 45(10):903-909.

Wise GE, Ren Y, Yao S. 2003. Regulation of osteoprotegerin gene expression in dental follicle cells. J Dent Res. 82(4):298-302.

Wise GE, Yao S. 2003. Expression of tumour necrosis factor-alpha in the rat dental follicle. Arch Oral Biol. 48(1):47-54.

References

Wise GE, Yao S. 2006. Regional differences of expression of bone morphogenetic protein-2 and rankl in the rat dental follicle. Eur J Oral Sci. 114(6):512-516.

Wise GE, Yao S, Henk WG. 2007. Bone formation as a potential motive force of tooth eruption in the rat molar. Clin Anat. 20(6):632-639.

Wise GE, Yao S, Odgren PR, Pan F. 2005. Csf-1 regulation of osteoclastogenesis for tooth eruption. J Dent Res. 84(9):837-841.

Wise GE, Yao S, Zhang Q, Ren Y. 2002. Inhibition of osteoclastogenesis by the secretion of osteoprotegerin in vitro by rat dental follicle cells and its implications for tooth eruption. Arch Oral Biol. 47(3):247-254.

Yang H, Li J, Sun J, Guo W, Li H, Chen J, Hu Y, Tian W, Li S. 2017. Cells isolated from cryopreserved dental follicle display similar characteristics to cryopreserved dental follicle cells. Cryobiology. 78:47-55.

Yao S, Liu D, Pan F, Wise GE. 2006. Effect of vascular endothelial growth factor on rank gene expression in osteoclast precursors and on osteoclastogenesis. Arch Oral Biol. 51(7):596-602.

Yao S, Norton J, Wise GE. 2004. Stability of cultured dental follicle cells. Cell Prolif. 37(3):247-254.

Yao S, Pan F, Prpic V, Wise GE. 2008. Differentiation of stem cells in the dental follicle. J Dent Res. 87(8):767-771.

Yao S, Pan F, Wise GE. 2007. Chronological gene expression of parathyroid hormone-related protein (pthrp) in the stellate reticulum of the rat: Implications for tooth eruption. Arch Oral Biol. 52(3):228-232.

Yao S, Prpic V, Pan F, Wise GE. 2010. Tnf-alpha upregulates expression of bmp-2 and bmp-3 genes in the rat dental follicle--implications for tooth eruption. Connect Tissue Res. 51(1):59-66.

Young MF. 2003. Bone matrix proteins: Their function, regulation, and relationship to osteoporosis. Osteoporos Int. 14 Suppl 3:S35-42.

Yu X, Tang X, Gohil SV, Laurencin CT. 2015. Biomaterials for bone regenerative engineering. Adv Healthc Mater. 4(9):1268-1285.

Zappitelli T, Aubin JE. 2014. The "connexin" between bone cells and skeletal functions. J Cell Biochem. 115(10):1646-1658.

Zhao M, Xiao G, Berry JE, Franceschi RT, Reddi A, Somerman MJ. 2002. Bone morphogenetic protein 2 induces dental follicle cells to differentiate toward a cementoblast/osteoblast phenotype. J Bone Miner Res. 17(8):1441-1451.

APPENDIX

I.   **Uribe P,** Ransjö M, and Westerlund A. Clinical predictors of maxillary canine impaction: a novel approach using multivariate analysis. European Journal of Orthodontics, 2017, 153–160. doi: 10.1093/ejo/cjw042.

II.   **Uribe P,** Larsson L, Westerlund A, and Ransjö M. Gene expression profiles in dental follicles from patients with impacted canines. *Submitted for publication*

III.   **Uribe P,** Plakwicz P, Larsson L, Czochrowska E, Westerlund A, and Ransjö M. Local patterns of regulatory factors expressed in human dental follicles. *Submitted for publication*

IV.   **Uribe P,** Johansson A, Westerlund A, Larsson L, Magnusson C, and Ransjö M. Effect of soluble Silica on Cx43 gap junction communication and osteogenic differentiation in human dental follicle cells. *In manuscript*