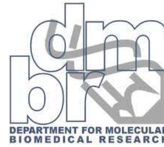

THE CADHERIN-CATENIN COMPLEX DURING DEVELOPMENT AND REPLACEMENT OF TEETH IN THE ZEBRAFISH (*DANIO RERIO*)

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Thesis submitted to obtain the degree of
DOCTOR IN SCIENCES: BIOLOGY
Academic year 2011-2012

Dit werk werd mogelijk gemaakt door een beurs van het Instituut voor de Aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen (IWT-Vlaanderen).

This work was supported by a grant of the Institute of Promotion of Innovation through Science and Technology in Flanders (IWT-Flanders).

DANKWOORD

Wie mij aan het begin van mijn studie ‘Licentiaat in de Biologie’ had gezegd dat ik 8 jaar later ‘Doctor in de Biologie’ zou zijn, had ik eens raar aangekeken. Graag wil ik hier enkele mensen bedanken die er deze hele fijne tijd altijd voor mij waren.

Ik zou graag willen beginnen met het bedanken van mijn promotoren: Ann en Jolanda.
Ann, bedankt voor de afgelopen vier jaar. Ik ben met heel veel plezier een deel geweest van je lab. Door de vrijheid die je mij gaf, ben ik kunnen worden wie nu ben. Maar als het nodig was, was je er altijd om dingen te bespreken en bij te sturen. Ook en vooral tijdens deze laatste periode is jouw input voor mij van heel groot belang geweest, op alle vlakken.

Jolanda, ook jij heel hard bedankt. Niet alleen voor de laatste vier jaar maar ook om mij als master student te laten proeven van moleculair werk. Zonder die aanzet had ik hier niet gestaan. Ik mocht altijd langskomen en jullie labo gebruiken alsof ik er elke dag werkte. Een hele fijne en leerrijke omgeving.

Dit proefschrift heb ik niet alleen gemaakt. Elke dag ben ik met veel plezier komen werken en dit door een hele fijne groep van collega’s die dit gebouw telt!
Sam, Jeroen, Jasper, Anabela en Tho: bedankt voor de vele fijne momenten, zowel op werk als prive vlak. Speciale dank voor Jeroen en Sam die ik nooit niets kon misvragen, zeker niet tijdens deze laatste maanden vol figuur- en Photoshop-‘plezier’!

Ook onze laboranten verdienen een dikke pluim. Zonder hun technische expertise en ongelofelijk vermogen voor het maken van histologisch perfecte coupes, zou dit proefschrift en de gepubliseerde manuscripten niet van deze kwaliteit zijn. Alle eer gaat hiervoor dus naar Tommy, Ellen, Mieke en Dennis!

De rest van de derde verdieping is natuurlijk ook niet te verwaarlozen. Door de ‘social events’ leerden we elkaar allemaal ook buiten het werk kennen: kwissen, films, eten,... . Echt een hele toffe bende allemaal samen!

Hanne en Pam, een meer dan welverdiende speciale vermelding voor deze twee top-madammen! Altijd klaar met woord en daad (en leesbril). Hanne, meer dan alleen mijn trein-mate en Pam, meer dan mijn volley/groententuin-

mate! Bedankt dat jullie er ook voor mij waren tijdens de moeilijkere momenten.

Niet enkel op de Ledeganck was ik actief, ook in Zwijnaarde kwam ik af en toe over de labo-vloer. Daarom bedank ik ook graag het hele team van Frans van Roy en Jolanda van Hengel voor de fijne werksfeer daar!

En dan is er natuurlijk nog het thuisfront.
Eerst en vooral wil ik mijn mama en papa bedanken om mij al deze kansen te geven en onvoorwaardelijk in mij te geloven. Jullie zijn er altijd voor mij en dat doet ongelofelijk goed. Mama, die positieve ingesteldheid en dat babbeltje betekenen zoveel. Bedankt voor jullie warme nest!

In dat nest zat ik niet alleen: sissie, bedankt om gewoon mijn zusje te zijn. Altijd fijne en zotte momenten samen, goed lachen en regelmatig shoppen hebben ervoor gezorgd dat ik niet op een boomknuffelaar ben gaan lijken en met de beide voetjes op de grond ben gebleven.

Het nest is in de loop der jaren wat uitgebreid en daarom is het niet meer dan normaal dat ik ook mijn 'schijnpapa' Peter, Sam, Inge, Vince en Carlos, Thijs, Valerie, Arne en mijn toekomstig metekindje en nog eens een Peter bedank. Omdat het nest nog toffer en gezelliger is met jullie allemaal erbij! Heel veel bewondering en hele grote dank aan Peter voor de fantastisch geslaagde zebravis tekening die mijn cover siert. Juist zoals ik hem wou!

Ook bobon wil ik bedanken. Je kon dit net niet meer meemaken maar ik ben ervan overtuigd dat jij ergens samen met pépé fier bent. Dit is dan ook een beetje voor jullie.

Mieke en Freddy, Bart en Jessica en Pieter en Justine ook jullie bedankt voor de fijne momenten en goeie zorgen tijdens de afgelopen jaren.

En dan is er Jan, een aparte categorie. Zonder jou had ik dit nooit gekund. Jouw positieve ingesteldheid en onvoorwaardelijke liefde voor mij en het leven hebben mij op alle vlakken een beter mens gemaakt. Je bent er altijd voor mij zowel op de zonnige dagen als op de dagen dat het ietsje minder gaat. Er zijn geen woorden om jou te bedanken. Ik kijk er al enorm naar uit om samen met jou ons nestje uit te breiden!

The cadherin-catenin complex during development and replacement of teeth in the zebrafish (*Danio rerio*)

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TABLE OF CONTENT

LIST OF FIGURES **ix**

LIST OF TABLES **xi**

LIST OF ABBREVIATIONS **xii**

SECTION I INTRODUCTION **1**

CHAPTER I: GENERAL INTRODUCTION **1**

1. TOOTH DEVELOPMENT 1

1.1. Introduction **1**

1.2. Tooth development can be subdivided into multiple stages **2**

1.2.1. Tooth development starts morphologically with the formation of an epithelial placode 6

1.2.2. Morphogenesis stage is marked by the invagination of the epithelial cells into the underlying mesenchyme 10

1.2.3. Cytodifferentiation involves ameloblasts, odontoblasts and matrix deposition 12

1.2.4. Attachment makes the tooth functional and elicits the formation of a next tooth generation 14

1.3. Tooth development is regulated by several signalling pathways **17**

2. TEETH AS INTEGUMENTAL APPENDAGES 20

3. THE CADHERIN-CATENIN COMPLEX..22

CHAPTER II: LITERATURE REVIEW **25**

1. CELL ADHESION AND THE CADHERIN-CATENIN COMPLEX............................25

1.1. Introduction to intercellular adhesion **25**

1.1.1. Tight junctions are responsible for intercellular sealing 27

1.1.2. Desmosome plaques provide adhesion strength by linking to the intermediate filaments 30

1.1.3. Adherens junctions: a combination of two basic units 31

1.2. Classical cadherin-based adherens junctions: structure and organization **33**

1.3. Cadherin-mediated cell adhesion is regulated in many ways **35**

1.3.1. By interaction with β-catenin 35

1.3.2. Through α-catenin activity 36

1.3.3. By endocytosis and recycling 38

1.4. Cadherins and their involvement in signalling pathways **39**

1.4.1. Introduction 39

1.4.2. Cadherins can form signalling units by interacting with several binding partners 41

1.4.3. Cleavage of the ectodomain or cytoplasmic tail of cadherins can mediate signalling pathways 42

1.4.4. Cell adhesion molecules as a component of a mechanosensory complex 43

2. BETA-CATENIN AND PLAKOGLOBIN..44

2.1. β-catenin **44**

2.1.1. Introduction 44

2.1.2. β-catenin switches between cell adhesion and canonical Wnt signalling 45

2.1.3. The importance of β-catenin during development 48

2.1.4. β-catenin in zebrafish 49

2.2. Plakoglobin **51**

2.2.1. Introduction 51

2.2.2. Convergence of plakoglobin and β-catenin during Wnt signalling 53

2.2.3. The importance of plakoglobin during development 53

2.2.4. Plakoglobin in zebrafish 54

3. P120CATENIN 55

3.1. Introduction **55**

3.2. p120ctn regulates cadherin stability and trafficking **57**

3.3. p120ctn regulates Rho GTPases **60**

3.4. p120ctn interacts with Kaiso and regulates transcription **62**

3.5. p120ctn and knockout studies **63**

4. CELL ADHESION IN INTEGUMENTAL APPENDAGES 67

4.1. Morphogenesis of integumental appendages **67**

4.1.1. Mammary gland development 67

4.1.2. Salivary gland development 69

4.1.3. Hair follicle development 70

4.1.4. Tooth development 71

4.2. Cell adhesion molecules in integumental appendages **73**

4.2.1. Cell adhesion molecules in the mammary gland 73

4.2.2. Cell adhesion molecules in the salivary gland 76

4.2.3. Cell adhesion in hair development 78

4.2.4. Cell adhesion during tooth development 81

4.3. Conclusion **86**

SECTION II RESEARCH AIMS **88**

SECTION III MATERIAL & METHODS 92

1. INTRODUCTION 93

2. MATERIAL 93

3. CONCISE OVERVIEW OF METHODS USED 94

4. WHOLE MOUNT IMMUNOHISTOCHEMISTRY AND IN SITU HYBRIDIZATION OF LARVAL AND ADULT ZEBRAFISH DENTAL TISSUES 96

4.1. Summary 96

4.2. Introduction 97

4.3 Materials 100

4.3.1. Anaesthesia 100

4.3.2. Fixation of zebrafish embryos, larvae and adults 100

4.3.3. Depigmentation 100

4.3.4. Whole mount immunohistochemistry 100

4.3.5. Whole mount *in situ* hybridization 101

4.3.6. Epon embedding 103

4.4. Methods 103

4.4.1. Fixation of zebrafish embryos, larvae and dissected pharyngeal jaws of juveniles and adults 103

4.4.2. Depigmentation 104

4.4.3.Whole mount immunohistochemistry 104

4.4.4. Whole mount *in situ* hybridization 106

4.4.5. Epon embedding 108

4.5. Notes 111

4.6. Acknowledgements 114

SECTION IV RESULTS 115

1. ZEBRAFISH TEETH AS A MODEL FOR REPETITIVE EPITHELIAL MORPHOGENESIS: DYNAMICS OF E-CADHERIN EXPRESSION 117

1.1. Abstract 118

1.2. Background 119

1.3. Results 123

1.3.1. Distribution of E-cadherin during the development of first-generation teeth 123

1.3.2. Distribution of E-cadherin during the development of replacement teeth 127

1.4. Discussion 133

1.5. Conclusions 138

1.6. Methods 139

1.6.1. Fish strains 139

1.6.2. Tissue processing 139

1.6.3. Immunohistochemistry 140

1.6.4. Probe synthesis and *in situ* hybridization 140

1.7. Authors' contributions 141

1.8. Acknowledgements 141

2. N-CADHERIN EXPRESSION AND DISTRIBUTION DURING TOOTH DEVELOPMENT AND TOOTH REPLACEMENT IN THE ZEBRAFISH 142

2.1. Abstract 143

2.2. Introduction 144

2.3. Results 148

2.3.1. N-cadherin expression during tooth development and replacement 148

2.3.2. Tooth development in N-cadherin deficient *Parachute* zebrafish mutants 153

2.4. Discussion 155

2.5. Material and Methods 160

2.5.1. Zebrafish collection 160

2.5.2. Toluidine blue staining of *Parachute* mutants 160

2.5.3. Tissue processing 161

2.5.4. Immunohistochemistry and *in situ* hybridization 161

2.6. Aknowledgements 162

3. BETA-CATENIN AND PLAKOGLOBIN EXPRESSION DURING TOOTH DEVELOPMENT AND TOOTH REPLACEMENT 163

3.1. Abstract 164

3.2. Introduction 165

3.3. Results 169

3.3.1. Distribution of plakoglobin during zebrafish tooth development and tooth replacement 169

3.3.2. Tooth development in plakoglobin morphant zebrafish 174

3.3.3. Distribution of β-catenin during zebrafish tooth development and tooth replacement 176

3.4. Discussion 179

3.5. Material and Methods 186

3.5.1. Zebrafish collection 186

3.5.2. Plakoglobin morphant zebrafish 186

3.5.3. Histological analysis of plakoglobin morphants 186

3.5.4. Immunohistochemistry and *in situ* hybridization 187

3.6. Acknowledgments 188

4. Molecular cloning and developmental expression of p120catenin (Ctnnd1) in zebrafish 189

4.1. Abstract 190

4.2. Introduction 191

4.3. Results 195

4.3.1. zfp120ctn mRNA expression throughout the different stages of zebrafish tooth development 195

4.3.2. Overexpression of zf p120catenin in MCF7 cells 198

4.4. Discussion 200

4.5. Material and Methods 203

4.5.1. Zebrafish 203

4.5.2. Probe design 203

4.5.3. Whole mount *in situ* hybridization 204

4.5.4. Generation of polyclonal ctnnd1 antibody 204

4.5.5. MCF7 cell culture, transfection and immunostainings 204

4.6. Acknowledgements 205

Appendix 206

Section V General Discussion and Perspectives 214

Section VI Summary 234

English Summary 235

Nederlandse samenvatting 242

Section VII References 250

SECTION VIII CURRICULUM VITAE 289

PUBLICATION LIST ... 290

CONTRIBUTION TO INTERNATIONAL CONFERENCES: ... 290

LIST OF FIGURES

Figure 1. Comparison of homologous stages of tooth development between zebrafish and mouse

Figure 2. The zebrafish dentition

Figure 3. Schematic representation of the different stages of zebrafish tooth development combined with histological sections and orientation

Figure 4. Schematic representation of a transverse section through the pharyngeal epithelium and mesenchyme of a left tooth placode showing zebrafish tooth gene expression

Figure 5. The sequential and reciprocal regulatory signalling interactions between the epithelium and mesenchyme in the mouse dentition

Figure 6. Schematic representation of the cadherin-catenin complex between two neighbouring epithelial cells

Figure 7. Schematic representation of cell-cell adhesion complexes

Figure 8. Biosynthesis, endocytosis and recycling of E-cadherin

Figure 9. Overview of the signalling pathways regulated by cadherins

Figure 10. β-catenin primary structure

Figure 11. Simple overview of the canonical Wnt signalling pathway

Figure 12. Plakoglobin primary structure

Figure 13. Schematic representation of the human p120catenin protein

Figure 14. Overview of p120ctn in cadherin trafficking

Figure 15. Different functions of p120ctn in the cell

Figure 16. Schematic overview of the key developmental steps of four major integumental appendages

Figure 17. Orientation of plane of sectioning

Figure 18. Whole mount immunohistochemistry and whole mount *in situ* hybridization results obtained using the protocols described in this chapter

Figure 19. Expression pattern of E-cadherin in first-generation teeth

Figure 20. Uneven distribution of E-cadherin in the crypt epithelium

Figure 21. E-cadherin expression pattern during tooth replacement

Figure 22. Beta-catenin immunolocalization in the successional lamina

Figure 23. N-cadherin protein distribution during development of first-generation teeth

Figure 24. N-cadherin protein distribution during tooth replacement in an adult zebrafish

Figure 25. N-cadherin and MMP-13 are both expressed in differentiating odontoblasts

Figure 26. Semithin toluidine blue stained section of the pharyngeal region of *pac*^{tm101b} mutant zebrafish

Figure 27. Plakoglobin distribution during the development of first-generation teeth

Figure 28. The distribution of plakoglobin during later-generation teeth

Figure 29. Comparison of control and plakoglobin morpholino-injected zebrafish at different times during tooth development

Figure 30. β-catenin distribution during the development of later-generation teeth

Figure 31. mRNA expression of zebrafish p120 catenin during development of first-generation teeth and the first replacement tooth

Figure 32. Overexpression of zebrafish p120ctn tagged to a Myc-epitope in MCF-7 cell line

Figure 33. Predicted amino acid sequence of zebrafish p120ctn

Figure 34. RT-PCR experiments used to examine alternative splicing events in zebrafish p120ctn mRNA

Figure 35. Schematic overview of the alternative splicing of exon 21 and the use of a splice donor sequence within exon 21

Figure 36. Analysis by RT-PCR of alternative splicing events occcuring in the 3’ region of the zebrafish p120ctn mRNA

Figure 37. Overview of expression patterns of the cell adhesion molecules analyzed in this PhD thesis during the different stages of both first-generation and later-generation teeth

LIST OF TABLES

Table 1. Overview of concentration and duration of proteinase K (PK) treatment in relation to larval age (in hours or days post-fertilization, hpf and dpf, resp.) and dissected pharyngeal jaws of juvenile and adult zebrafish

Table 2. Overview of expression of E-cadherin in the different cell layers of a first-generation tooth on the basis of mRNA and protein distribution

Table 3. Overview of expression of E-cadherin in the different cell layers of a replacement tooth

Table 4. Relative expression of the different zebrafish p120ctn mRNA isoforms

LIST OF ABBREVIATIONS

AJs	adherens junctions
APC	adenomatous polyposis coli
Arm	armadillo
ARVCF	armadillo repeat gene deleted in velo-cardio-facial syndrome
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BMP	bone morphogenetic protein
bp	base pair
BSA	bovine serum albumin
BTB	broad complex, tramtrak and bric à brac
CKII	casein kinase II
cKO	conditional knockout
ctnnb1	β-catenin-1
ctnnb2	β-catenin-2
D	dorsal row
DAB	diaminobenzidine
DIG	digoxigenin
dkk	dickkopf
DMF	dimethylformamide
DMSO	dimethylsulfoxide
dpf	days post-fertilization
DSG-1	desmoglein-1
E	embryonic day
EC	ectodomain
EC1	extracellular domain
ED	early cytodifferentation
EM	early morphogenesis
ER	endoplasmatic reticulum
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GEFs	guanine exchange factors
GSK3	glycogen synthase kinase 3β
hpf	hours post-fertilization
HRP	horseradish peroxidase

IDE	inner dental epithelium
IP	immunoprecipitation
IRS	inner root sheet
JAM-C	junctional adhesion molecule C
JMD	juxtamembrane domain
KO	knockout
LD	late cytodifferentation
LEF	lymphoid enhancer factor
LM	late morphogenesis
MD	medio-dorsal row
MMP	matrix metalloproteinase
MMTV	mouse mammary tumor virus
MO	morpholino
MS222	3-aminobenzoic acid ethyl ester
NBT	nitro blue tetrazolium
NES	nuclear export signal
NLS	nuclear localization signal
ODE	outer dental epithelium
ORS	outer root sheet
p120ctn	p120 catenin
pac	parachute
PBS	phosphate buffered saline
PECAM	platelet endothelial cell adhesion molecule
PFA	paraformaldehyde
PI3K	phosphoinositide 3-kinase
PK	proteinase K
PM	plasma membrane
POZ	pox virus zinc finger
RhoGAP	Rho GTPase activating protein
RNAi	RNA interference
RT	room temperature
RTK	receptor Tyr kinase
shh	sonic hedgehog
SI	stratum intermedium
SR	stellate reticulum
Src	sarcoma
TCF	T-cell factor

TEA	triethanolamine
TGFβ	transforming growth factor-β
TJs	tight junctions
V	ventral row
VEGFR2	vascular endothelial growth factor receptor 2
WB	western blots
Wnt	wingless
Wt	wild-type
zfp120ctn	zebrafish p120 catenin
ZO	zonula occludens

SECTION I

INTRODUCTION

CHAPTER I: GENERAL INTRODUCTION

1. TOOTH DEVELOPMENT

1.1. Introduction

The vertebrate dentition is predominantly studied using the mouse as an animal model. These studies have provided information on many levels including the genetic control of tooth development (Järvinen et al., 2006; Thesleff, 2006; Tummers and Thesleff, 2009), underlying mechanisms of evolutionary differences in tooth shape, reduction and tooth loss (Harada and Ohshima, 2004; Jernvall and Thesleff, 2000). Yet, mouse molars are permanent teeth and their incisors are continuously growing, which makes this model organism unsuitable for studying tooth replacement. Over the years, more interest has been gained for tooth development in other species such as for example the zebrafish, *Danio rerio*. Other non-mammalian species have also become the subject of studies concerning the dentition (Huysseune et al., 2007; Shaw, 1979; Sire et al., 2002; Stembirek et al., 2010). Not only has tooth development in zebrafish been studied in the past, it is also a species that replaces its teeth throughout life, which makes it an excellent model for studies on tooth replacement (Van der heyden and Huysseune, 2000; Van der heyden et al., 2001). This polyphyodont condition implies repeated initiation of tooth germs, which offers interesting possibilities of experimentation. Moreover, the development of zebrafish teeth morphologically resembles the different stages of murine tooth development (Borday-Birraux et al., 2006). An important array of molecular and genetic tools are available for zebrafish providing the possibility to advance our understanding of the mechanisms that govern the epithelial-mesenchymal interactions underlying tooth development (Dahm and Geisler, 2006).

1.2. Tooth development can be subdivided into multiple stages

Teeth always develop as a result of reciprocal interactions between the dental epithelium and the underlying mesenchymal cells. The latter have been shown to be derived from neural crest cells, first in amphibians (Sellman, 1946) and later in mammals (Chai et al., 2000) and this is generally assumed to be the case in other vertebrates. The origin of the epithelium is less well unequivocal. In mammals, it is believed to form from the ectoderm (Imai et al., 1998) but in vertebrates that develop teeth on their posterior branchial arches it is suggested that the endoderm can also participate in tooth development (Yelick and Schilling, 2002). Recently Soukup et al. (2008) demonstrated that the epithelium participating in tooth formation in Urodeles can have a dual origin at least under experimental conditions, i.e. both ectoderm and endoderm.

In zebrafish, tooth development passes through different, yet partially overlapping stages, starting with initiation, followed by morphogenesis and cytodifferentiation. Finally, the tooth becomes attached and thereby functional and a replacement tooth will start to form (Van der heyden and Huysseune, 2000). Each developmental stage is linked to characteristic histogenesis of the different cells involved (discussed below). Likewise, mouse (molar) tooth development passes through different stages, referred to as lamina, bud, cap and bell stage (Lesot and Brook, 2009). In order to facilitate comparisons between zebrafish (teleost) and mouse (mammalian) tooth development,

Borday-Birraux et al. (2006) have attempted to equate the stages in both taxa (Fig. 1). Thus, mouse dental lamina and bud stage is equal to early morphogenesis stage in zebrafish. Cap stage in the mouse can be equalled to zebrafish late morphogenesis stage. Early bell stage in the mouse corresponds with zebrafish early cytodifferentiation stage, while murine late bell stage resembles late cytodifferentiation of zebrafish teeth. Finally, attachment of a mouse tooth can be equalled to eruption of a zebrafish tooth (Borday-Birraux et al., 2006). In the description below, we will focus on tooth development in zebrafish and its characteristics but, if necessary, the comparison or link will be made to other species.

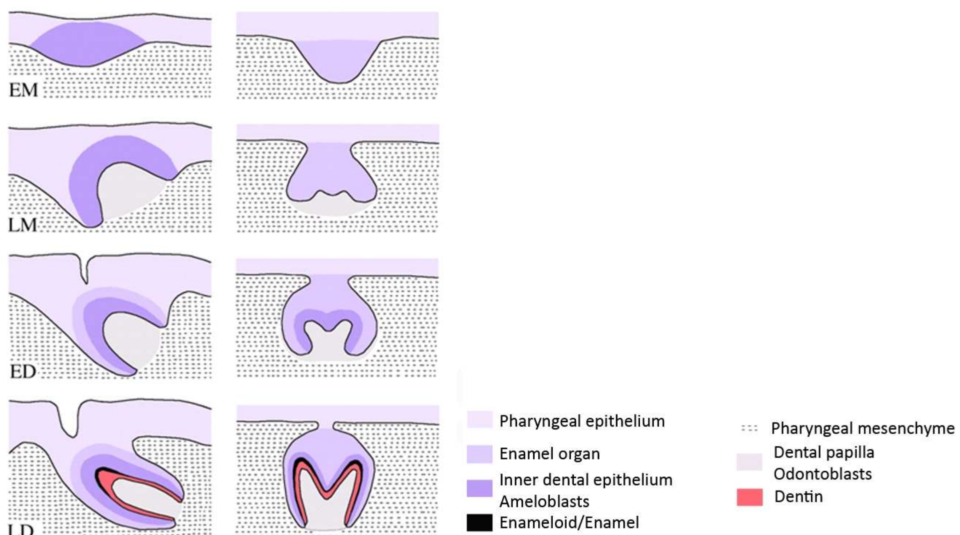


Figure 1. Comparison of homologous stages of tooth development between zebrafish and mouse

Early morphogenesis (EM) in zebrafish corresponds to initiation and bud stage in mouse and zebrafish late morphogenesis (LM) to mouse cap stage. Early cytodifferentiation (ED) of zebrafish is similar to early bell stage in murine tooth development while zebrafish late cytodifferentiation (LD) stage is comparable with late bell stage in mouse (after Borday-Birraux et al., (2006)).

One of the most striking differences compared to mouse or human dentition is that zebrafish do not have oral teeth but they develop pharyngeal teeth on the fifth branchial arch (Huysseune et al., 1998).

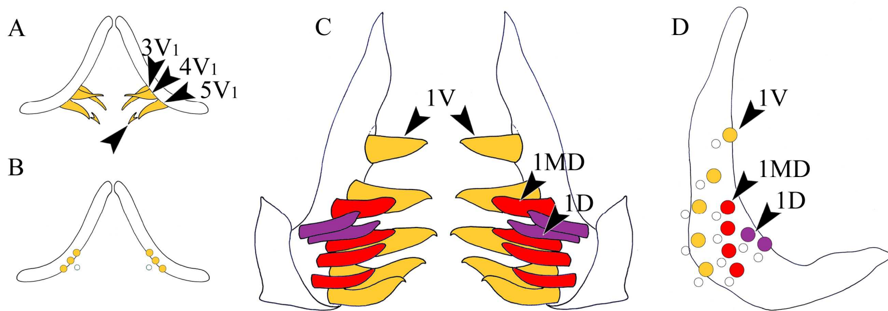


Figure 2. The zebrafish dentition

A, B) The pharyngeal dentition of a larval zebrafish (6 dpf) from a dorsal and slightly posterior view, showing the first three teeth developing ($3V^1$, $4V^1$, $5V^1$) and their position on the fifth branchial arch. The replacement tooth for $4V^1$ ($4V^2$) is indicated by an arrowhead. C, D) The complete zebrafish dentition (adult zebrafish) with all tooth positions occupied. It contains a ventral row (yellow) with five teeth, a medio-dorsal row (red) with four teeth and a dorsal row (purple) with two teeth. C: Dorsal view of both jaws; D: Medial view of right pharyngeal jaw. B, D: Schematized drawing of dentition shown in A and C, respectively. The small grey circles in (D) represent the replacement teeth each associated with a functional tooth (large colored circles) (after Huysseune et al. (2005)).

The dentition of an adult zebrafish consists of three tooth rows with a total of 11 teeth on each body side. The ventral row (V) has five teeth, the medio-dorsal row (MD) has four and the dorsal row (D) bears two teeth (Fig. 2). At 48 hours post-fertilization (hpf), the first tooth starts to develop (position 4V). Shortly after (at 56 hpf), the two neighbouring teeth on the same row (tooth $3V^1$ and $5V^1$) start to develop at approximately the same time and continue to develop near-simultaneously. At that time point, tooth $4V^1$ is already in early cytodifferentiation stage. At 80 hpf tooth $4V^1$ has become functional and the replacement tooth $4V^2$ (the second generation tooth at the fourth position on

the ventral row) forms from the epithelial fold that surrounds the erupted part of a functional tooth (called crypt, cf. Huysseune and Sire (2004)). At 6 days post-fertilization (dpf), tooth $3V^1$ and $5V^1$ are attached and their replacement tooth has started to form (Fig. 2A,B). The two remaining teeth on the ventral row, tooth $2V^1$ and $1V^1$, appear at 12 dpf and 16 dpf, respectively. Even before the last tooth on the ventral row can be observed, the first tooth on the medio-dorsal row starts to develop (tooth $3MD^1$). At 20 dpf, the two adjoining teeth on the medio-dorsal row develop followed by the onset of tooth formation in the last tooth position on the medio-dorsal row together with the development of the first tooth of the dorsal row. At four weeks post-fertilization all 11 tooth positions are occupied (Fig. 2C,D) (Van der heyden and Huysseune, 2000). From the foregoing description, it is clear that some teeth start to develop at the same moment and pass through the different developmental stages together. This has been called an odontogenic wave, during which teeth at even positions on the ventral row develop in alternation with teeth at odd positions. Teeth that develop consecutively at the same position (i.e. a tooth and all of its successors) comprise a tooth family (Van der heyden and Huysseune, 2000; Van der heyden et al., 2001).

Murine tooth development starts during embryonic development. At E11.5 (embryonic day 11.5) the first visual sign, thickening of the oral epithelium, can be observed. For a long time this thickening has been assumed to be the anlage of the first molar. Only recently it has been shown this first thickening is the remnant of a vestigial tooth placode that will be incorporated in the development of the first molar (Peterkova et al., 2000; Peterkova et al., 2002; Viriot et al., 2002). Next, the mesenchyme starts to condense around the epithelial bud at E12, which is completely formed at E13.5. During the cap stage at E14.5, the epithelium starts to fold and the primary enamel knot is active. This stage is followed by the typical bell-shaped enamel organ with two

secondary enamel knots at E16.5. Mice are born (E20) with teeth at late bell stage in which ameloblasts and odontoblasts are producing enamel and dentin, respectively (Caton and Tucker, 2009).

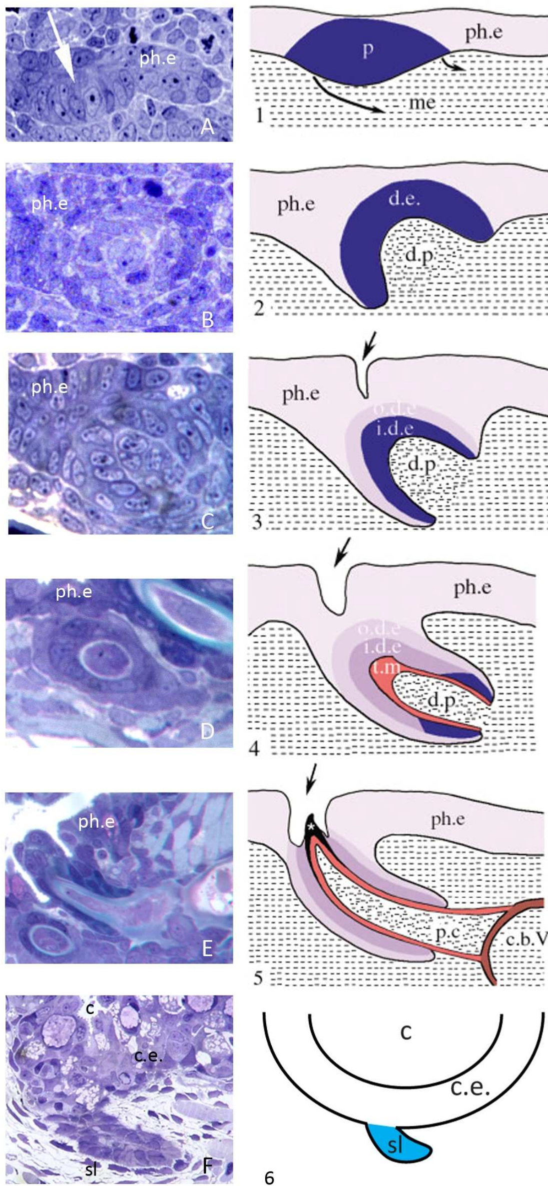
1.2.1. Tooth development starts morphologically with the formation of an epithelial placode

The epithelium lining the floor of the pharyngeal cavity and the underlying mesenchyme contribute to the formation of first-generation teeth, i.e., the first tooth at each tooth position. Prior to the onset of tooth formation, the pharyngeal epithelium is composed of a basal layer of cylindrical cells facing the basal lamina, an intermediate layer with more cuboidal cells covered by a layer of flattened, superficial cells. The initiation of the first tooth that develops, tooth 4V[1], already starts at 48 hpf. The onset is clearly visible by the elongation of the epithelial cells of the basal layer of the pharyngeal epithelium, creating a local thickening called the tooth placode (Fig. 3). This placode constitutes a disc of four or five cylindrical epithelial cells, overlain by four or five rather cuboidal cells. These placodal cells have been previously described as polarized cells (Huysseune et al., 1998; Van der heyden et al., 2000). Cell polarity involves the asymmetric organization of most of the physical aspects of the cell, including the cell surface, intracellular organelles and the cytoskeleton (Bryant and Mostov, 2008). At present, there are not enough data available concerning these parameters in the placodal cells. Therefore, it is better to appoint these cells as elongated.

The formation of a placode starts with the determination of a preplacodal region. This region comprises a group of cells that becomes segregated from the surrounding cells in order to further develop into a placode. The neighbouring cells preserve their characteristics while only the cells constituting the placode will further differentiate and pass through morphogenesis.

The induction of the preplacodal region is a multi-step process that requires the integration of different signals produced by different tissues (Streit, 2007). The segregation of the placodal cells is based on the formation of a border and reflected by differential expression of several signalling pathways such as FGF, BMP and Wnt signaling pathways (Mikkola and Millar, 2006; Streit, 2007). The cells within the preplacodal region respond differently to the signalling events compared to the surrounding tissue. Models have been proposed on the determination of the signals constituting the formation of placodes of hairs, mammary glands (Mikkola and Millar, 2006). Additionally to difference in signalization between the placode cells and the cells surrounding them, other mechanisms have also been proposed to create border formation and cell segregation. The signalization can generate actomyosin-mediated contractions which results in cortical tension at the border. A second possible mechanism is based on differences in cadherin-mediated cell-cell adhesion that establishes interfacial tension at the border between distinct cell populations (Batlle and Wilkinson, 2012). Irrespective of the exact signalization and underlying mechanisms of placode formation, it is very likely that the cells that will form the placode become segregated from the surrounding tissue enabling them to acquire specific properties.

In most teleosts the first tooth germ develops directly from the superficial epithelium. This is in contrast to replacement teeth, which bud off from a non-permanent and discontinuous dental lamina, called successional lamina, formed from the enamel epithelium of the predecessor (Berkovitz and Shellis, 1978; Huysseune, 1983; Huysseune and Sire, 1997a; Huysseune and Sire, 1997b). The formation of mammalian teeth involves the formation of a dental lamina, which is a ridge-like epithelial invagination. This dental lamina is continuous between tooth families and persists to give rise to both first-generation and replacement teeth (Fraser et al., 2006; Reif, 1982).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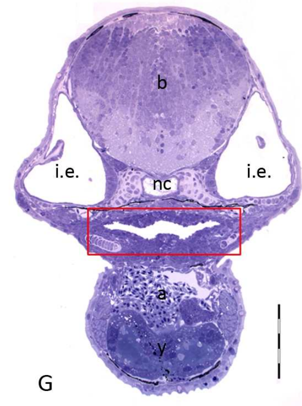
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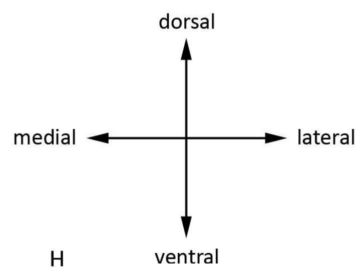
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Figure 3. Schematic representation of the different stages of zebrafish tooth development combined with histological sections and orientation (left page)

Initiation of a tooth starts with the formation of an epithelial thickening, the tooth placode (A;1). These epithelial cells will invaginate into the underlying mesenchyme during morphogenesis stage. The epithelial part is called the enamel organ and the condensed mesenchyme the dental papilla (B;2). When cytodifferentiation starts, two distinct layers are detectable in the enamel organ, the inner dental epithelium (IDE) and outer dental epithelium (ODE). The cells of the IDE will start to differentiate into ameloblasts while the cells of the dental papilla will differentiate into odontoblasts (C;3). Late cytodifferentiation stage is marked by the presence of a noticeable amount of tooth matrix, deposited by both the ameloblasts and the odontoblasts (D;4). When the tooth attaches to the fifth branchial arch, the tooth tip erupts into the pharyngeal cavity and the tooth becomes functional (E;5). This event coincides with the start of the formation of a replacement tooth. A replacement tooth does not arise directly from the pharyngeal epithelium but a successional lamina is formed from the crypt surrounding the functional tooth (F;6). G: Section through the head of a zebrafish at the level of the area in which teeth will develop (red box). H: Orientation cross for A-E and 1-5. a: atrium; b: brain; c: crypt; c.b.V, ceratobranchial Vth; c.e.: crypt epithelium; d.e., dental epithelium; d.p, dental papilla; i.d.e, inner dental epithelium; i.e.: inner ear; me, mesenchyme; nc: notochord; o.d.e, outer dental epithelium; p, placode; p.c, pulp cavity; ph.e, pharyngeal epithelium; sl: successional lamina; t.m, tooth matrix; y:yolk *, enameloid cap (after Laurenti et al. (2004)).

The amount of data on the molecular control of zebrafish tooth development stands in sharp contrast to the knowledge in mouse tooth development. The earliest sign of zebrafish tooth development are bilateral patches of *pitx2* expression in the pharyngeal epithelium (Fig. 4). The expression of *pitx2* persists in the bell-shaped enamel organ (Jackman et al., 2004). In mouse, the earliest marker in the dental epithelium is *Dlx2*. The two zebrafish orthologues *dlx2a* and *dlx2b* become expressed in the zebrafish tooth placode from 48 hpf onwards, in close relation with but after *pitx2* (Fig. 4) (Borday-Birraux et al., 2006; Jackman et al., 2004). The tooth placode also expresses *dlx3b, dlx4b* and *dlx5a*. The same dlx genes remain expressed during morphogenesis stage but additionally *dlx4a* becomes also expressed (Borday-Birraux et al., 2006).

The zebrafish tooth placode expresses *fgf3* and *fgf4*, simultaneously with *dlx2a* and *dlx2b* in the dental epithelium. The dental mesenchyme also expresses *dlx2a* and *dlx2b* and the transcription factors *lhx6* and *lhx7* (Fig. 4) (Jackman et al., 2004). *Shha* is also expressed in the placode while *shhb* is not (Jackman et al., 2010). Although *bmp2b* or *bmp4* are expressed in the developing tooth germ, their loss does not affect zebrafish tooth development and therefore are considered to be dispensable (Wise and Stock, 2010).

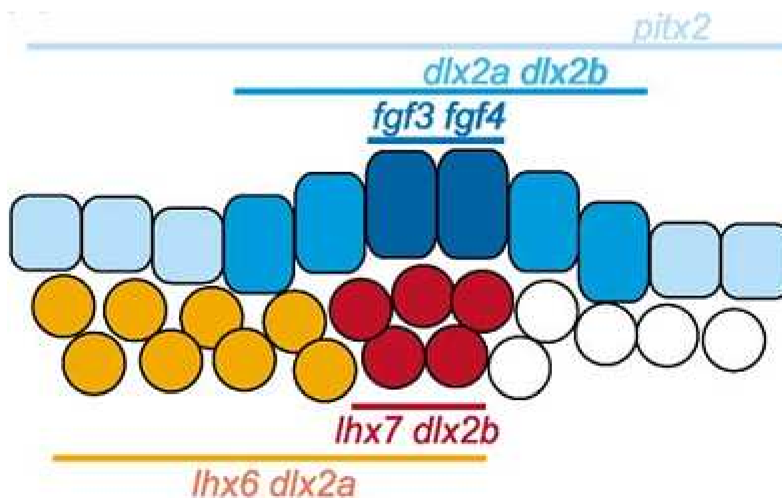

Figure 4. Schematic representation of a transverse section through the pharyngeal epithelium and mesenchyme of a left tooth placode (dorsal up, medial to the right), showing zebrafish tooth gene expression (after Jackman et al. (2004)).

1.2.2. Morphogenesis stage is marked by the invagination of the epithelial cells into the underlying mesenchyme

The epithelial cells of the placode will protrude into the underlying mesenchyme. This results in the formation of a bell-shaped epithelial organ with two epithelial arms parallel to the pharyngeal epithelium (Fig. 3).

In that way, a dorsal and ventral side of the developing tooth can be distinguished (Huysseune and Sire, 1998).

This epithelial part of the tooth is called the enamel organ and the 'leading edge' of epithelial cells invaginating the mesenchyme is called the cervical loop. The enamel organ envelops the condensing mesenchymal component of the tooth. In first-generation teeth, the mesenchymal contribution consists of only a few cells; in later generation teeth considerably more cells will be present constituting the so-called dental papilla (Huysseune et al., 1998; Van der heyden et al., 2000). This stage of tooth development is called morphogenesis stage. At the cellular level, it is possible to distinguish two separate layers in the enamel organ of a developing zebrafish tooth: the inner dental epithelium (IDE) and the outer dental epithelium (ODE) (Fig. 3) (Huysseune et al., 1998). These two distinct layers have their own characteristics and function, as will become clear later during tooth development. The cells of the IDE will transform into ameloblasts while the cells of the ODE, rather flattened cells, are involved in the transport of ions. Some of the ODE cells will give rise to the onset of the replacement tooth (Huysseune, 2006; Sasagawa, 1997; Van der heyden and Huysseune, 2000). Different from tooth development in mammals, zebrafish teeth have their IDE and ODE closely apposed. In mammals, these two cell layers are separated by two additional layers referred to as the stellate reticulum (SR) and stratum intermedium (SI) (Butler, 1956). It has been suggested that the SR prevents distortion of the crown shape of the tooth under influence of the growing dental papilla (Butler, 1956; Butler, 1995). The function of the SI is not fully known but based on previous studies this layer is suggested to be involved in the differentiation of ameloblasts and enamel formation (Harada et al., 2006; Kawano et al., 2004; Sasagawa and Ishiyama, 2002; Wise and Fan, 1989).

1.2.3. Cytodifferentiation involves ameloblasts, odontoblasts and matrix deposition

Cytodifferentiation stage is the stage of tooth development in which the cells of the enamel organ and dental papilla become differentiated and tooth matrix is deposited. The cells of the IDE differentiate into functional ameloblasts while the mesenchymal preodontoblasts are transformed into matrix-depositing odontoblasts. During cytodifferentiation the first matrix (enameloid) is laid down (Fig. 3). Enameloid is built by both the ameloblasts and the odontoblasts, in contrast to enamel which is a purely epithelial product (Huysseune and Sire, 1998). Enameloid is known to contain collagen, that has been suggested to be produced by the ameloblasts. At least in salmon, the ameloblasts express high levels of collagen type I (Huysseune et al., 2008).

Both the cells of the IDE and the dental papilla undergo multiple changes to become functional ameloblasts and odontoblasts, respectively. The proliferating dental epithelial cells, after having become localized in the IDE, undergo successive changes into preameloblasts, presecretory ameloblasts and secretory ameloblasts that synthesize and secrete enamel/enameloid matrix proteins. These functional changes are coupled to morphological changes in the cells. In preameloblasts, the nucleus shifts away from the dental papilla, the cell elongates and the cytoplasm becomes filled with organelles needed for later production of matrix proteins such as mitochondria, RER cistern and free ribosomes. When the preameloblasts become functional ameloblasts, they lengthen and increasingly polarize while depositing matrix. Finally, to obtain a hard substance, the ameloblasts resorb much of the water and organic material from the enamel/enameloid after which the ameloblasts undergo final regression and shrinkage in height to become classical, cuboidal-shaped reduced ameloblasts (Bei, 2009; He et al., 2010; Sasagawa, 1995; Sasagawa and Ishiyama, 2005a; Smith, 1979; Van der heyden et al., 2000).

During the stage of enameloid maturation where the ameloblasts function to resorb much of the water and organic matrix, the ameloblasts in teleost teeth have ruffled apical cell membranes. These are suggested to play a role in calcium transport and protein resorption (Prostak and Skobe, 1986; Prostak and Skobe, 1988; Sasagawa, 1988; Sasagawa, 2002; Sasagawa and Ishiyama, 2005b). In contrast to mammalian enamel, the mineralization of enameloid only starts when the complete enameloid cap and a thin layer of predentine is formed (Huysseune and Sire, 1998; Prostak and Skobe, 1986).

The formation of predentine (which later mineralizes into dentine) takes place after the enameloid deposition is completed. This matrix is only produced by the odontoblasts (Huysseune and Sire, 1997a). In first-generation teeth, which house only a few mesenchymal cells, all these mesenchymal cells differentiate into odontoblasts. In later-generation teeth only those cells of the dental papilla adjacent to the ameloblasts become odontoblasts (Huysseune and Sire, 1998; Van der heyden et al., 2000). Odontoblasts are originally derived from neural crest cells and go through different stages before becoming a terminally differentiated odontoblast. Preodontoblasts are withdrawn from the cell cycle and become elongated and polarized odontoblasts. Subsequently, they start to produce predentine which later is turned into dentine. This differentiation process is induced by the IDE both in Teleosts (Huysseune et al., 1998; Van der heyden et al., 2000) and in mouse (Lesot et al., 2001; Ruch et al., 1995).

As the cervical loop penetrates even more deeply into the mesenchyme, a gradient of differentiation becomes visible from the tooth tip towards the tooth base. The cells closest to the superficial epithelium are more differentiated than the cells closest to the cervical loop. This explains why tooth developmental stages are considered to partially overlap: while the cervical loop can still be considered in morphogenesis stage, the cells surrounding the future tooth tip have entered cytodifferentiation.

This gradient of differentiation is maintained as development proceeds. Thus, once the enameloid cap is formed, predentine deposition will also spread from the tip to the tooth base, paralleling the differentiation of the cells. Similarly, mineralization will spread from the tooth tip to the base. Because of this gradient of development and the resulting overlap in stages, it is hard to assign a developing tooth to a given developmental stage. Thus, we use a rather pragmatic subdivision. When a small amount of matrix (slightly thicker than the basement membrane) is visible on sections, we assign this tooth to early cytodifferentiation stage; yet, the tooth tip will already have passed early cytodifferentiation while at the base early cytodifferentiation is still ongoing. The same holds for defining a tooth in late cytodifferentiation stage.

1.2.4. Attachment makes the tooth functional and elicits the formation of a next tooth generation

In the final stage of tooth development, the tooth becomes functional. To be able to attach to the fifth branchial arch, zebrafish teeth form attachment bone (Fig. 3) (Huysseune et al., 1998). This attachment bone is deposited as a cylinder in the prolongation of the most basal predentine towards the fifth branchial arch. The matrix of this attachment bone is deposited by cells of the dental papilla that are clearly distinguishable from functional odontoblasts (apparent cell processes penetrating into the matrix) and differentiated outer mesenchymal cells (Van der heyden et al., 2000). Eventually, the attachment bone fuses with the bone of the pharyngeal jaw. Mineralization of the attachment bone continues and eventually dentine, attachment bone and supporting bone can no longer be distinguished (Van der heyden et al., 2000). This mode of tooth attachment, without root structures, is a basal character (Huysseune and Sire, 2004).

Eruption of an attached tooth in zebrafish, is accomplished by the remodelling of the overlying epithelial cell layers: the epithelial cells overlying the tooth tip fold back hereby exposing the tip into the pharyngeal cavity. Prior to this epithelial remodelling an epithelial crypt has formed, provoked by the growth of the developing tooth. Such an epithelial crypt starts as a shallow depression of the pharyngeal epithelium which becomes progressively deeper. The exposure of the tooth tip in this crypt is achieved by the disconnection of, first, the cells of the pharyngeal epithelium from the ODE, and then the loss of contact between IDE and ODE itself (Huysseune and Sire, 2004). After eruption, the tooth tip remains covered in part with a very flattened lining of cells, issuing from the IDE. The tooth tip does not protrude above the general level of the pharyngeal epithelium but rather sits in the crypt into which it has erupted, and which has been enlarged by the eruption process itself. Thus, after eruption, the wall of the crypt is constituted by the former ODE.

Once attached and erupted, the functional tooth continues to mature. Thus, there is a clear histological difference in young, mature and old functional teeth, which is only visible in larger teeth as first-generation teeth only contain very few cells. Young functional teeth still have polarized odontoblasts at the tip of the pulp cavity, which contains a fair amount of cells. Mature functional teeth are marked by a more empty-looking pulp cavity with a network of loose stellate cells and some polarized odontoblasts remaining at the tip of the pulp cavity. In old functional teeth the cavity looks almost empty and is lined by flattened cells (Huysseune, 2006).

From the moment the tooth has attached and has become functional, a replacement tooth starts to develop from it, i.e. at the same tooth position. A replacement tooth in zebrafish, in contrast to a first-generation tooth, does not develop directly from the pharyngeal epithelium but from an outgrowth of the epithelial crypt surrounding the tip of the functional tooth.

This outgrowth that will give rise to the successor is called the successional lamina. In young zebrafish, teeth are very small and contain only a small amount of cells, making this outgrowth barely visible. In later-generation teeth, the successional lamina consists of a larger number of cells and therefore its presence is much more distinctive. It has been suggested that the formation of the successional lamina, and the formation of the tooth germ itself, are two distinct phases of the replacement process. Indeed, it has been observed that the successional lamina can persist for a while without giving rise to the successor (Huysseune, 2006). This delay between onset and further development is also observed in mammals. For example, in humans the dental lamina exists for months before the primary teeth start to develop. Moreover, the permanent molars, which are not replaced, have been reported to develop a dental lamina which eventually disappears (Berkovitz et al., 1992).

1.3. Tooth development is regulated by several signalling pathways

Tooth development is controlled by ligands, receptors and downstream effectors that belong to multiple pathways. Signalling pathways such as Bmp (bone morphogenetic protein), Fgf (fibroblast growth factor), Wnt, TGFβ (transforming growth factor-β), Hedgehog and Eda pathways are used reiteratively during advancing tooth development (Fig. 5). Deletion of a component of these pathways often results in defects in tooth formation (Thesleff, 2003; Thesleff and Tummers, 2008). The first signalling centre during tooth development is the dental placode. Via Fgf and Bmp signalling, the epithelium induces morphogenesis of the odontogenic mesenchyme. The mesenchyme responds by sending reciprocal signal molecules, including Fgfs and Bmps (Fig. 5) (Thesleff, 2003; Thesleff and Sharpe, 1997). An attractive model in mouse for which evidence is growing, states that tooth initiation is a fine balance between activators and inhibitors of tooth formation (Mandler and Neubuser, 2001; Neubuser et al., 1997). In the odontogenic epithelium, Fgf ligands are expressed before any morphological appearance of the new tooth. Ligands of the BMP family on the other hand inhibit the process of tooth initiation. Thus, a tooth develops in a region where Fgfs are expressed and Bmps are absent (Mandler and Neubuser, 2001; Neubuser et al., 1997). Blocking Fgf signalling in the mouse showed that both molar and incisors require this signalling pathway to proceed beyond an early stage of tooth development (Mandler and Neubuser, 2001). Fgf8 is found in the pre-dental epithelium and its function is required for initiation of tooth formation in mouse (Abu-Issa et al., 2002). Further research showed that Fgf8 regulates Dlx2, Pax9 and Pitx2 during murine tooth development (Abu-Issa et al., 2002; Kettunen and Thesleff, 1998).

When these Fgfs are examined in the light of tooth development in zebrafish, it was found that there are extensive similarities but also remarkable differences in their expression compared to their mouse orthologs. Blocking Fgf signalling revealed that this pathway appears to be required for early tooth morphogenesis in zebrafish. Moreover, all odontogenic gene expression is eliminated except for *pitx2* expression. When knocking down *fgf4* or *fgf3* by morpholino injection, only a relative mild effect on tooth development was observed (Jackman et al., 2004).

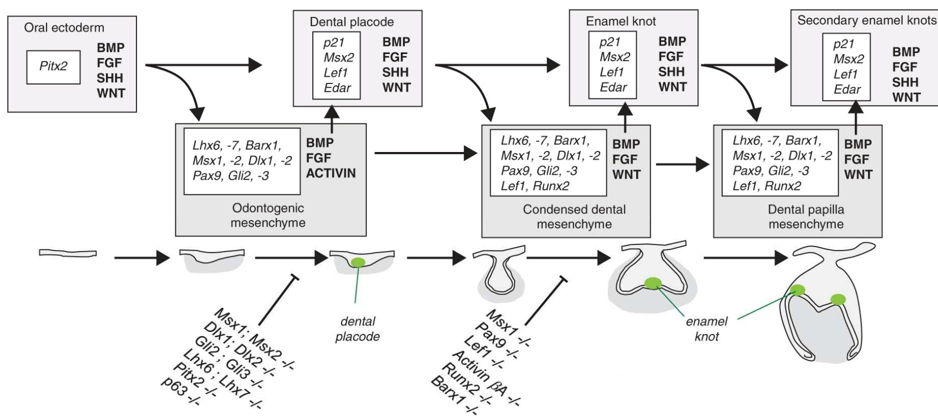

Figure 5. The sequential and reciprocal regulatory signalling interactions between the epithelium and mesenchyme in the mouse dentition

The various signalling molecules involved are shown in the different boxes in bold while the transcription factors expressed are depicted in italics. Each box represents a tissue compartment. Note that the same gene families are repeatedly used during the different steps of tooth development (after Tummers and Thesleff (2009)).

In mouse, shh signalling is required for tooth initiation and subsequent morphogenesis and differentiation of the dental epithelium into ameloblasts (Gritli-Linde et al., 2002; Hardcastle et al., 1998).

Targeted disruption of S*hh* in transgenic mice results in an early craniofacial phenotype resembling holo-prosencephaly (Chiang et al., 1996).

Shh expression in the early dental epithelium appears to follow that of *Fgf8* and *Fgf9*, suggesting that Fgfs may be upstream of shh signalling. The role of shh signalling in the early epithelial thickenings is unclear but its highly localized expression suggests an involvement in tooth bud initiation (Fig. 5) (Thesleff and Sharpe, 1997). In zebrafish, the hedgehog signalling pathway is shown to be essential for proper tooth initiation and further development (Jackman et al., 2010).

The importance of the Wnt signalling pathway during tooth development has been suggested multiple times (Chen et al., 2009; Järvinen et al., 2006; Sarkar and Sharpe, 1999; Wang et al., 2009). *Lef1* knockout (KO) mice lack teeth as their development is arrested in bud stage (van Genderen et al., 1994) while overexpression of *Lef1* in epithelial cells of transgenic mice results in an increased invagination of the epithelium and the formation of extra tooth like structures (Zhou et al., 1995). In other cycling structures such as hairs, Wnt signalling plays an important role (Andl et al., 2002). In 2006, it was shown that mouse tooth buds expressing stabilized β-catenin in the epithelium give rise to dozens of teeth (Järvinen et al., 2006). In humans on the other hand, conflicting results are obtained regarding the involvement of Wnt signalling in tooth development. A mutation in *AXIN2* is found in patients with extreme hypodontia (Lammi et al., 2004), while adenomatous polyposis coli (APC) mutations result in the formation of supernumerary teeth (Wijn et al., 2007). Yet, both are negative regulators of the canonical Wnt signalling pathway. In zebrafish, the masterblind mutant which is found to contain a point mutation in *axin 1* and thus mimics Wnt overexpression has an overall dramatic phenotype but teeth are replaced in a normal way (van de Water et al., 2001 and A. Huysseune, pers. communication).

2. TEETH AS INTEGUMENTAL APPENDAGES

Not only teeth but many other organs such as lung, pancreas or kidney depend on interactions between the epithelium and mesenchyme, not only during embryonic development, but also postnatally (Thesleff et al., 1995).

In mammals, the embryonic ectoderm gives rise to several integumental appendages including teeth, hairs and glands such as the mammary and salivary gland and in birds, it gives rise to feathers. All of these appendages similarly develop as a result of epithelial-mesenchymal interactions (Mikkola, 2007; Pispa and Thesleff, 2003). Although these structures all have a different shape, structure and function, they share at least one characteristic: i.e. they all start their development with the formation of an epithelial thickening. The further formation of these appendages is characterized by morphogenetic movements and depends on the interaction of the epithelial cells with the surrounding mesenchymal cells.

Studies on the molecular control underlying the formation of different integumental appendages have revealed a common set of molecules regulating the formation of these appendages. Also the function of growth and transcription factors are widely conserved across the appendages (Chuong, 1998; Jernvall and Thesleff, 2000; Mikkola, 2007; Pispa and Thesleff, 2003; Thesleff and Tummers, 2008). By comparing the first stages of zebrafish tooth development with these other integumental appendages, it is clear that zebrafish teeth can be an equally valuable model for epithelial morphogenesis. Moreover, the early developmental stages of the integumental appendages mentioned above, can be compared in order to get a better, more overall view of the underlying mechanisms that drive these remarkable cellular changes.

In chapter II.4, we examine the different cell adhesion systems during the development of hair, mammary and salivary gland, as well as teeth from different species, including zebrafish. It is apparent that the distribution of

components of the cadherin-catenin complex closely matches the distribution of this complex in the other appendages. Additionally, literature research showed that the regulation of different cell adhesion systems plays a significant role in proper development of these integumental appendages. Because of the high resemblance of these appendages to zebrafish tooth development, this suggests that each of the components of the cadherin-catenin complex can influence normal tooth development in zebrafish.

3. THE CADHERIN-CATENIN COMPLEX

Cell-cell adhesion is accomplished by several cell adhesion systems, each with its own specific functions and characteristics. One of these systems is the cadherin-catenin complex at the basolateral membranes of the cell (Fig. 6). This complex provides a strong adhesive force between two neighbouring cells by keeping the adjacent membranes closely together. The cadherin-catenin complex consists of different components with a specific function within the complex (Alberts et al., 2002; Green et al., 2010).

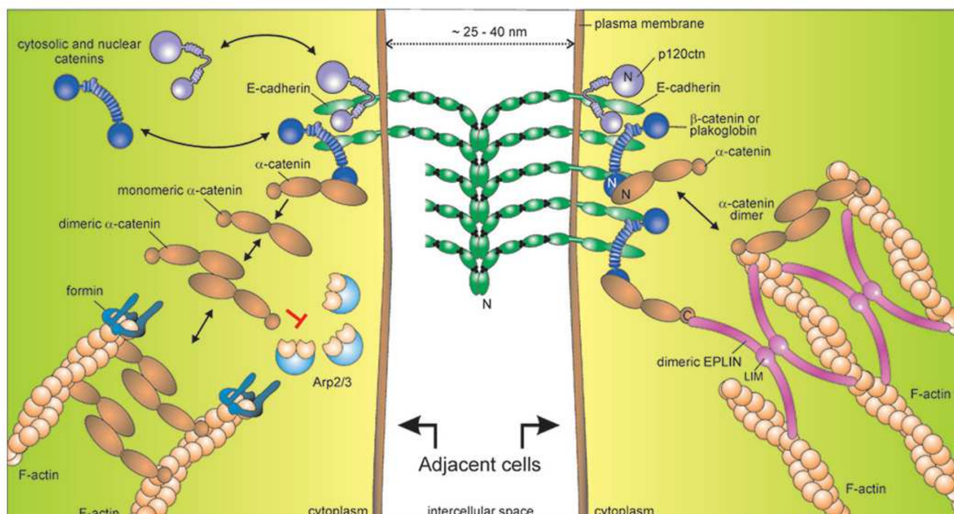

Figure 6. Schematic representation of the cadherin-catenin complex between two neighbouring epithelial cells

In the intercellular space, the extracellular domains of the cadherins in adjacent cells connect to each other. The cytoplasmic tail of the cadherin is coupled to p120ctn at the juxtamembrane domain (JMD) while more at the C-terminal end the tail is coupled to β-catenin. β-catenin can provide a link to the actin cytoskeleton (after van Roy and Berx (2008).

At the cell surface, the transmembrane proteins, cadherins, link with their extracellular part to the extracellular part of the cadherins at the neighbouring

cell. In this way, the two adjacent cell membranes are brought closely together. The intracellular part of the cadherins is linked to multiple cadherin-associated molecules, providing the link with the actin cytoskeleton (Fig. 6) (van Roy and Berx, 2008). Via this connection, tension can be transmitted from the cytoskeleton to the plasma membrane which can result in cell shape changes and contractions typical for tissue bending and invagination (Hogan and Kolodziej, 2002; Martin et al., 2010; Odell et al., 1981; Sawyer et al., 2010). Cadherins are more than just cell adhesion molecules, they can also play a signalling role. This can be accomplished by the destruction of the cadherin-catenin complex and the subsequent release of the associated molecules or parts of the cadherin (Bartlett et al., 2011; Latefi et al., 2009; Niessen et al., 2011; Shoval et al., 2007). The cadherins can also interact with other binding partners and therefore regulate signalling processes (Cavallaro and Dejana, 2011; Knights and Cook, 2010; Shay-Salit et al., 2002).

β-catenin and plakoglobin are close homologues with resembling molecular structure. Therefore, they are known to overlap in certain functions (Zhurinsky et al., 2000). Although plakoglobin is mainly present as a component of another cell adhesion system, the desmosomes, and β-catenin couples to the catenin-binding domain of the cytoplasmic tail of the cadherin (Fig. 6), they can both influence the Wnt signalling pathway (Delva et al., 2009; Garrod and Chidgey, 2008; Meng and Takeichi, 2009; Shapiro and Weis, 2009; Zhurinsky et al., 2000). Under certain circumstances, they can substitute each other resulting in plakoglobin linked to a classical cadherin and β-catenin located at the desmosomes (Bierkamp et al., 1999; Delva et al., 2009; Hatzfeld et al., 2003; Thomason et al., 2010).

Also at the cytoplasmic tail but closer to the membrane, at the juxtamembrane domain, p120catenin is bound the cadherin. Its main function is to stabilize the complex at the cell membrane, preventing internalization (Fig. 6) (Ireton et al.,

2002; Pieters et al., 2012b). When not associated to a cadherin, p120ctn regulates small Rho GTPases and therefore cell motility and cell shape (Lecuit and Lenne, 2007; Noren et al., 2000; Pieters et al., 2012b).
Following chapters II.1-3 extensively describe the different components of the cadherin-catenin complex and provide more detail of the mechanisms underlying the functions of each.

Because of the little amount of data on cell adhesion molecules during tooth development in general, we embarked a study on the distribution and possible role of the components of the cadherin-catenin complex during tooth development. Tooth development is a process resulting from reciprocal interactions between epithelial and mesenchymal cell layers. It starts with the formation of an epithelial thickening, the tooth placode, that will protrude into the underlying mesenchyme. As a model we chose the zebrafish which not only starts tooth development with the formation of a placode, it also replaces its teeth continuously throughout life. Replacement teeth in zebrafish do not develop directly from the pharyngeal epithelium, as first-generation teeth do, but from a successional lamina issuing from the epithelial crypt surrounding the functional tooth. This polyphyodont condition implies repeated initiation of tooth germs, which offers interesting possibilities of experimentation. Moreover, this hallmark enables the comparison of tooth replacement with other cycling structures such as hairs. Teeth and hairs but also other integumental appendages start their development with the formation of a placode. Therefore, parallels can be drawn between their initial development.

CHAPTER II: LITERATURE REVIEW

1. CELL ADHESION AND THE CADHERIN-CATENIN COMPLEX

1.1. Introduction to intercellular adhesion

Every tissue consists of different cell types which need to be able to communicate with each other in order to obtain information about their position, shape and function. Depending on their position and fate they need to interact to get information concerning proliferation, changes in cell shape, migratory stimuli, differentiation, polarization and many more. Cell-cell interactions are thus of paramount importance in order to obtain a fully functional organ.

Epithelia are a classical example of polarized tissue known to separate another type of tissue (mostly connective tissue) from an opening or lumen. An epithelial cell, at least of a single layered epithelium, lining a lumen or cavity has an apical cell membrane directed towards the opening, a basal cell surface adjacent to the basement membrane and the lateral surfaces connecting both. Each surface has different functions and hence has specialized molecules according to its different roles. The basal surface of the cells makes contact with the basement membrane and the extracellular matrix. The lateral surfaces make contact with neighbouring cells and contain specialized junctions while the apical surface is specialized in absorbing and secreting materials such as nutrients and ions. In an epithelial cell layer two adjacent cells adjoin with their lateral surface. To prevent intracellular leakage, to promote transport through the cell, and to mechanically link the two cells, the membranes are joined closely together by means of different cell adhesion systems. From apical to basal on the lateral cell surface there are tight junctions (TJs), adherens junctions (AJs), and desmosomes (Fig. 7).

The connection of the basal surface with the underlying basement membrane is formed by hemi-desmosomes (Alberts et al., 2002; Green et al., 2010).

All of these cell-cell adhesion systems (AJs, TJs and desmosomes) are present during one or multiple stages of tooth development (Barron et al., 2008; Fausser et al., 1998; Kieffer-Combeau et al., 2001; Unda et al., 2003; Verstraeten et al., 2010b; Yoshida et al., 2010). Therefore, they will be briefly discussed separately below.

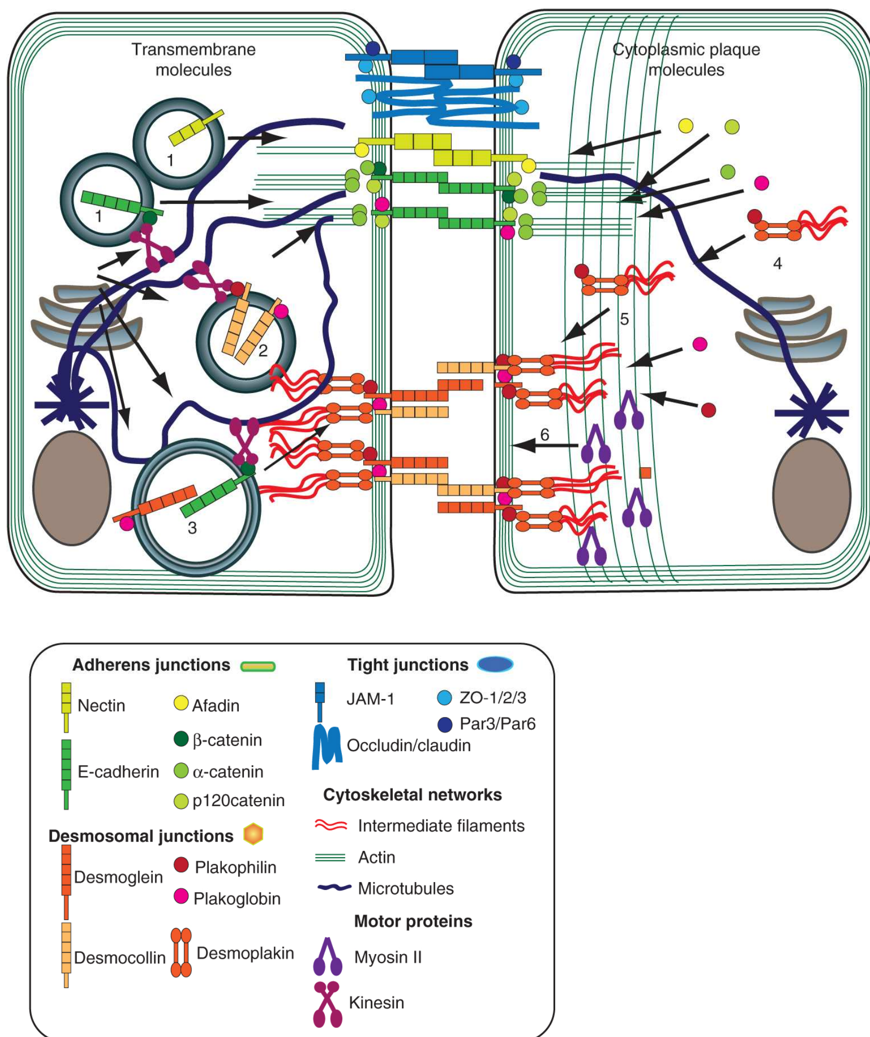

Figure 7. Schematic representation of cell-cell adhesion complexes (left page)

Tight junctions (TJs) occupy the most apical position on the lateral membrane. TJs form a continuous belt around the apical margins of the epithelial cells forming a circumferential seal. TJs are multiprotein complexes comprising transmembrane proteins such as claudin, occludin and tricellulin which are intracellularly linked to a scaffolding protein from the zonula occludens (ZO)-family, linking this complex to the actin cytoskeleton. JAMs, a distinct group of integral, tight junction membrane proteins are characterized by the presence of IgG-like domain which can bind both homo- and heterophilic but do not induce tight junctional strands.
Two basic units form adherens junctions (AJs): nectin-afadin complex and the cadherin-catenin complex. The nectin-afadin complex is a connection between the membrane-bound nectin family and the scaffolding protein afadin. The cadherin-catenin protein complex is the second major component of AJs. This complex is composed of a transmembrane cadherin which is stabilized at the membrane by binding of p120 catenin (p120ctn) on the cytoplasmic tail, close to the membrane. The C-terminal end of the cytoplasmic tail of the cadherin comprises a binding site for β-catenin. This chain of molecules is linked to the actin cytoskeleton by the association of β-catenin with α-catenin, which binds F-actin.
Desmosomes are cellular junctions characteristic for cells subjected to high mechanical stress. At the plasma membrane, the desmosomal cadherins, desmocollin or desmoglein, are anchored. The intracellular tail is linked to several proteins providing the connection with the intermediate filaments via desmoplakin, plakoglobin, and plakophillin, so-called linker proteins (after Green et al. (2010)).

1.1.1. Tight junctions are responsible for intercellular sealing

TJs occupy the most apical position of the three types of cell-cell adhesion junctions at the lateral membranes. They closely join the plasma membranes of two adjacent cells, almost as if they would be fused together. Freeze fracture electron microscopy showed that the TJs form a continuous belt around the apical margins of the epithelial cells forming a circumferential seal (Staehelin, 1974). This seal prevents free diffusion of molecules between two cells, favouring active transport through the cells (transcellular), but it is not absolute since small ions and uncharged molecules can still pass (paracellular).

It is necessary to balance out the transcellular and paracellular transport in order to fulfil the needs of the tissue (O'Neill and Garrod, 2011). Not only do TJs control the passage of ions and solutions between or through cells, they also fulfil a fence function preventing the mixing of membrane proteins of the apical and basolateral membranes. This way, TJs also show to be an important factor for polarisation and therefore are involved in the maintenance of cell polarity (Shin et al., 2006; Umeda et al., 2006).

TJs are multiprotein complexes comprising transmembrane proteins such as claudin and occludin which are intracellularly linked to a scaffolding protein from the zonula occludens (ZO)-family that provides the link of this complex to the actin cytoskeleton (Fig. 7) (Ebnet, 2008; Hartsock and Nelson, 2008).

Both claudins and occludins are tetraspan proteins with two extracellular loops and both the C- and N-terminal end are located in the cytoplasm. TJs are formed by the interaction of these extracellular loops of the proteins expressed at adjacent plasma membranes (O'Neill and Garrod, 2011; Piontek et al., 2008). It has been shown by overexpression and knock-down studies that claudins (of which there are at least 24 family members in human) are responsible for the permeability of the TJs. Some claudins have ‘tightening’ properties and by overexpressing these isoforms, paracellular transport is reduced (Krause et al., 2008). In contrast, overexpressing the ‘pore-forming’ claudin isoforms results in an enhanced paracellular ion transport (Krause et al., 2008; O'Neill and Garrod, 2011). In turn, occludins are recruited to the TJs by claudins (Furuse et al., 1998) and influence the permeability of the TJs. Only recently, tricellulin was discovered (Ikenouchi et al., 2005), which has high structural similarity to occludin and is specifically enriched at tricellular contact sites. A distinct group of integral, tight junction membrane proteins is characterized by the presence of IgG-like domain and comprises the closely related molecules JAM-A, -B and -C (Fig. 7) (Niessen, 2007; Williams and Barclay, 1988).

JAMs can bind both homo- and heterophilic but do not induce tight junctional strands. The latter are also found in cells that do not express TJs such as leucocytes where they contribute to the transendothelial migration (Ebnet et al., 2004).

To connect these transmembrane proteins to the actin cytoskeleton, scaffolding proteins are present and the typical tight junctional strands are formed. An important group of scaffolding proteins is the ZO-family consisting of ZO-1, ZO-2 and ZO-3 (Fig. 7). These proteins interact directly with claudins and occludins via their N-terminal PDZ-domains while their C-terminal domain provides the link with the actin cytoskeleton (Schneeberger and Lynch, 2004). Since ZO-1 can also bind α-catenin, it is believed to be the possible link between TJs and AJs. Simultaneous binding of ZO-1 to both α-catenin and actin has not been proven (Hartsock and Nelson, 2008). MUPP1 and MAGI proteins also contain the PDZ-domain and can therefore also interact with the transmembrane components of the TJs (Schneeberger and Lynch, 2004). In contrast, the non-PDZ tight junctional plaque protein cingulin is known to interact with actin, ZOs and JAMs via its head domain while homodimerization and interaction with myosin chains is conducted by the central rod domain of this protein (Fig. 7) (Clayburgh et al., 2005; Niessen, 2007).

The molecules discussed above are the main components of the TJs but the number of proteins localized at the TJs is much higher. Not only scaffolding and adapter proteins but also regulatory proteins such as small GTPases, kinases and phosphatases, as well as transcription factors are present at the TJs.

This suggests that TJs are very dynamic and can interact with the signals surrounding them.

1.1.2. Desmosome plaques provide adhesion strength by linking to the intermediate filaments

Desmosomes are cellular junctions characteristic for cells subjected to high mechanical stress. They are very electron-dense structures, less than 1 µm in diameter, comprising two cytoplasmic plaques that are each associated with the intermediate filaments. At the plasma membrane, the desmosomal cadherins, desmocollin or desmoglein, are anchored. Their extracellular part provides the coupling of two halves of the desmosome together in the intercellular space (Fig. 7). Desmocollins and desmogleins are always co-expressed in tissues that develop desmosomes and are needed for the formation of a functional desmosome (Marcozzi et al., 1998). Under different circumstances, they can form both homophilic and heterophilic interactions (Amagai et al., 1994; Chitaev and Troyanovsky, 1997). However, it has been indicated that adhesive binding under normal conditions in desmosomes is homophilic and isoform-specific (Garrod and Chidgey, 2008).

The intracellular tail is linked to several proteins providing the connection with the intermediate filaments via desmoplakin, plakoglobin, and plakophillin, the so-called linker proteins (Fig. 7) (Garrod and Chidgey, 2008; Yin and Green, 2004). The central Armadillo (Arm) domain of plakoglobin interacts with the cytoplasmic tail of the desmosomal cadherin (Troyanovsky et al., 1994a; Troyanovsky et al., 1994b). The N-terminal domain of desmoplakin binds plakoglobin itself while the C-terminal domain of desmoplakin is linked to the intermediate filaments (Kouklis et al., 1994; Kowalczyk et al., 1997). It is also possible that desmoplakin interacts directly with the cytoplasmic tail of the cadherin, without the interposition of plakoglobin (Troyanovsky et al., 1994b). Plakophilins can bind to all members of this complex (i.e. the cadherin, plakoglobin, desmoplakin and the intermediate filaments), and serve as a linker protein (Bonne et al., 2003).

However, it is also possible that plakophilins are responsible for lateral interactions in the same way as suggested for plakoglobins (Garrod and Chidgey, 2008; Kowalczyk et al., 1999; Thomason et al., 2010).
The intracellular binding of one half of a desmosome with the intermediate filaments consists of two dense plaques, an outer dense plaque and an inner dense plaque (Bornslaeger et al., 2001; Delva et al., 2009; Yin and Green, 2004). The outer dense plaque comprises the intracellular tail of the desmosomal cadherins, the two proteins associated with that tail (plakoglobin and plakophilin) and the N-terminal domain of desmoplakin, providing the link with the intermediate filaments. The inner dense plaque is thinner and includes the intermediate filaments and the C-terminal domain of desmoplakin.

1.1.3. Adherens junctions: a combination of two basic units

Two basic units form adherens junctions (AJs): nectin-afadin complex and the cadherin-catenin complex (Fig. 7). The first major adhesive protein complex at the AJs is a connection between the membrane-bound nectin family and the scaffolding protein afadin. The nectin family of IgG-like adhesion receptors consists of four members, nectin-1 to -4. They consist of an extracellular domain with three IgG-like loops, a transmembrane domain and a cytoplasmic domain with a PDZ binding domain (Irie et al., 2004). Nectins form lateral, *cis*-homodimers at the membrane but can form both homo- and heterophilic *trans*-interactions with other nectins or with members of the nectin-related family nectin-like receptors (Sakisaka et al., 2007). To provide the link with the cytoskeleton, nectins couple to afadin (Reymond et al., 2001). Afadin has different binding sites for nectin and actin which suggests a direct linkage between the nectin-based adhesion site and the cytoskeleton (Mandai et al., 1997).

However, an indirect link can also be established both with the actin cytoskeleton (via vinculin or α-actinin) (Mandai et al., 1999; Ooshio et al., 2004) and with the cadherin-catenin complex (via α-catenin) (Pokutta et al., 2002). Because of this interplay it is very likely that these two cell adhesion complexes are physically linked and therefore able to influence each other's activity and localization (Sakisaka et al., 2007).

Nectin-2, like E-cadherin, is present in the so-called primordial AJs or punta which typically form at newly created sites of cell adhesion at the tips of the protrusions of two contacting cells (Asakura et al., 1999; Perez-Moreno and Fuchs, 2006). Moreover, nectins and cadherins both share the ability to activate small GTPases like Cdc42 and Rac1 which is known to facilitate junction formation. This suggests that both nectins and cadherins regulate the actin cytoskeleton at sites of cell adhesion (Kawakatsu et al., 2005).

Since the cadherin-catenin protein complex is the second major component of AJs and the major topic of this PhD-thesis, the different components, their role in adhesion and how they influence cell signalling will be elaborately discussed below. Generally, the cadherin-catenin complex consists of a transmembrane cadherin which is stabilized at the membrane by binding of p120 catenin (p120ctn) on the cytoplasmic tail, close to the membrane. The C-terminal end of the cytoplasmic tail of the cadherin comprises a binding site for β-catenin. This chain of molecules is linked to the actin cytoskeleton by the association of β-catenin with α-catenin, which binds F-actin (Fig. 7). The image of this rigid structure providing strong cell adhesion strength was assumed for many years until it was shown that it was impossible for α-catenin to simultaneous bind β-catenin and actin (Drees et al., 2005; Yamada et al., 2005).

1.2. Classical cadherin-based adherens junctions: structure and organization

The superfamily of cadherins consists of the classical or type-I cadherins, the atypical or type-II cadherins, desmosomal cadherins, protocadherins and Flamingo cadherins. All subfamilies share the presence of several ectodomains (EC) and a short transmembrane domain. The amount of EC, the presence of a cytoplasmic tail, and the intracellular linkage with other proteins differs between the subfamilies (Angst et al., 2001; Hulpiau and van Roy, 2009; Nollet et al., 2000).

The subfamily of classical or type-I cadherins is one of the components of the well known adherens junctions. Classical cadherins themselves constitute a group including E-cadherin (cdh1), typical for epithelial cells; N-cadherin (cdh2), first identified in neural cells; and C-cadherin, only found in muscles and epithelia of *Xenopus laevis* embryos. Characteristic for this subfamily is the presence of five EC, a short transmembrane domain and a cytoplasmic tail (Nollet et al., 2000). The extracellular part of the cadherin is a rigid structure due to the binding of Ca^{2+}-ions. Without the binding of Ca^{2+} this extracellular part is limp and cannot function properly. The function of this extracellular part is to link to the extracellular part of a classical cadherin localized on the membrane of the neighbouring cell. Cadherins prefer homophilic binding: they prefer the linkage with a cell expressing the same cadherin family member (Ebnet, 2008; Halbleib and Nelson, 2006; Meng and Takeichi, 2009; Niessen et al., 2011). Similar cadherins bind with different strength: C-cadherin/C-cadherin binding is the strongest, N-cadherin/N-cadherin binding the weakest, and E-cadherin/E-cadherin binding is of intermediate strength (Katsamba et al., 2009; Prakasam et al., 2006). Cell sorting in cell culture experiments has shown that cells expressing the same type of cadherin cluster together. The segregation of cells into tissues is based on the differential expression of different cadherins in

different cell types (Halbleib and Nelson, 2006; Steinberg and Takeichi, 1994). The binding of two extracellular domains, each on an adjacent cell, is carried out by the first N-terminal extracellular domain (EC1). Indeed, the replacement of the native EC1 by the EC1 of another classical cadherin converts the preferential binding of this cadherin to the cadherin to which the new EC1 belongs. This implicates that the EC1 domain contains a binding site necessary for specific homophilic adhesion. The role of the other four ectodomains remains unknown though it is suggested to play a role in homophilic binding (Nose et al., 1990; Yap et al., 1997). The other ectodomains together can serve as a spacer between the two neighbouring cell membranes and it has been shown that without those other domains no functional binding or adhesive strength can be maintained (Shapiro et al., 1995; Yap et al., 1997). Furthermore, also heterophilic binding does occur, since not all classical cadherins bind solely to equal cadherins but also to other types of cadherins. The magnitude of the heterophilic adhesion, with the exception of N-cadherin/C-cadherin and N-cadherin/E-cadherin bounds, is equal to homophilic adhesion. These two exceptions were stronger than homophilic N-cadherin/N-cadherin interaction (Katsamba et al., 2009; Prakasam et al., 2006). The cadherin ectodomains are more stable when functioning as parallel, lateral dimers. Monomers are considered to be less active and therefore the lateral clustering of cadherins (*cis*-interaction) is necessary to provide strong adhesive force. Formations of *cis*-homodimers of either E- or N-cadherin has been previously reported in various epithelial systems (Gumbiner, 2005; Wheelock and Johnson, 2003b; Zhang et al., 2009). Only recently it has been reported that E- and N-cadherin can form *cis*-heterodimers in several endoderm-derived tissues and tumors (Straub et al., 2011). These E-N heterodimers can even form stable interaction with N-cadherin homodimers of various mesenchymal cells.

1.3. Cadherin-mediated cell adhesion is regulated in many ways

The components of the cadherin-catenin complex mutually influence and regulate each other. When the cadherin-catenin complex is formed, possible transcriptional active proteins such as β-catenin and p120ctn are bound to the cadherin and retained at the membrane. This implies that cadherins can repress or induce gene transcription (Cavallaro and Dejana, 2011) as explained below. On the other hand, cadherins themselves, they get also regulated by the protein it gets bound to: an overview.

1.3.1. By interaction with β-catenin

β-catenin is constitutively bound to cadherins and is also a key player in Wnt signalling (Bienz, 2005; Clevers, 2006; Huelsken and Held, 2009). In absence of Wnt, β-catenin is rapidly inactivated when it is released for some reason into the cytoplasm. This inactivation includes phosphorylation and ubiquitylation by a destruction complex which includes axin and adenomatous polyposis coli (APC). This complex is dismantled when Wnt is present which results in the translocation of β-catenin to the nucleus. Nuclear β-catenin will bind lymphoid enhancer factor/T-cell factor (LEF/TCF) and thereby modulate gene transcription (Gordon and Nusse, 2006; van Amerongen and Nusse, 2009). In addition, cadherins can influence β-catenin signalling by promoting its degradation through a membrane-associated degradation complex (Heuberger and Birchmeier, 2010; Maher et al., 2009). Cadherins do not only influence transcriptional activity of β-catenin by holding it at the plasma membrane, they also increase the degradation at the membrane thereby increasing the turnover of cytoplasmic β-catenin.

β-catenin can regulate the adhesive state of the cadherins by changing its own phosphorylation status.

For example, phosphorylation of tyrosine 142 results in the loss of cadherin adhesion and thus in an increased dissociation of β-catenin and α-catenin (Piedra et al., 2003). There are several other studies on where phosphorylation of β-catenin always results in a downregulation of cadherin-mediated adhesion (Rhee et al., 2002; Roura et al., 1999). Not only does it affect its interaction with the cadherins, phosphorylation of β-catenin does also influence the binding strength with α-catenin. A stronger interaction between these two catenins, which improves the strength of the cadherin-mediated adhesion, is obtained by the phosphorylation of β-catenin by casein kinase II (CKII) (Bek and Kemler, 2002).

Phosphorylation also determines the strength of association of β-catenin with the cadherin. The phosphorylation of the cadherin-serine residues takes place during biosynthesis of the cadherin at the endoplasmatic reticulum and leads to an augmented affinity of cadherins for β-catenin (Lickert et al., 2000; Simcha et al., 2001). The subsequent binding of β-catenin masks the PEST sequence region of the cadherin, which is responsible for its degradation (Huber et al., 2001).

1.3.2. Through α-catenin activity

α-catenin can interact with different actin-binding proteins such as IQGAP1 and formin-1, which regulates the strength of the adhesion. The binding of α-catenin and formin-1 leads to actin polymerization at the sites of emerging cadherin contacts resulting in a functional link to the actin cytoskeleton and consequently in a strong adhesive force (Bershadsky, 2004).

In contrast to formin-1, IQGAP1 activity results in weaker adhesive properties because it competes with α-catenin for the binding of β-catenin. When IQGAP1 is bound to β-catenin, there is no linkage of the cadherin with the actin cytoskeleton which results in a weak adhesion (Kuroda et al., 1998).

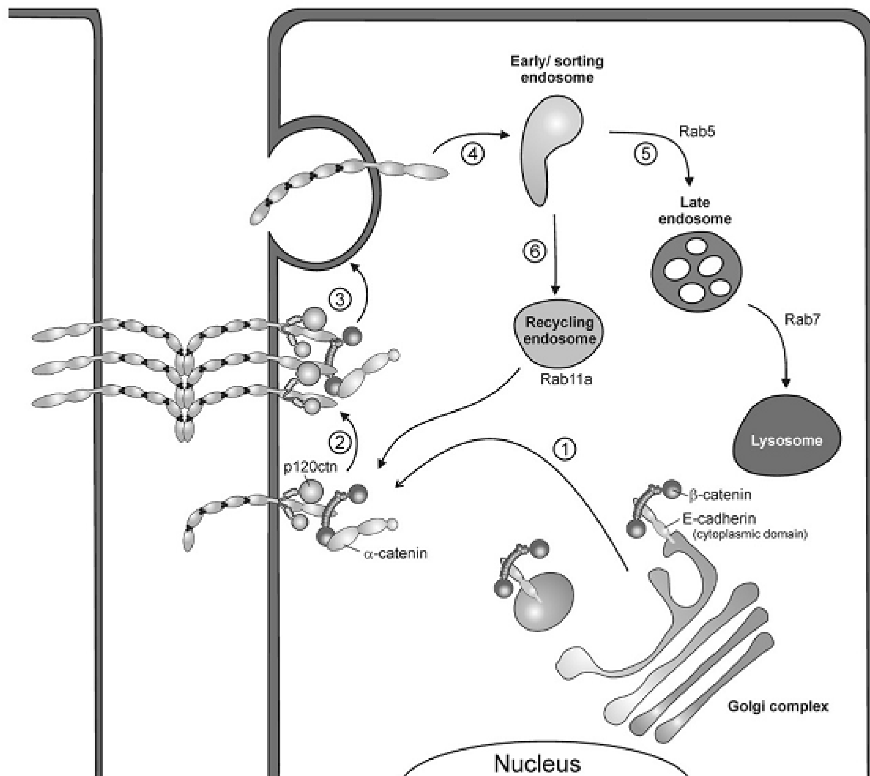

Figure 8. Biosynthesis, endocytosis and recycling of E-cadherin

Biosynthesis of E-cadherin occurs in the Golgi apparatus where it becomes bound to β-catenin (1). Together, they transfer to the basolateral plasma membrane where the cadherin is bound by p120ctn, stabilizing the complex at the membrane (2). At the membrane, the functional cadherin-catenin complex is formed. When endocytosis starts (3), E-cadherin is internalized and transported to the early sorting endosome (4). From the early endosome, E-cadherin can be recycled back to the membrane via the recycling endosome (6) or is marked for final degradation via the late endosome (5) (after van Roy and Berx (2008)).

1.3.3. By endocytosis and recycling

In order to adapt to internal and external signals, adhesive complexes are highly dynamic. Biosynthesis of E- and N-cadherin occurs in the endoplasmatic reticulum (ER) after which the predomain of the cadherin molecule is cleaved off in the Golgi apparatus (Fig. 8). Subsequently, β-catenin binds to E-cadherin and they are transported to the AJ at the plasma membrane together (Chen et al., 1999; Miranda et al., 2001). Upon delivery at the cell surface, the cadherin is regulated by phosphorylation, ubiquitination and proteolysis (Halbleib and Nelson, 2006) and bound by p120ctn, which is necessary for stabilizing this complex at the membrane (Thoreson et al., 2000). p120ctn already binds to N-cadherin during its biosynthesis (Wahl et al., 2003). For recycling, the cadherins are transported to recycling endosomes, trafficked to late endosomes and finally returned to the cell membrane. When cell-cell junctions are disrupted in any way, the cadherins are actively internalized and marked for degradation (Delva and Kowalczyk, 2009; Meng and Takeichi, 2009; Xiao et al., 2007).

1.4. Cadherins and their involvement in signalling pathways

1.4.1. Introduction

Cadherins are proteins with much more influence than mere adhesion during multiple processes. Not only are they fundamental during cell growth, apoptosis and cell differentiation as important mediators of mechanical adhesion between cells, they also cause intracellular signals. As cadherins are linked to multiple important signalling networks, they can serve as sensors to adapt cell behaviour in relation to the surrounding environment.

Indirectly, cadherins recruit signalling proteins to the membrane such as p120ctn, β-catenin and plakoglobin in order to incorporate them into the cadherin-catenin complex. Once these molecules are released from their cytoplasmic tail, they can transfer to the nucleus and regulate transcription. How p120ctn influences transcriptional levels is described in section I, Chapter II.3.4., β-catenin and plakoglobin in section I, Chapter II.2.

In this section, an overview will be given on how cadherins can form signalling units through interaction with growth factor receptors, with intracellular signalling partners and adaptor proteins. In addition, part of the cytoplasmic tail or the ectodomain of the cadherin can be cleaved and translocated to the nucleus (Riedle et al., 2009). Finally, a flow sensor complex that transfers intracellular signals can also be formed (Fig. 9).

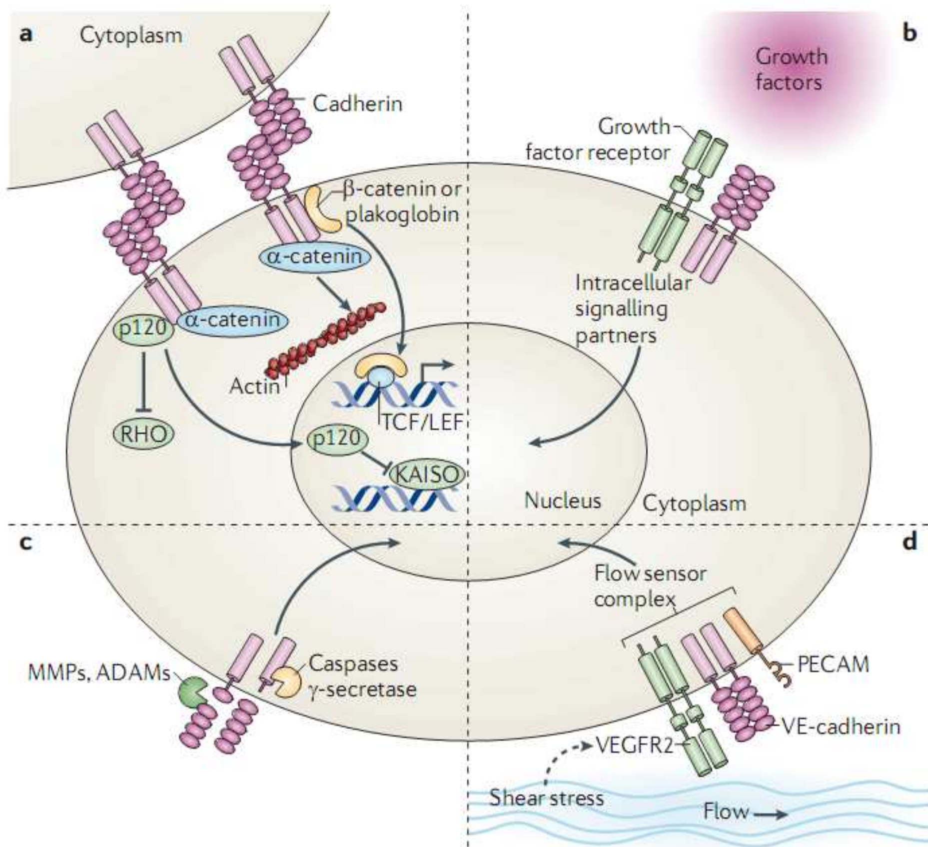

Figure 9. Overview of the signalling pathways regulated by cadherins

a) Cadherins can indirectly influence signalling by recruiting signalling proteins to the membrane such as p120ctn, β-catenin and plakoglobin in order to incorporate them into the cadherin-catenin complex. Once these molecules are released from the cadherin cytoplasmic tail, they can transfer to the nucleus to influence gene transcription or interact with other molecules in the cytoplasm. b) Cadherins can form signalling units by interacting with growth factor receptors or with intracellular signalling partners such as kinases. c) Cleavage of the ectodomain or intracellular domain of cadherins can influence several processes. The cleaved cytoplasmic tail can even translocate to the nucleus and manipulate transcription. d) VE-cadherin can form a flow sensor complex when bound to PECAM and VEGFR2. In this way, the cells can adapt to shear stress by transferring intracellular signals (after Cavallaro and Dejana (2011)).

1.4.2. Cadherins can form signalling units by interacting with several binding partners

Several studies have shown that cell adhesion molecules can modulate growth factor receptor signalling. In general, cadherins affect the interaction of the growth factor with its receptor or influence the response of the receptor on the stimulation of the growth factor itself (Fig. 9B). A few examples are clarified below.

When FGFR (a typical receptor Tyr kinase (RTK)) is recognized by its ligand FGF, binding occurs. Subsequently, FGFR undergoes dimerization and autophosphorylation and multiple signal transduction signals are triggered (Knights and Cook, 2010). Because of the *cis*-dimerization of N-cadherin, FGFR dimerization is facilitated, thus initiating growth factor-independent signalling (Utton et al., 2001; Williams et al., 2001). On the other hand, N-cadherin-mediated invasion is repressed by downstream inhibitors of Fgf signalling (Nieman et al., 1999). In addition, FGFR can interact with the extracellular domain of N-cadherin thereby preventing internalization of the receptor. This results in a bigger amount of FGFR at the cell surface and consequently a constant activation of the FGFR by FGF (Suyama et al., 2002).

VE-cadherin is known to modulate signalling by interacting with vascular endothelial growth factor receptor 2 (VEGFR2) which requires the association of the cytoplasmic domain of VE-cadherin with the receptor. When bound to each other, VEGFR2 can remain at the plasma membrane as it is dephosphorylated and therefore loses its mark for degradation (Grazia Lampugnani et al., 2003). Intracellularly, the MAPK pathway (important for proliferation) is influenced by VEGFR2 and as a result of the dephosphorylation, proliferation is inhibited. This way, VE-cadherin results in cell growth inhibition (Lampugnani et al., 2006).

A third example of interplay was found by studying the role of the desmosomal cadherin desmoglein-1 (DSG-1). A mutant version of DSG-1, lacking the N-terminal ectodomain, does not have any adhesive function but is able to start keratinocyte differentiation (Getsios et al., 2009), which is linked to the suppression of the EGFR-mediated extracellular signal-regulated kinase 1 (ERK1) and ERK2 pathway (Lacouture, 2006). DSG-1 and EGFR co-localize at the plasma membrane suggesting that they might be a component of the same protein complex, but the exact mechanism has not yet been clarified.

1.4.3. Cleavage of the ectodomain or cytoplasmic tail of cadherins can mediate signalling pathways

Cell adhesion molecules do not only contribute to multiple signalling pathways by interactions at the plasma membrane, they can also influence nuclear processes. This either directly, through their activity in the nucleus or indirectly, by regulating nuclear trafficking of certain transcriptional activators (Fig. 9C).

Multiple studies have shown that proteolytic processing of cell adhesion molecules can influence signalling. The cytoplasmic domain or the ectodomain of cadherins can be cleaved by several proteases such as caspases, MMPs (matrix metalloproteases) and members of the ADAM (a disintegrin and metalloprotease) family. However, there is still no clarity on the complete biological function of the resulting separated cytoplasmic fragment (Dusek et al., 2006; Steinhusen et al., 2001; Vallorosi et al., 2000).

A study reported an increased β-catenin-dependent transcription due to the cleaved cytoplasmic domain of N-cadherin (Shoval et al., 2007). N-cadherin is cleaved by ADAM10 which is induced by Bmp4 (bone morphogenetic protein 4) from the TGFβ family (Cavallaro and Dejana, 2011; Shoval et al., 2007).

In addition, the C-terminal fragment of desmoglein-2, cleaved by Cys protease, is shown to be pro-apoptotic (Nava et al., 2007). Only recently it was shown that the cleavage of E-cadherin by MMP20 is necessary for ameloblast development in mouse tooth development (Bartlett et al., 2011). The hydrolysis of E-cadherin results in the release of intracellular transcription factors, such as β-catenin, which are important for enamel development (Bartlett et al., 2011).
Another possibility to obtain a signalling function for cell adhesion molecules is by shedding their ectodomains, resulting in soluble ligands with various biological functions (van Kilsdonk et al., 2010). The soluble ectodomain of JAM-C (junctional adhesion molecule C) has been reported to promote angiogenesis (Rabquer et al., 2010) and the cleaved N-cadherin ectodomain has also been related to angiogenesis and stimulation of neurite outgrowth (Latefi et al., 2009; Niessen et al., 2011).

1.4.4. Cell adhesion molecules as a component of a mechanosensory complex

As described above in 2.4.2. VE-cadherin can interact with VEGFR2 thereby contributing to cell growth inhibition. In addition, VE-cadherin can also act as an adaptor between PECAM (platelet endothelial cell adhesion molecule) and VEGFR2 (Shay-Salit et al., 2002). The formation of this protein complex appeared important during exposure to shear stress (Fig. 9D) (Tzima et al., 2005). PECAM transmits mechanical force through SRC family kinase activity and VEGFR2 activates phosphoinositide 3-kinase (PI3K) through which the formation of this complex leads to rearrangements of the actin cytoskeleton, integrin activation and activation of the nuclear factor-κB pathway (Shay-Salit et al., 2002).

2. BETA-CATENIN AND PLAKOGLOBIN

2.1. β-catenin

2.1.1. Introduction

Primarily, β-catenin is an essential part of the cadherin-catenin complex. It is known to bind the catenin binding domain at the C-terminal end of the cadherin cytoplasmic tail. In the classical view, β-catenin provides a dynamic link with α-catenin that regulates the underlying actin cytoskeleton. The linkage with F-actin gives the cadherin-catenin complex strong adhesive force (Meng and Takeichi, 2009; Nelson, 2008; Shapiro and Weis, 2009). Not only is β-catenin a key player in cell-cell adhesion, it is also known as a downstream effector of the Wnt signalling pathway (Bienz, 2005; Clevers, 2006; Nelson and Nusse, 2004).

β-catenin is a highly conserved protein; there is only a six amino acid difference between the human and *Xenopus* protein and it is 67% identical to armadillo, the *Drosophila* homolog. Moreover, β-catenin, unlike other catenins, does not have tissue-specific variants and thus only one β-catenin protein exists in mammals and insects (Shapiro and Weis, 2009). The 781 AA β-catenin protein contains a central structural core region of 12 Arm-repeats, an amino-terminal domain of about 150 AA and a carboxy-terminal 100 residue region (Huber et al., 1997; Xing et al., 2008).

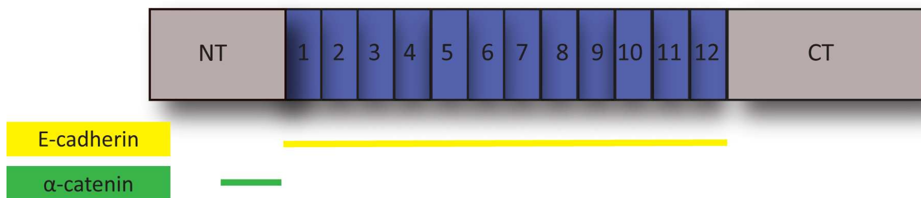

Figure 10. β-catenin primary structure

β-catenin protein contains a central structural core region of 12 Arm-repeats, an amino-terminal domain of about 150 AA and a carboxy-terminal 100 residue region. α-catenin binds β-catenin in the region just before the first Arm-domain. The link between E-cadherin and β-catenin is provided by all 12 Arm-domains of β-catenin.

β-catenin can be bound by α-catenin at the region just before the Arm-domain while the Arm-domain itself enables the linkage with E-cadherin (Fig. 10) (Aberle et al., 1996a; Aberle et al., 1996b). A positively charged groove spans the entire superhelical Arm-repeat region that constitutes the binding surface for the majority of β-catenin partners, many important for cell adhesion (E-cadherin) and Wnt signalling (APC, TCF, LEF) (Graham et al., 2002; Poy et al., 2001; Xing et al., 2004).

Sequences of the N- and C-terminal domains of β-catenin are less conserved than the Arm-domain. Nevertheless, they also mediate protein-protein interaction such as the interaction with α-catenin or with ligases (Shapiro and Weis, 2009; Wu et al., 2003).

2.1.2. β-catenin switches between cell adhesion and canonical Wnt signalling

β-catenin plays a central role in both cell adhesion and as one of the main components of the AJs and in the canonical Wnt signalling pathway, which is known for its importance during multiple developmental processes.

These two different but essential concepts depend on the same pool of β-catenin. For example, overexpression of cadherins in *Xenopus* resulted in the inhibition of dorsal axis formation, which is a function of the canonical Wnt pathway (Fagotto et al., 1996). Sanson et al. (1996) showed that cadherin overexpression in *Drosophila* embryos mimicked the wingless (Wnt) phenotype. Inversely, Wnt-induced increase in β-catenin levels may lead to saturation of β-catenin binding to E-cadherin and an increase in cell adhesion (Hinck et al., 1994).

Adherens junctions and more specific the cadherin-catenin complex is extensively discussed in Section I, Chapter II.1. β-catenin also fulfils a function during Wnt signalling. The soluble cytoplasmic pool of β-catenin is highly unstable in the absence of a Wnt-signal as a result of several phosphorylations at the N-terminus. As β-catenin is marked for degradation, it will bind APC which results in a complex including the scaffolding protein Axin and two serine/threonine protein kinases, i.e. glycogen synthase kinase 3β (GSK3) and casein kinase 1. This complex will destroy the redundant β-catenin and the transcription factors LEF and TCF, and will interact with Grouchos in the nucleus to repress Wnt-specific target genes (Klaus and Birchmeier, 2008). In the presence of a Wnt-signal, GSK3 cannot be recruited to the complex and as a result this complex fails to form. Accumulation of β-catenin in the cytoplasm follows, after which β-catenin will translocate to the nucleus and bind LEF/TCF there. This results in the transcription of several Wnt-target genes, such as L1-CAM, Snail, Slug and Matrilysin, that have an important role during normal and malignant development (Fig. 11) (Klaus and Birchmeier, 2008; Nelson and Nusse, 2004).

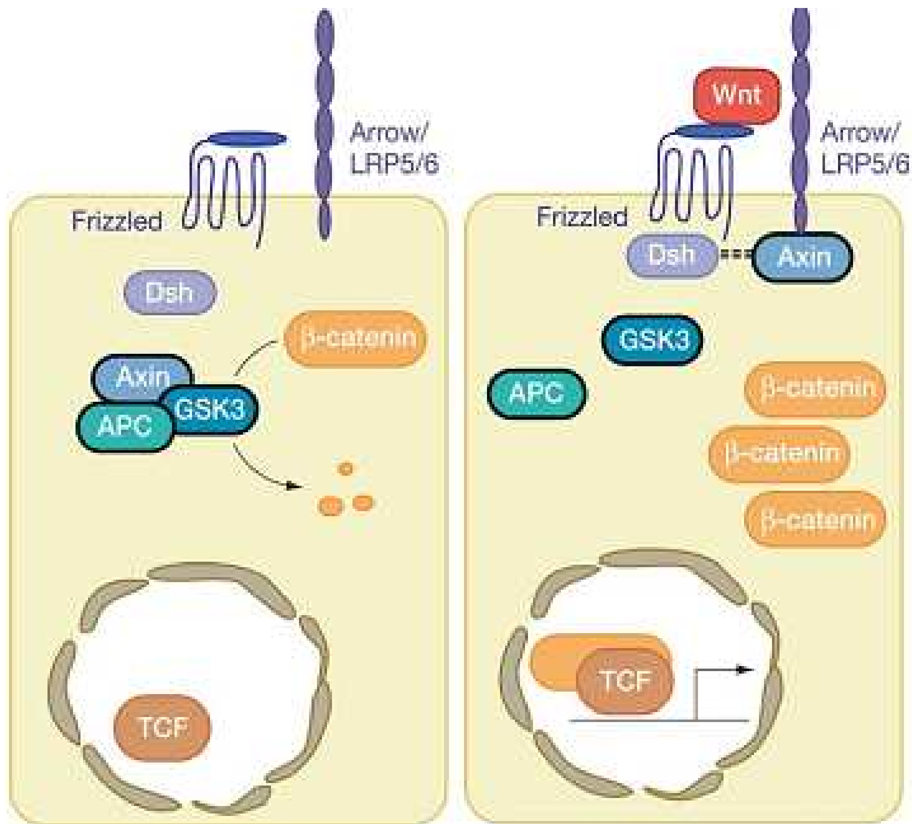

Figure 11. Simple overview of the canonical Wnt signalling pathway

Left panel: β-catenin becomes degraded by the degradation complex (consisting of Axin, APC and GSK3), when the cell is not exposed to a Wnt-signal. Right panel: When a Wnt-protein binds the Frizzled/LRP complex, Axin is recruited to the membrane and the degradation complex fails to form. Therefore, cytoplasmic β-catenin is not degraded and accumulates in the cytoplasm and transfers to the nucleus to bind LEF/TCF (after Logan and Nusse (2004)).

The main principle of the pivoting of β-catenin between cell adhesion and Wnt signalling depends on the stability and availability of β-catenin as well as regulatory loops that allow the coordination between the two systems. Phosphorylation is an important process in favouring one system over the other.

Depending on the site of phosphorylation of the C-terminal domain of the cadherins, they can either strengthen or weaken the interactions with β-catenin (Choi et al., 2006; Qi et al., 2006). But also the phosphorylation of β-catenin itself can influence its binding to the cadherin and therefore influence adhesion functions (Rhee et al., 2002; Roura et al., 1999). Moreover, components of the Wnt pathway such as axin, APC and TCF also become phosphorylated which influences the binding capacity to β-catenin (Ha et al., 2004; Lee et al., 2001; Rubinfeld et al., 1996; Willert et al., 1999) and therefore the availability of β-catenin in the cytoplasm. If for some reason the cadherin-catenin complex becomes distorted, for example through cleavage of the cadherin, β-catenin is released from the complex and is able to translocate to the nucleus (Cavallaro and Dejana, 2011; Heuberger and Birchmeier, 2010).
It is not desirable that when Wnt signalling is active, cell adhesion would be weakened. In which of the two systems β-catenin will be active, is affected by other signals such as phosphorylation and competition between the interacting proteins E-cadherin, APC and TCF (Bienz, 2005; Nelson and Nusse, 2004).

2.1.3. The importance of β-catenin during development

As a result of its multiple functions in the cell and therefore during development in general, it is no surprise that the loss of β-catenin in an organism is embryonic lethal. During early development in mouse, trophectoderm formation is not affected by the loss of β-catenin probably because of the presence of a sufficient amount of maternal β-catenin. The augmented morphogenetic movements and cell proliferation in the embryonic ectodermal cell layer is the primary defect in this β-catenin null mouse. The absence of β-catenin results in defects of the adhesive system leading to improper integration of the dividing cells.

Alternatively, β-catenin may have a signalling role in the ectodermal cell layer that can no longer be executed (Haegel et al., 1995). Multiple studies target β-catenin in specific mouse tissues, all showing the importance of this protein. Huelsken and co-workers (2001) introduced a conditional mutation of the gene in the epidermis and hair follicles of mice. When β-catenin is lost during embryogenesis, the formation of hair placodes is blocked. Additionally, they showed that when β-catenin is deleted after the formation of hair follicles, all hair is completely lost after the first hair cycle (Huelsken et al., 2001). When deleting β-catenin in mouse limb and head mesenchyme, it was found that β-catenin is required for osteoblast lineage differentiation. Osteoblast precursors lacking β-catenin are blocked in differentiation and developed into chondrocytes (Hill et al., 2005). Mice with impaired formation of the coronary arteries resulted from the conditional deletion of β-catenin in the proepicardium (Zamora et al., 2007). Inactivation of β-catenin in the domain of *Wnt1* expression (neural crest cells) in mice results in dramatic brain malformation and failure of craniofacial development (Brault et al., 2001).

2.1.4. β-catenin in zebrafish

For several years, research has been conducted to unravel wether β-catenin in zebrafish has the same essential roles as in mouse development. It has been found that β-catenin mediates Wnt signalling and that this mechanism is important for axis formation and neural induction (Haegel et al., 1995; Kelly et al., 2000). The loss of maternal β-catenin, as in the spontaneous *ichabod* mutation, results in severe ventralization, since β-catenin is unable to localize to the dorsal yolk syncytial layer and blastomere nuclei. Perturbation of β-catenin regulation in zebrafish leads to the loss of dorsal axial structures (Kelly et al., 2000).

In zebrafish, two β-catenin genes have been discovered, β-catenin-1 (*ctnnb1*) and β-catenin-2 (*ctnnb2*) (Bellipanni et al., 2006; Kelly et al., 1995). Paralogs occur regularly in the zebrafish because of genome duplication during evolution (Taylor et al., 2003). When comparing the two β-catenin cDNAs, no significant homology was found in the 5'UTR and 3'UTR. The two resulting proteins are nevertheless highly similar, only the C-terminal region shows differences. The only zebrafish β-catenin mutant known, the *ichabod* mutant, is a consequence of the maternal loss of β-catenin-2. When only β-catenin-1 function is inhibited, no early patterning defects are observed (Bellipanni et al., 2006). When blocking the function of both genes at the same time, so-called *ciuffo* embryos develop that are dorsalized and express all neurectodermal markers. This means that both genes work with functional redundancy to restrict formation of the neurectoderm (Bellipanni et al., 2006).

2.2. Plakoglobin

2.2.1. Introduction

Plakoglobin (also known as γ-catenin) is a multifunctional protein known to be important for cell adhesion and signalization (Choi et al., 2009; Delva et al., 2009; Zhurinsky et al., 2000). It belongs, together with β-catenin, to the β-catenin subfamily (Zhao et al., 2011). Plakoglobin is highly homologous with β-catenin as they both posses a central region of 12 Arm-repeats that enables them to interact with the cadherins (Troyanovsky et al., 1996; Zhurinsky et al., 2000), flanked by distinct N- and C-terminal domains (Fig. 12) (Garrod and Chidgey, 2008; Garrod et al., 2002; Huber et al., 1997).

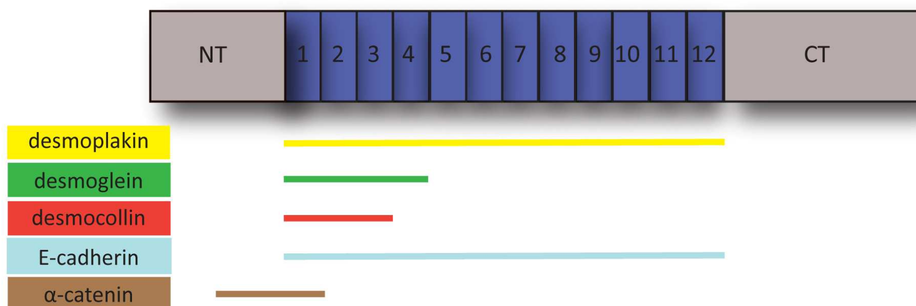

Figure 12. Plakoglobin primary structure

Plakoglobin contains 12 Arm-repeats flanked by distinct amino- and carboxy-terminal domains. The N-terminal domain and several Arm-repeats near the C-terminal domain are key for desmosomal cadherin binding. The central Arm-domain is necessary for the interaction of plakoglobin with desmoplakin. Coloured bars indicate binding region.

Plakoglobin is particularly known to interact with desmosomal cadherins, which is why it is an important component of desmosomes (Delva et al., 2009). Because of their resemblance, it is not surprising that plakoglobin, just as β-catenin, has also been detected while coupled to classical cadherins.

Indeed, in AJs plakoglobin and β-catenin compete for binding to the catenin-binding site on the intracellular tail of classical cadherins and each of them can link with α-catenin to anchor the complex to actin. In desmosomes, plakoglobin binds to the desmosomal cadherins and to desmoplakin to provide a link with the intermediate filament cytoskeleton (Delva et al., 2009; Zhurinsky et al., 2000). The binding sites in plakoglobin for desmosomal or classical cadherins are distinct. At least six Arm-repeats are necessary for the binding of a classical cadherin while only three N-terminal Arm-repeats are required for linkage to desmosomal cadherins (Troyanovsky et al., 1996). Interestingly, when plakoglobin is incorporated into desmosomes, it loses its ability to bind with α-catenin. Therefore, the interactions of plakoglobin with α-catenin and desmosomal cadherins appear to be mutually exclusive (Chitaev et al., 1998; Troyanovsky et al., 1996; Zhurinsky et al., 2000). The cadherin-plakoglobin complex is an important intermediate complex, since first adherens junctions need to be formed before desmosomes can start to be assembled (Lewis et al., 1997; Yin and Green, 2004). If plakoglobin is not present, other molecules such as β-catenin and p0071 can substitute for its presence, both in adherens junctions and in desmosomes (Hatzfeld et al., 2003). When not incorporated in a junction, plakoglobin is marked for degradation by the proteosome machinery, but the efficiency is less than compared to the degradation of β-catenin (Choi et al., 2009; Zhurinsky et al., 2000).

2.2.2. Convergence of plakoglobin and β-catenin during Wnt signalling

The Wnt signalling pathway is of major importance during several developmental processes. Since the interaction of β-catenin with transcription factors is mediated mostly by the Arm-domain of the protein, which is highly homologous to that of plakoglobin, plakoglobin might also interact with the same nuclear partners such as TCF (Cowin and Burke, 1996; Zhurinsky et al., 2000). Indeed, plakoglobin can influence the Wnt signalling pathway in different ways. Plakoglobin is able to shuttle to the nucleus and act as a transcriptional activator on its own. The target genes, however, are different from the target genes of β-catenin (Garrod and Chidgey, 2008; Kolligs et al., 2000; Zhurinsky et al., 2000). A second possibility is that plakoglobin interferes with the cytoplasmic degradation of β-catenin hence allowing β-catenin to transfer to the nucleus (Merriam et al., 1997; Zhurinsky et al., 2000). A third possibility is that plakoglobin will replace β-catenin in the cadherin-catenin complex of the adherens junctions, allowing β-catenin to transfer to the nucleus (Garrod and Chidgey, 2008).

2.2.3. The importance of plakoglobin during development

It is clear from the above studies, mostly on mice, that plakoglobin is a protein with multiple functions and therefore extremely interesting to understand its role during development and morphogenesis. The total KO of plakoglobin in mouse results in embryonic lethality (Bierkamp et al., 1996). Plakoglobin null mice die because of a heart defect as a result of impaired cell adhesion of the cardiomyocytes. Desmosome morphology is defective and the overall number of desmosomes present is significantly decreased (Bierkamp et al., 1996; Grossmann et al., 2004). Some plakoglobin KO mice survive longer but show blistering of the skin. A possible reason for the longer survival of plakoglobin -/-

mice is that β-catenin can substitute for plakoglobin in the desmosomes, or that desmoplakin can directly bind to the desmosomal cadherins without the linkage with plakoglobin (Bierkamp et al., 1996; Bierkamp et al., 1999; Thomason et al., 2010). In contrast, when overexpressing plakoglobin under control of the K14 promoter, targeting the skin, these transgenic mice display lower hair growth and a decrease in the period of the hair growth phase (Charpentier et al., 2000).

2.2.4. Plakoglobin in zebrafish

Plakoglobin has also been studied in zebrafish, where it has been detected in all stages of zebrafish development with a remarkable increase in protein level from 12 to 24 hpf (Maurin et al., 2004). Two plakoglobin genes have been discovered in zebrafish: one with a longer transcript, plakoglobin-1a and one with a shorter, plakoglobin-1b (Martin et al., 2009). Plakoglobin-1a contains an additional 1089 bp in the 3' end, which is absent in plakoglobin-1b. Furthermore, plakoglobin-1a contains 13 Arm repeat domains in contrast to only four repeats in plakoglobin-1b. The loss of plakoglobin-1a in zebrafish results in a disturbed midbrain-hindbrain border, reduction in heart size, oedema, and a kinked tail. The loss of plakoglobin-1a cannot be compensated by plakoglobin-1b. It was also shown that in the absence of plakoglobin, Wnt signalling is increased. β-catenin expression was also elevated as a consequence of higher β-catenin translation or protein stability. β-catenin also augmented its binding strength with E-cadherin. In contrast, β-catenin did not substitute for plakoglobin in the desmosomes. In general, it was found that the loss of plakoglobin resulted in a reduction in the amount of adhesion junctions, which had a more diffuse structure (Martin and Grealy, 2004; Martin et al., 2009).

3. P120CATENIN

3.1. Introduction

Initially, p120ctn was identified as a Src (sarcoma) substrate whose phosphorylation correlated with transformation (Reynolds et al., 1992; Reynolds et al., 1989). Further analysis revealed that this molecule contains an Armadillo (Arm) domain, like β-catenin does. Therefore, it was suggested that, like β-catenin, this newly discovered protein had the ability to bind a classical cadherin (Reynolds, 2007; Reynolds and Roczniak-Ferguson, 2004).

p120ctn belongs to the p120ctn subfamily including ARVCF, δ-catenin, and p0071 which all share similar features. One remarkable characteristic is the presence of 9 Arm-repeats that are organized identically into a central domain (Choi and Weis, 2005; Ishiyama et al., 2010). Furthermore, the p120ctn family members also possess a highly conserved N-terminal motif, which encodes for a possible coiled-coil domain. These types of domains are known to be involved in protein-protein interactions. All members of this family, with the exception of p120ctn itself, share a third region of similarity in the C-terminal end: a so-called PDZ-binding motif (Anastasiadis and Reynolds, 2000).

Studies in both human and mouse have revealed the presence of multiple p120ctn isoforms as a result of alternative splicing (Keirsebilck et al., 1998; Mo and Reynolds, 1996). Alternative splicing can occur both in the N-terminal and C-terminal region. The use of four different start codons N-terminally gives rise to p120ctn isoform type 1, 2, 3 or 4 according to which one of the start codons is used as translational start (Keirsebilck et al., 1998; Pieters et al., 2012b). Splicing at the C-terminal end leads to the incorporation of exon A, exon B, both exon A and B, or none of them. Sometimes a third exon, exon C, is included. This exon is positioned between Arm repeat 5 and 6 (Choi and Weis, 2005).

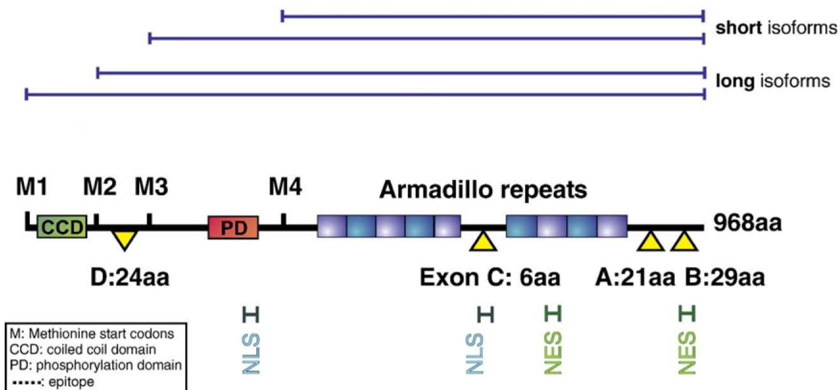

Figure 13. Schematic representation of the human p120catenin protein

p120ctn contains 9 Arm-repeats, the central Arm-repeat domain, flanked by an N- and C-terminal region. There are four possible translation initiation sites (M1-4) and four alternatively spliced exons (exons A-D). Combinations of translational starts and alternative splicing results in several possible isoforms which display tissue- and cell-specific distribution. p120ctn can shuttle in and out of the nucleus. Therefore, two nuclear localization signal (NLS) and two nuclear export signal (NES) sequences are present (after van Hengel and van Roy (2007)).

Combinations of a specific start codon and exons generate a considerable number of possible p120ctn isoforms (Fig. 13) (Keirsebilck et al., 1998). In contrast to the N- and C-terminal domain, which are subjected to splicing, the central Arm domain remains intact and thus free to interact with cadherins. Depending on the cell type, different p120ctn isoforms are expressed, which implies functional differences between the isoforms. Motile cells such as fibroblasts preferentially express p120ctn 1A isoform, in contrast to epithelial cells, which express smaller isoforms such as p120ctn 3A. This differential expression of isoforms suggests a specific function for each of them, e.g. recruiting different cadherins to the membrane (Aho et al., 1999; Keirsebilck et al., 1998; Mo and Reynolds, 1996; Montonen et al., 2001; van Hengel and van Roy, 2007).

3.2. p120ctn regulates cadherin stability and trafficking

Based on the presence of an Arm domain, as in β-catenin, and the co-immunoprecipitation of both catenins, it was first suggested that p120ctn could bind a classical cadherin (Reynolds et al., 1994). All classical cadherins display a highly conserved region near the membrane, the juxtamembrane domain (JMD) for binding this protein, indicating that all classical cadherins bind p120ctn (Anastasiadis and Reynolds, 2000).

Ireton et al. (2002) showed that without the coupling of p120ctn to E-cadherin, the cadherin cannot be stabilized at the membrane and is internalized and degraded. p120ctn loss decreases the expression levels of the components of the cadherin-catenin complex (Davis et al., 2003; Dohn et al., 2009; Ireton et al., 2002). This loss of p120ctn can partially or completely be rescued by the function of another p120ctn family member, but only when they are overexpressed (Davis et al., 2003; Mariner et al., 2000; Yang et al., 2010). None of the family members can substitute for p120ctn at endogenous levels (Davis and Reynolds, 2006; Smalley-Freed et al., 2010).

The biosynthesis of the cadherin occurs within the ER/Golgi apparatus where it becomes coupled to β-catenin, which is essential for the trafficking to the plasma membrane (Chen et al., 1999). If not bound to p120ctn at the membrane, the cadherin will enter a degradative endocytic pathway (Fig. 14). Different internalization pathways can mediate the endocytosis of a cadherin. E-cadherin is typically internalized by a clathrin-dependent pathway. During the internalization process, p120ctn dissociates from the cadherin cytoplasmic tail. This suggests that the release of p120ctn exposes a dileucine motif in the cytoplasmic tail, close to the JMD, by which the cadherin will enter the endocytic pathway. This model suggests that p120ctn serves as a 'cap' that prevents the binding of clathrin adaptor proteins to the tail, necessary for clathrin-mediated endocytosis (Xiao et al., 2007).

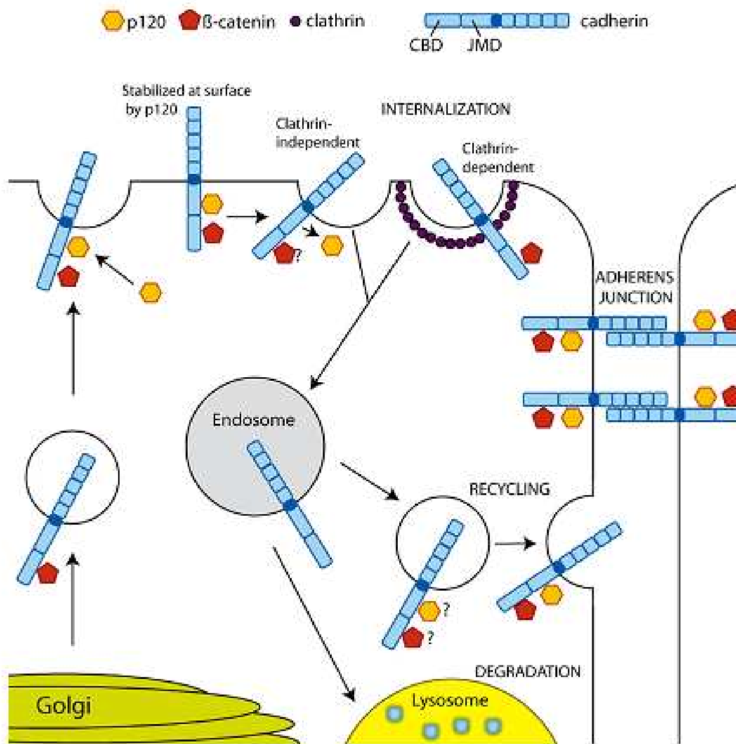

Figure 14. Overview of p120ctn in cadherin trafficking

Biosynthesis of E-cadherin occurs in the Golgi apparartus from which it is transported to the cell membrane. p120ctn binds to the juxtamembrane domain (JMD) of the cadherin cytoplasmic tail at or near the membrane. Cadherins can be internalized via different pathways, including clathrin-dependent endocytosis, but the choice of a specific pathway depends on the tissue or cell type. It was shown that clathrin-dependent endocytosis is blocked when p120ctn is associated with the JMD of VE-cadherin. Moreover, when cells express p120ctn-uncoupled E-cadherin mutants or p120ctn is depleted by RNAi, increased endocytosis is observed. There are several possibilities proposed on how p120ctn can prevent cadherin endocytosis. First, it is possible that p120ctn serves as a cap, preventing the exposure of a dileucine motif close to the JMD. E-cadherin is not internalized when the cadherin tail is not present or when the dileucine motif is mutated. Second, p120ctn competes with Hakai and Presenilin-1 for binding the JMD of the cadherin intracellular part. When Presenilin-1 binds to the cadherin, it becomes degraded by proteolytic cleavage of the cytoplasmic tail. Hakai binding results in ubiquitination and endocytosis of the cadherin (after Xiao et al. (2007)).

Another possibility on how p120ctn prevents endocytosis is by competing with other proteins such as Hakai and Presenilin-1. Hakai functions as an E3 ubiquitin-ligase and interacts with E-cadherin after phosphorylation of the cadherin complex. This leads to internalization of E-cadherin (Fujita et al., 2002; Pece and Gutkind, 2002). Presenilin-1 can also cleave E-cadherin and hence favors E-cadherin degradation (Marambaud et al., 2002).

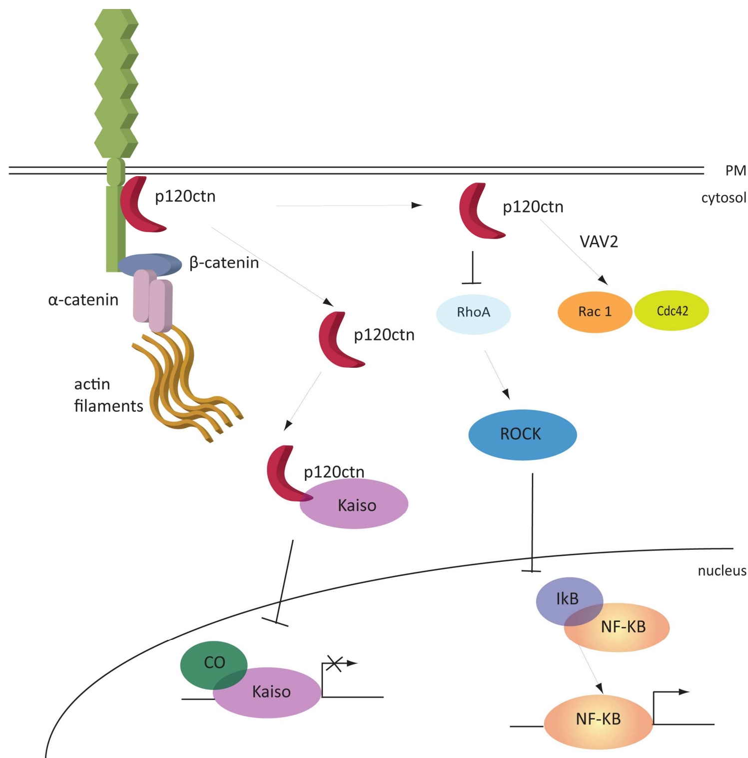

Figure 15. Different functions of p120ctn in the cell

p120ctn is a component of the adherens junctions via the binding to the juxtamembrane domain. However, besides its role at the plasma membrane (PM), p120ctn also displays different functions in the cytoplasm. This catenin can regulate the activity of RhoA, Rac1 and Cdc42 (Rho GTPases) via direct or indirect interaction, resulting in different downstream effects such as the regulation of the NF-κB pathway. The interaction of p120ctn with Kaiso results in the repression of the function of Kaiso. CO, co-repressor; ROCK, Rho kinase.

3.3. p120ctn regulates Rho GTPases

p120ctn has multiple functions besides the association with the plasma membrane-bound cadherin. One of the functions is the interaction with small GTPases (Fig. 15) (Anastasiadis, 2007; Noren et al., 2000). The Rho family of GTPases are known to play a role in cell proliferation, reorganization of the actin cytoskeleton and gene expression (Braga and Yap, 2005). From this family, RhoA, Rac1, and Cdc42 have been well studied and have been suggested as regulators of cadherin-based cell adhesion (Anastasiadis, 2007; Ellis and Mellor, 2000; Xiao et al., 2007). The activation of Cdc42 and Rac will enhance the ability of the cadherin-catenin complex to bind α-catenin and to reorganize the actin cytoskeleton (Noren et al., 2000). Rac is essential for cellular migration while Cdc42 regulates the polarity of the migrating cells. The clustering of the cadherins at the site of first cell-cell contacts is based on the activity of RhoA, implicating that RhoA activity inhibits cell migration (Nobes and Hall, 1999).

Overexpression of p120ctn in different cell types results in a dose-dependent incidence of a branching phenotype (Reynolds et al., 1994; Reynolds et al., 1996). This phenotype describes an array of cell morphologies starting from cells showing the augmented formation of lamellipodia to cells showing severe branching. This branching is the result of the perturbation of actin stress fibers, structures that are dependent on RhoA activity, and the reorganization of the actin cytoskeleton. This suggests that p120ctn inhibits RhoA activity (Noren et al., 2000). Indeed, other assays where RhoA activity has been blocked, such as overexpression of p190 RhoGAP (Rho GTPase activating protein), also results in this branching phenotype (Anastasiadis et al., 2000). Dominant negative Rac1 and Cdc42 expression results in the inhibition of the p120ctn-induced phenotype (Grosheva et al., 2001; Noren et al., 2000). In contrast to RhoA, Rac1 and Cdc42 appear to be activated by p120ctn and result in actin polymerization (Noren et al., 2000).

When overexpressing p120ctn in normal cells, the p120ctn-binding sites of the cadherins available on the membrane are rapidly occupied. The remaining unbound p120ctn molecules then accumulate in the cytoplasm. This uncoupled cytoplasmic p120ctn influences the branching morphology and cell contractility (Reynolds et al., 1996). Indeed, p120ctn, which has a rather weak affinity to the cadherin compared to that for β-catenin, can shuttle between the membrane-bound and cytoplasmic state. In this way, the binding of p120ctn to the cadherin results in the disability to affect Rho GTPases (Noren et al., 2000; Thoreson et al., 2000). The overexpression of p120ctn results in the inhibition of the migration antagonist RhoA and activation of the migratory promoters Rac1 and Cdc42 (Noren et al., 2000). Therefore, it is no surprise that overexpression of p120ctn leads to the stimulation of migration of the cell. How p120ctn inhibits RhoA is still unclear, but two mechanisms have been proposed. The first mechanism is supported by the observation that RhoA is inhibited by Rac1 and Cdc42 in NIH3T3 cells (Sander et al., 1999). The cytoplasmic pool of p120ctn does not react directly with Rac1 and Cdc42 but via the binding of Vav2, a guanine nucleotide exchange factor which can lead to activation of Rac1 and Cdc42 (Noren et al., 2000). Therefore, p120ctn can inhibit RhoA by Rac1-mediated activation of p190RhoGAP via the 'Bar-Sagi' pathway (Niessen and Yap, 2006; Nimnual et al., 2003). The second proposed mechanism states that RhoA can be directly bound by p120ctn and therefore prevents it activation by Rho guanine exchange factors (GEFs) (Anastasiadis, 2007; Grosheva et al., 2001; Noren et al., 2000).

After the first reports on the dendritic-like branching induced by cytoplasmic p120ctn, it turned out that the effect of p120ctn on Rho GTPases varies with cell type, assay conditions, micro- and macro-environments, and types of cadherins expressed (Pieters et al., 2012b).

3.4. p120ctn interacts with Kaiso and regulates transcription

Apart from a membrane and cytoplasmic localization, p120ctn was also found to be able to shuttle in and out of the nucleus (Aho et al., 2002; Kelly et al., 2004; van Hengel et al., 1999). A yeast two-hybrid approach with p120ctn as bait has shown that p120ctn can link to a transcription factor in the nucleus, called Kaiso (Daniel, 2007; Daniel and Reynolds, 1999). The nuclear localization of p120ctn is hard to reveal and has rarely been detected in normal cells. Only when cell lines were treated with an agent blocking the nuclear export, p120ctn accumulation occurred in the nucleus and could be detected (van Hengel et al., 1999). The localization of nuclear p120ctn appears to be a very dynamic and tightly regulated process.

Kaiso is a member of the BTB/POZ (Broad complex, Tramtrak, Bric à brac/Pox virus and zinc finger subfamily of zinc-finger proteins (Daniel and Reynolds, 1999). Members of this family of transcription factors are implicated in embryogenesis and cancer (Albagli et al., 1995; Maeda et al., 2005; van Roy and McCrea, 2005). p120ctn can interact with Kaiso by entering the nucleus and binding the zinc finger domains, suggesting that the binding of p120ctn to Kaiso inhibits its interaction with the DNA (Fig. 15). Therefore, the formation of this complex abolishes the repression of the Kaiso-mediated promoter. This shows that p120ctn can influence gene expression, like β-catenin does (Kelly et al., 2004; Spring et al., 2005). In addition, target genes of Kaiso include the β-catenin/TCF targets *cyclinD1* and *matrilysin* implicating that Kaiso and p120ctn are indirect regulators of the Wnt signalling pathway (Daniel et al., 2002; Park et al., 2005). It is also possible that p120ctn and Kaiso associate in the cytoplasm which also prevents Kaiso from entering the nucleus and thereby acting in the nucleus (van Roy and McCrea, 2005).

3.5. p120ctn and knockout studies

Different KO mice are generated to study the role of the multifunctional p120ctn protein. Global loss of p120ctn results in embryonic lethality (Reynolds and Roczniak-Ferguson, 2004). Therefore, several conditional knockout (cKO) mice have been generated to examine the loss of p120ctn in specific tissues or organs (Pieters et al., 2012a).

In a first study, the salivary gland has been targeted by the use of the Cre/LoxP system under the Mouse mammary tumor virus (MMTV) promoter (Davis and Reynolds, 2006). The loss of p120ctn in the developing salivary gland resulted in a severely aberrant phenotype. The developing acini were not differentiated while cell adhesion and polarity were also missing. Tumor-like protrusions developed and formed unpolarized epithelial masses resulting in the death of the mice shortly after birth. Further examination of the epithelial masses showed that E-cadherin expression was decreased. The general loss of polarity and differentiation is suggested to be caused by the reduction of E-cadherin combined with the possible effect of the loss of p120ctn on Rho GTPases and Wnt signalling (Davis and Reynolds, 2006). The MMTV-Cre effects on the lacrimal gland were consistent with the effects on the salivary gland. The effect on the skin on the other hand, was minimal at early developmental stages. (Davis and Reynolds, 2006).

Ablation of p120ctn in the skin has been accomplished through the use of K14-Cre -mediated recombination (Perez-Moreno et al., 2006). The skin of newborns did not show any histological defects and the typical barrier function was maintained. As the mice aged, a striking phenotype developed characterized by skin hyperplasia, an increased number of blood vessels in the dermis, hair loss, increased subdermal infiltration of immune cells, and loss of subcutaneous fat. This phenotype is consistent with the image of an elevated inflammatory response.

Further investigation revealed that the activated NFκB pathway was responsible for this inflammatory response rather than the reduction of cell adhesion (Perez-Moreno et al., 2006).

A third cKO mouse has been generated to delete p120ctn from the dorsal forebrain (Elia et al., 2006). The components of the cadherin-catenin complex are known to be involved in the formation, maintenance, and remodelling of synapses. The loss of p120ctn resulted in the reduction of cadherins present at the membrane and had an effect on the Rho family GTPases. This was mostly reflected in the reduction in spine density along the dendrites and the altered neck length and head width of the spines. Additionally, a reduction in synapse densities in the hippocampus was also observed. These defects have been linked to the altered expression of N-cadherin and to the activity of Rho and inhibition of Rac (Elia et al., 2006).

Recently, a study was performed on the cKO of p120ctn in the epithelial part of the teeth in mice. Without p120ctn the enamel was distorted whereas the development of the teeth itself appeared normal. Only when the ameloblasts became secretory, they lost their cell polarity and became disorganized (Bartlett et al., 2010). The cKO of p120ctn in the liver is also viable, although these mice show severely disrupted bile duct development but normal hepatocyte differentiation. Cell adhesion is not impaired (van Hengel, personal communication).

When p120ctn is knocked-out in the cells of the colon, the cKO mice die within three weeks. The loss of p120ctn in the colon results in the disruption of the epithelial barrier and increased inflammation events. The levels of the components of the adherens junctions are reduced (Smalley-Freed et al., 2010).

By using the Tie2-Cre system, p120ctn is deleted specifically from the vascular endothelium.

These embryos die during embryonic development (11.5 dpc) and display overall disorganized vasculature. VE-cadherin and N-cadherin levels are reduced and proliferation is inhibited (Oas et al., 2010).
Not only in mice but also in *Xenopus* does the loss of p120ctn (by morpholino injection) result in severe abnormal development. Gastrulation is disrupted and axial elongation inhibited when p120ctn is knocked-down in the whole *Xenopus* embryo. Moreover, a decrease in C-cadherin was observed (Fang et al., 2004). When p120ctn is targeted solely in the anterior neural ectoderm, evagination of the optic vesicles fails, which results in defective eye formation. In addition, cranial neural crest cells are perturbed which influences migration of these cells and results in malformation of the craniofacial cartilage (Ciesiolka et al., 2004).
The loss of p120ctn does not always result in negative effects for the organism. p120ctn RNAi knock-down in *C. elegans* did not result in a lethal phenotype (Pettitt et al., 2003). During the development of *C. elegans*, the p120ctn homologue JAC-1 appeared dispensable as the nematodes developed normally. A more detailed study revealed the contribution of this molecule to the cadherin and cytoskeletal functions (Pettitt et al., 2003). In *Drosophila*, the loss of p120ctn also fails to have a severe impact on the development. Viable and fertile flies developed with a normal expression of adherens junctions. The fly mutant for p120ctn did nevertheless show defective cells at the fronts participating in dorsal closure, such as alterations in cell shape and actin organization. However, these minor defects were eventually resolved and normal dorsal closure occurred. It is remarkable that the levels of p120ctn at the junctions varied significantly more than the other components of the cadherin-catenin complex. Therefore, it has been suggested that p120ctn in *Drosophila* is no essential component of the adherens junctions in general but its expression is modulated especially in tissues subjected to movement or mechanical stress. p120ctn is not essential during *Drosophila* development but

it is a positive regulator of the cadherin-catenin complex formation (Myster et al., 2003). When depleting both zygotic and maternal p120ctn in *Drosophila* embryos by RNAi, severe morphogenetic events did occur, particularly in head involution (Magie et al., 2002). Until recently, there have been no studies performed on p120ctn in zebrafish.

4. CELL ADHESION IN INTEGUMENTAL APPENDAGES

4.1. Morphogenesis of integumental appendages

4.1.1. Mammary gland development

During mouse embryonic development a bilateral ectodermal ridge develops in a rostral-caudal orientation between the fore and hind limb. First the ridge becomes multilayered, second the ectodermal cells within the ridge become columnar-shaped and eventually the mammary line results into five pairs of placodes. Each pair of placodes develops symmetrically while between the placode pairs, development is not simultaneous (Veltmaat et al., 2003; Veltmaat et al., 2004). Each placode is a lens-shaped thickening of the surface ectoderm and consists of several cell layers with typical columnar and larger cells compared to the cells of the surrounding epidermis. In addition, the cells of the placode are not uniformly orientated (Robinson, 2007). At E (embryonic day) 12.5 of mouse embryonic development, the placode expands and starts to form a mammary bud which is embedded in dermal mesenchyme (Fig. 16C). When invagination starts, the placode cells re-organize into a concentric organization and pile up at the surface resulting in an elevated bud above the surrounding epidermis. Not only the epithelial cells but also the mesenchymal cells undergo dramatic changes (Foley et al., 2001; Veltmaat et al., 2004; Watson and Khaled, 2008). The cells adjacent to the developing epithelial cells will elongate and condense. Eventually, the mature bud consists of a sphere of epithelial cells connected to the cell surface by epidermal-like cells and is surrounded by multiple layers of condensed mesenchyme (Fig. 16C) (Cowin and Wysolmerski, 2010).

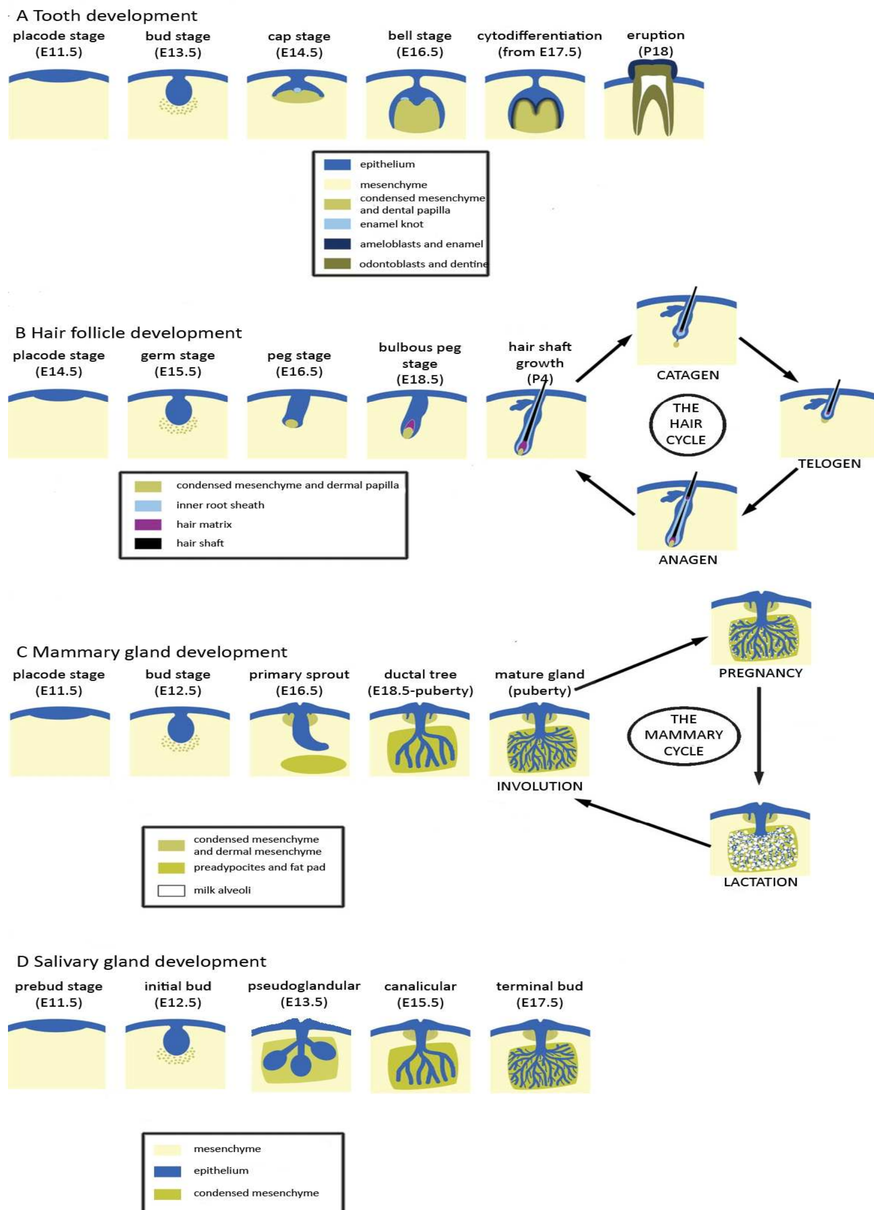

Figure 16. Schematic overview of the key developmental steps of four major integumental appendages

A) Development of the first molar in mouse. B) Body hair development in mouse. C) Mouse mammary gland development, including changes that occur during pregnancy and lactation. D) Mouse salivary gland development. E: embryonic day; P: postnatal. (Figure adapted from Duverger and Morasso (2008)).

This mammary bud is the starting point of differential development between male and female mice. In male mice, the mesenchyme proceeds condensation leading to the disruption of the connection of the bud with the epidermis. This is followed by apoptosis of, first, the mesenchymal compartment and afterwards the epithelial bud, resulting in reduced or no mammary epithelial ducts in male mice (Veltmaat et al., 2003). Female mammary gland development is totally different as it does not show any progress in development after the formation of the mammary bud for two days. Next, the developmental stage in female mice is the ductal branching from E16 onwards. A string of epithelial cells develops from the bud constituting the mammary sprout. This grows down into the deeper located mammary fat pad. Once reached the loose collection of mesenchymal-derived adipocytes of the fat pad, the mammary sprout starts to branch (Fig. 16C). This is the start of the initial round of branching which results in a primary duct and around 15 initial branches (Hens and Wysolmerski, 2005; Watson and Khaled, 2008). The formation of the epithelial outgrowth from the mammary bud coincides with lumen formation and the formation of a nipple structure. At the end of embryonic development, the mouse mammary gland is a short primary duct that ends in a branched tree embedded in the mammary fat pad (Foley et al., 2001). Further development of the mammary gland will occur during puberty (second round of rapid growth) and pregnancy (milk-producing glands) (Fig. 16C) (Cowin and Wysolmerski, 2010).

4.1.2. Salivary gland development

The formation of a salivary gland in mouse starts with the prebud stage, i.e. a thickening of the epithelium next to the tongue (Fig. 16D).

This prebud (or placode) develops into the initial bud by the subsequent protrusion of the epithelial cells into the underlying mesenchyme. The bud remains in contact with the oral surface by a duct that eventually becomes the main duct of the salivary gland (Fig. 16D) (Tucker, 2007). This stage is rapidly followed by the pseudoglandular stage in which the bud undergoes branching and the formation of new buds which will also start to branch. This process leads to the formation of a multi-lobed gland (Jaskoll and Melnick, 1999). The ducts will start to form their lumen as the saliva needs to be able to pass through the ducts from the acini to the oral surface. The formation of the lumen is linked to the canalicular stage of salivary gland development (Fig. 16D). The epithelial cells around the developing lumina continue proliferating while the cells from the presumptive lumina undergo apoptosis. In the end, terminal bud stage is reached when the end buds and branches are hollowed out to form functional ducts and acini (Tucker, 2007). Although well-developed lumina are present, the connection between the ducts and lumina are not yet completed (Melnick and Jaskoll, 2000). The salivary gland continues differentiating postnatally (Gresik, 1994).

4.1.3. Hair follicle development

Hair follicle morphogenesis in mouse is initiated by the formation of a focal thickening in the basal layer of the epidermis. This first stage of development is called the hair germ or hair placode, and is characterized by typical vertically oriented cells (Fig. 16B) (Botchkarev and Paus, 2003).

The dermal cells underneath the epithelial thickening become more dense and form a cluster (hair germ stage). These cells will become the follicular papilla (Paus et al., 1999). Subsequently, the cells of the hair placode will grow down into the dermis resulting in the formation of a column of epithelial cells covered

by mesenchymal cells at the proximal end. As the downgrowth of the epithelial cells progresses, they start to envelope the mesenchymal cells. The epithelial cells have a high proliferation rate (peg stage) (Paus et al., 1999). The next stage is characterized by the incorporation of the follicular papilla into the epithelial hair bulb. The cells of the hair bulb have a high proliferation rate and differentiate into the inner root sheath and hair shaft cells (Fig. 16B) (Paus et al., 1999). The following developmental stages include the development of the hair shaft above the follicular papilla, the appearance of the hair canal at the distal side of the hair follicle and finally the elongation of the follicle to its maximal length and protrusion of the hair shaft through the epidermis (bulbous peg stage) (Botchkarev and Paus, 2003; Paus et al., 1999; Schmidt-Ullrich and Paus, 2005). Hairs are continuously renewing structures that pass through different stages of the hair cycle (Fig. 16B). The follicle proceeds from the resting (telogen phase) to an active growth phase (anagen phase), followed by the involution (catagen phase) and finally the hair gets shed (exogen phase) (Botchkarev and Paus, 2003).

4.1.4. Tooth development

Teeth develop from the oral epithelium or the pharyngeal epithelium in combination with the neural crest-derived mesenchyme. The first sign of tooth development in the mouse is a local thickening of the epithelium, the tooth placode (Fig. 16A). These epithelial cells will start to invaginate into the underlying mesenchyme while the mesenchyme condenses. In the following developmental steps, bud, cap and bell stage, the epithelium undergoes extensive folding, resulting in the establishment of the tooth crown (Fig. 16A).

During cap stage, the mesenchyme becomes enveloped by the epithelium forming the dental papilla which will give rise to the odontoblasts and the tooth pulp. The epithelial part of the tooth is called the enamel organ in which four distinct cell layers will develop: the inner and outer dental epithelium with in between the stratum intermedium and stellate reticulum (Lesot and Brook, 2009; Thesleff and Sharpe, 1997). Moreover, when the bud transforms into a cap, the enamel knot appears. This cluster of epithelial cells was shown to be a signalling centre (Thesleff and Sharpe, 1997; Vaahtokari et al., 1996). It was demonstrated that this enamel knot lacks cell division but undergoes cell-cell interactions and apoptosis (Coin et al., 1999; Lesot et al., 1996; Matalova et al., 2004; Obara and Lesot, 2004; Shigemura et al., 1999). It has been proposed that the enamel knot determines the site of the first cusp in molar teeth (Jernvall and Thesleff, 2000). An enamel knot is also present in the incisors during the cap stage. In molars, new enamel knots appear at the sites of new cusps (Jernvall et al., 1994). Another specific region that starts to develop from cap stage onwards is the cervical loop, which grows into the mesenchyme and delimitates the dental papilla. These epithelial areas are highly proliferative and consist of the cells of the inner and outer dental epithelium.

This cervical loop structure is maintained in continuously growing teeth and constitutes the epithelial part of the adult stem cell niche in mouse incisors (Harada et al., 1999; Thesleff and Tummers, 2008). Bell stage is characterized by the differentiation of the epithelial ameloblasts and the mesenchymal odontoblasts. The odontoblasts secrete a collagenous matrix that will subsequently mineralize into dentin. The ameloblasts deposit enamel matrix. Cusps will start to form and the size of the tooth crown increases. The roots of the tooth start to develop after crown morphogenesis, followed by the eruption of the tooth (Fig. 16A) (Lesot and Brook, 2009; Thesleff and Sharpe, 1997).

4.2. Cell adhesion molecules in integumental appendages

4.2.1. Cell adhesion molecules in the mammary gland

For many years, the mammary gland has been an important model for epithelial morphogenesis. Moreover, the rising incidence of breast cancers has augmented the interest in mammary gland development in order to understand the reason of onset of a mammary tumor and related metastasis. Cell adhesion has for a long time been correlated to the development of cancer and tumors, resulting in multiple studies concerning cell adhesion during normal mammary gland development and breast cancer (Knudsen and Wheelock, 2005; Paredes et al., 2007; Reynolds and Roczniak-Ferguson, 2004; van Roy and Berx, 2008). Below, a short overview of these studies will be presented.

Adherens junctions

The early developmental stages of the mammary gland serve as a model for studying epithelial morphogenesis. Mouse embryos have been examined from E13 to E17, given that the first morphogenetic stages of the mammary gland take place during this timeframe. Immunofluorescent staining has shown the colocalized distribution of E-cadherin and β-catenin at the plasma membrane of the mammary epithelium, in all stages examined (Nanba et al., 2001). The colocalization of these two molecules at the cell boundaries suggests the presence of the cadherin-catenin complex in the adherens junctions. No change in expression of components of the adherens junction has been observed in the mammary epithelial cells during the different stages of mouse mammary gland development. The mammary gland in young mice is branched with terminal buds at the end. Once pregnant and lactating, hormones will induce proliferation of the end buds, differentiation of the alveoli for milk production and more branching (Cowin and Wysolmerski, 2010).

The cells lining the ducts and alveoli are highly polarized epithelial cells expressing all components of the E-cadherin-catenin complex. Myoepithelial cells that support the luminal epithelial cells at their basal side express P-cadherin. The linkage between luminal cells and myoepithelial cells is not provided by classical cadherins but by desmosomes (Knudsen and Wheelock, 2005).

Desmosomes

The observations regarding the AJs are in contrast to the distribution of the most important desmosomal components such as plakoglobin, desmoplakin and the two desmosomal cadherins, desmocollin and desmoglein (Nanba et al., 2001). The expression pattern of these components shows fluctuations during mammary gland development. The mammary bud at E13 shows expression of the desmosomal molecules whereas at E15 this expression in the bud has decreased dramatically and is almost unexisting. While the desmosomal molecules are being restored in most parts of the epithelium, the distal region of the mammary bud remains negative. An aggregation assay has shown that the E15 bud cells form small buds or remain single cells. The overlying epithelial cells on the other hand were able to form large clusters. This indicates that the overlying epithelial cells have strong intercellular adhesion, in contrast to the cells of the mammary bud that possess much weaker cell-cell adhesion. The difference in adhesive properties might be the mechanism underlying the segregation of the mammary bud cells from the overlying epithelial cell layer (Nanba et al., 2001).

Tight junctions

Occludin, a marker for tight junctions, shows a completely different expression pattern compared to the desmosomal proteins. It is completely absent from the mammary bud cells during the period of E13 to E15 (Nanba et al., 2001).

Only the uppermost layer of the epidermis showed the presence of tight junctions. When the mammary epithelium starts to form cavities, from E17 onwards, the inner region of this epithelium starts to express occludin. It is remarkable that occludin becomes newly expressed together with cavity formation (Hieda et al., 1996; Nanba et al., 2000; Nanba et al., 2001).
The restoration of cell adhesion systems around E17 and the resulting cell polarization are likely to cause cell rearrangements during the epithelial morphogenesis of the mammary gland (Nanba et al., 2001).

Gene manipulations of cadherins in mice have elucidated the possible role of cadherins during development of different tissues or organs. General E-cadherin deletion in the mouse results in early embryonic lethality but E-cadherin disruption specific in the mammary epithelium results in substantial apoptosis and the loss of milk production. Therefore, E-cadherin appears to be essential for epithelial cell survival and proper functioning of the mammary gland (Boussadia et al., 2002). The P-cadherin null mouse is viable but also shows an abnormal mammary gland phenotype (Radice et al., 1997). The mammary gland in P-cadherin KO mice starts its development remarkably earlier and shows precocious epithelial growth and differentiation in comparison to wild-type mice. However, this mis-regulation of growth and differentiation does not influence the lactating ability of female mice (Radice et al., 1997). The transgenic expression of P-cadherin under control of the MMTV promoter results in the inappropriate expression of P-cadherin in the adult mammary epithelium. These transgenic mice display a normal developing and functioning mammary gland (Radice et al., 2003). This suggests that the potential increase in cell adhesion between the two different cell types, epithelial and myoepithelial cell layers, does not have an effect on mammary gland development (Radice et al., 2003).

Additionally, in another transgenic line the epithelial cells were forced to express N-cadherin (under control of MMTV promoter), normally only expressed in mesenchymal-derived cells. Similarly, this did not have any consequence on function, differentiation and proliferation of the mammary gland (Knudsen et al., 2005). Ectopic expression of N-cadherin or P-cadherin did not eliminate or radically affect endogenous E-cadherin expression, nor did it displace E-cadherin from cell-cell contacts. β-catenin staining showed no apparent difference in its pattern or levels when comparing transgenic and wild-type mice (Knudsen et al., 2005; Radice et al., 2003).

4.2.2. Cell adhesion molecules in the salivary gland

The salivary gland develops from the oral epithelium and undergoes major changes in shape by repetitive branching and cell rearrangements. This branching requires the interaction between epithelial cells and the surrounding mesenchyme (Tucker, 2007). Salivary gland development has been examined in mice from E12 until E18.

Adherens junctions

The early development of the salivary gland has been examined for the presence of adherens junctions by means of immunohistochemical staining. In all stages examined, E-cadherin and β-catenin show a similar distribution. At the initial bud stage (E12), both molecules are localized at the apical region of the stalk epithelium. During the next days, E-cadherin and β-catenin are detected at the cell borders of the entire epithelium. The diffuse immunostaining of E-cadherin in most of the epithelial tissue during the first days of development suggests that there is a pool of E-cadherin separate from that present in the adherens junctions.

When cytodifferentiation is complete (at E18), E-cadherin is found along tightly apposed cell-cell borders with no detectable diffuse staining (Menko et al., 2002). This change in localization of E-cadherin reflects its role in tissue organization and cytodifferentiation of the salivary cells (Hieda et al., 1996; Menko et al., 2002). N-cadherin expression in the epithelial cells is low during the first stages but at later stages (E16-E18) N-cadherin becomes prominent at the borders of the cells that are being organized into salivary tissue-specific structures and in the terminal end buds. When cytodifferentiation is completed, N-cadherin levels decrease at the salivary cell membranes except where the salivary cell membranes contact the myoepithelial cells. N-cadherin is only transiently expressed suggesting that this protein might play a role in cell-cell communication (Menko et al., 2002).

Desmosomes

Distribution of desmosomes during salivary gland development has been analyzed through the localization of desmoplakin, the protein responsible for the linkage of the desmosomal cadherin and plakoglobin with the intermediate filaments. Desmoplakin is present in the lateral region of the stalk epithelium (E12), in contrast to the lobule, which is not expressing desmoplakin at the most early stage examined. At E13, the entire epithelium lacks desmoplakin signal. Even at E14, the newly formed lobules do not express desmoplakin, while desmoplakin expression clearly augments in the epithelial cells of the recently formed lumina (Hieda et al., 1996).

Tight junctions

Similar to the distribution of desmosomes, tight junctions are lost or much reduced during early development. ZO-1, a marker for tight junctions, appears to be absent from the lobules whereas the apical region of the stalk epithelium is positive at E12.

At E13, ZO-1 is only faintly expressed in the lobules whereas the apical region of the epithelial cells of the forming lumina shows strong ZO-1 labelling by E14 (canalicular stage). Even the branched lobules display a patchy pattern of this tight junctions component (Hieda et al., 1996).

From the results above it is clear that the regulation of the cadherin-catenin complex in the developing salivary gland is different from that in the desmosomes and tight junctions. The cadherin-catenin complex remains present during all developmental stages examined, while the desmosomes and tight junctions are lost or extremely reduced in the epithelial cell mass, but reappear in the lumenized epithelium. The cavitated epithelium is fully differentiated with all junctional complexes present while the epithelial cell mass is in a histological undifferentiated state.

4.2.3. Cell adhesion in hair development

Like in other integumental appendages, early morphogenesis of a newly developing hair also includes the interaction of an epithelial cell layer with a mesenchymal cell layer. Several studies have shown dynamic changes in cell adhesion during hair follicle morphogenesis (Kurzen et al., 1998; Nanba et al., 2000; Nanba et al., 2003).

The mesenchymal-derived dermal papilla expresses cadherin-11. This molecule shows changes in its distribution pattern throughout hair development (Nanba et al., 2003). In the initial mesenchymal aggregate (E15), cadherin-11 is uniformly distributed along the cell surfaces. While further developing, the presumptive dermal papilla develops a dot-like pattern of cadherin-11 distribution that corresponds to recognizable intercellular junctions on an ultrastructural level. This dotted expression becomes more evident in the dermal papilla of more advanced hair follicles (Nanba et al., 2003).

The epithelial part also shows differential expression of the adherens junctions. E-cadherin expression is reduced in the hair placode while P-cadherin expression is increased. This might be a response to the mesenchymal message and cause the sorting out of cells of the hair placode from the neighbouring epidermal cells (Obara et al., 1998; Palacios et al., 1995).

Desmosomes

Desmosomes are essential cell adhesion systems in epidermal tissue. Staining for desmoglein, desmocollin, plakoglobin and desmoplakin, proteins that are part of the desmosomes, showed a significant reduction in the hair placode (Nanba et al., 2000). Although all of these desmosomal molecules are expressed in the epidermal cells, the hair plugs are completely negative for these molecules. The elongation phase of the follicles coincides with the re-expression of the different desmosomal components, except in the hair matrix. It is clear that the cell adhesion systems are dynamically regulated during hair follicle morphogenesis (Nanba et al., 2000). From studies on the development of human hair follicles, it is known that the composition of desmosomal cadherins differs according to the position of the desmosomes in both the foetal and the adult anagen hair follicle (Kurzen et al., 1998). The linker proteins desmoplakin, plakoglobin and plakophilin are weakly expressed in the hair bud. With further development, the expression of this set of molecules increases in the inner root sheet (IRS) cells and matrix cells. The cells of the outer root sheet (ORS) start expressing these desmosomal proteins even later. In the bulbous hair peg stage and in lanugo follicles, the IRS express Dsc1, Dsc3 weakly and Dsg1. The cells of the ORS in the bulbous hair peg mainly express Dsc3, Dsg2 and 3. In the lanugo hair follicle the expression pattern for these molecules persists and additional expression of Dsc2, Dsg1 and Dsc1 occurs in the upper half.

The matrix cells and trichocyts (differentiated matrix cells) show Dsc2 and 3 and Dsg2 and 3 expression in the hair peg. In the older hair follicle Dsc2 is not detected anymore while the expression pattern of the desmogleins persists, now also including weak Dsg1 expression. This clearly fluctuating expression of desmosomal components is typical for early development of a human hair follicle (Kurzen et al., 1998).

Cadherin-11 -/- mice did not show any abnormality concerning hair development. It is possible that other cadherins are also present in the dermal papilla (Manabe et al., 2000). Candidates are N-cadherin, which is simultaneously expressed with cadherin-11 during somitogenesis, and cadherin-8, known for possible heterophilic binding with cadherin-11. But none of those are detected in the dermal papilla (Nanba et al., 2003). The down-regulation of E-cadherin is hypothesized by Jamora et al. (2003) to be essential for the formation of an epithelial bud. cKO of E-cadherin in the skin resulted in defect in the hair coats of these cKO mice. The loss of E-cadherin did not influence the initiation and further progression of the hair follicle but the hairs were much smaller and more fragile. When the mice aged, they displayed sparse pelage hairs (Tinkle et al., 2004).

The loss of cell adhesion systems appears to have a serious effect on the normal development of hairs. For example, inactivating Dsg3 in mice results in impaired hair development, suggesting that desmosomal cadherin plays an essential role during hair growth and stability (Koch et al., 1997). Mice deficient for β-catenin in the skin show abnormal hair development. These mutant mice show hairless patches suggesting that β-catenin is involved in hair morphogenesis. β-catenin negative skin does not have hair placodes. Later, these β-catenin KO mice become completely hairless.

This result identifies β-catenin as a mediator of hair cycling (Enshell-Seijffers et al., 2010; Huelsken et al., 2001). A hair phenotype is also observed in mice deficient in plakophilin-3. These mutant mice display a ruffled and matted pelage. When examined more closely, they appear to have equal numbers of hair follicle compared to wild-type mice but many of these follicles are in a much earlier stage of morphogenesis. Mutant follicles are wavy and often misoriented (Sklyarova et al., 2008). The cKO of p120catenin in mouse results in smaller mice with patches of hair loss on the dorsal side of their skin surface. Normal hair follicles are absent, and the follicles present have large openings filled with keratinized material (Perez-Moreno et al., 2006).

4.2.4. Cell adhesion during tooth development

Mouse molars and incisors are the best studied models for tooth development over the past years. To a lesser extent, human deciduous and adult teeth have also been used to investigate various aspects of tooth development.

The knowledge on cell adhesion in human teeth is limited. Two important cell adhesion molecules have been studied during the development of deciduous tooth germs: E-cadherin and N-cadherin (Heymann et al., 2002). None of them have been detected in the dental bud but during cap stage, strong E-cadherin staining has been detected in the enamel organ. N-cadherin expression is hardly visible but some cells of the dental papilla and enamel organ show very weak expression. As development progresses, the enamel organ grows deeper into the mesenchyme while differentiating. E-cadherin has been detected in the proliferating cervical loop of the enamel organ, in contrast to the progressive reduction of E-cadherin signal in differentiating ameloblasts. On the other hand, N-cadherin signal increases in pre-ameloblasts, but cannot be demonstrated in the proliferative regions of the enamel organ.

The ODE and stellate reticulum only express E-cadherin, not N-cadherin. The mesenchyme of the dental papilla shows N-cadherin expression, but it is faint and limited to the cells at the cusp region. During the late bell stage, a decreasing gradient of E-cadherin staining has been observed following apical to coronal direction. N-cadherin shows the opposite expression pattern; it becomes strongly expressed in the differentiating ameloblasts and odontoblasts. The cells of the stratum intermedium are also expressing N-cadherin while the stellate reticulum is negative. Differentiating and functional odontoblasts are N-cadherin positive (Heymann et al., 2002).

The incisor of the rat was used to study the expression pattern of components of the cadherin-catenin complex in the light of amelogenesis and the life cycle of ameloblasts to produce enamel. α-catenin and plakoglobin were detected in the ameloblasts throughout the stages of amelogenesis. In stage 1, presecretory stage ameloblasts and the cells of the stratum intermedium show strong E-cadherin and β-catenin staining. The ameloblasts express p120ctn both in the cytoplasm and at the membrane. The odontoblasts exhibit β-catenin signal and a rather weak E-cadherin signal. Next, the ameloblasts start to produce enamel in the early secretory stage. The staining of E-cadherin, β-catenin and p120ctn greatly reduces in the ameloblasts from early to late secretory stage. This is in contrast to transition stage ameloblasts (stage 3), that show an intense staining of these three cell adhesion molecules at the lateral cell membranes. The other layers of the enamel organ are not responding to these antibodies. Finally, the ameloblasts reach maturation stage which is accompanied by a weak E-cadherin expression along the lateral borders of the ameloblasts. This expression strongly increases by late maturation stage during which β-catenin and p120ctn are also present (Sorkin et al., 2000).

A more expanded study has been performed by Kieffer-Combeau (2001) on different cell junctions during epithelial morphogenesis of the mouse incisor. The emphasis in this study is on the cytodifferentiation of the enamel organ rather than on early morphogenesis. E-cadherin appears to be homogeneously expressed in all cells of the enamel organ at cap stage (E14), and then decreases at the labial side of the cervical loop in the next couple of days. Desmoglein is not expressed in the enamel organ during cap stage but the expression strongly increases afterwards. This is in contrast to plakoglobin, that shows expression in the enamel organ. The enamel knot area also expresses plakoglobin but cells in contact with the basement membrane show a weaker signal. At early bell stage (E16), the cells of the SI at the labial side of the incisor intensely express desmoglein and plakoglobin, just like the epithelial cells in contact with the IDE on the lingual side. The IDE itself remains negative for desmoglein. The cervical loop displays differential expression of these two desmosomal proteins. The lingual side of the cervical loop is desmoglein positive while the labial side is negative. Plakoglobin has not been detected on either side of the cervical loop (Kieffer-Combeau et al., 2001).

In mouse molars, E- and P-cadherin show differential expression during morphogenesis. All cells of the tooth bud, except for the marginal cells, express only E-cadherin. The marginal cells express both E- and P-cadherin. During cap stage, the IDE and ODE show E- and P-cadherin expression, in contrast to the cells in between these two layers (SR and SI), which only express E-cadherin. The cells of the enamel knot display a lower staining intensity for E-cadherin, but express P-cadherin equally to the surrounding cells. During bell stage, when cytodifferentiation starts, the ODE maintains E-cadherin expression but lowers P-cadherin expression. The enamel knot is P-cadherin positive. SR and SI retain their E-cadherin signal. It is clear that differentiation to enamel-secreting ameloblasts is correlated to differential expression of cadherins.

Differentiating ameloblasts facing the newly formed dentin are strongly expressing E-cadherin. However, ameloblasts at the cusp tip do not express E-cadherin or P-cadherin (Obara et al., 1998; Palacios et al., 1995).

Classical cadherins are bound at their C-terminal end by β-catenin. Not only does β-catenin sustain the link between the membrane-bound cadherin clusters and the actin cytoskeleton, it also functions as a mediator in the canonical Wnt pathway. Therefore, β-catenin has been studied in many developmental processes including tooth development. At initiation stage of newly forming mouse molars, the odontogenic epithelium and mesenchyme both express β-catenin, although stronger expression has been observed in the tooth placode. Also when forming a bud, both the mesenchyme and epithelium maintain their β-catenin expression uniformly (Obara et al., 2006). It is not until cap stage that an upregulation of β-catenin is observed. This increased expression occurs in the enamel knot. The rest of the enamel organ maintains a constant level of expression, while β-catenin is slightly reduced in the mesenchyme. β-catenin protein has been found at the plasma membrane but also in the nucleus of the cells constituting the enamel knot. The IDE starts to show strong β-catenin expression from late cap stage onwards, persisting during early bell stage. Also in the IDE, nuclear β-catenin protein has been detected. Weak β-catenin expression has been found in the ODE and SR throughout bell stage. The cervical loops and ODE show β-catenin protein distribution in the cytoplasm and at the membrane but it has never been observed in the nucleus. The strength of β-catenin expression in the SI depends on the localization of the cells in the tooth germ. The protein has been detected mainly at the membrane but a low signal has been found in the nucleus. No expression of β-catenin has been detected in the differentiated odontoblasts; elsewhere the dental papilla displays low levels of β-catenin expression (Obara and Lesot, 2004; Obara et al., 2006).

The differences in subcellular localization of β-catenin suggest distinct roles for this cell adhesion molecule. When found close to the membrane, β-catenin is most probably involved in the adherens junctions. However, when located in the nucleus, it can be assumed that Wnt signalling is activated. The distribution of β-catenin suggests that, given the strong nuclear β-catenin signal in the enamel knot, Wnt signalling is active. This is not surprising as the enamel knot is known to be a signalling centre during tooth development and cusp formation (Obara and Lesot, 2004; Obara et al., 2006).

p120ctn catenin stabilizes classical cadherins at the cell border. The loss of p120ctn in the epithelium does not result in the absence of teeth. On the contrary, p120ctn seems dispensable in the epithelium for early tooth development. Only when the ameloblasts reach secretory stage, the effect of p120ctn loss shows. The ameloblasts display a disorganized arrangement and produce abnormal enamel (Bartlett et al., 2010).

Not only expression of components of adherens junctions, but also of other cell adhesion systems has been studied during tooth development. Plakoglobin is detected, during mouse molar development, from early cap until bell stage in the dental epithelial cells, but the medial side of the developing tooth displays a weaker signal. Later, both sides express plakoglobin equally. The IDE shows less plakoglobin signal compared to the SR and SI but this signal increases in time. Plakoglobin accumulates in the preameloblasts and ameloblasts. During early development of the enamel organ only the gubernaculum dentis (connective tissue uniting tooth sac with the gum) displays desmoglein expression. Desmoglein signal becomes stronger throughout development in the SR and SI. At E20 desmoglein expression decreases in the cells of the SR. Remarkable is the negative result in IDE and ODE (Fausser et al., 1998).

4.3. Conclusion

It is clear that the formation of integumental appendages requires regulation of the different cell adhesion systems. From their distribution during development of the mammary gland, salivary gland, hair and teeth several conclusions can be drawn. First, the comparison of these appendages suggests that adherens junctions are continuously expressed during their development. The hallmark, the presence of a classical cadherin at the membrane, is detected in every single developmental step. It is possible that there is a down-regulation of a component of the cadherin-catenin complex. However, this down-regulation appears to be compensated by the up-regulation of another protein. For example, the down-regulation of E-cadherin in the murine hair germ might be compensated by the up-regulation of P-cadherin. Accordingly, adherens junctions remain functional. As a consequence of the uninterrupted occurrence of the cadherin-catenin complex at the cell membrane, the loss of a component of this complex results in the absence or disrupted development of the structures mentioned above.

A second remarkable feature related to cell adhesion in the development of these appendages is that in contrast to the adherens junctions, the desmosomes show a remarkable up- and downregulation in their expression pattern. In early stages, desmosomal proteins are not or only faintly expressed. The occurrence of desmosomal components increases significantly in later developmental steps. This is not surprising as adherens junctions are always the first to form when new cell contacts are established (Baum and Georgiou, 2011; Bryant and Mostov, 2008; Yin and Green, 2004). Only afterwards, desmosomes become assembled in the cells.

From the limited amount of data on tight junctions in the appendages, it is apparent that tight junctions are not involved in the early development of these structures. They only assemble in later stages, when development is

already ongoing. This late expression can be understood knowing that the main function of tight junctions is to prevent intercellular leakage and to favour passage of molecules through the cell.

SECTION II

RESEARCH AIMS

Tooth development is a process resulting from reciprocal interactions between epithelial and mesenchymal cell layers. Tooth development starts with the formation of an epithelial thickening, the tooth placode, that will protrude into the underlying mesenchyme. Many integumental appendages such as hairs, feathers and glands, start their development with the formation of a placode. Some of these organs such as hairs and teeth are potentially cycling structures. Unlike mammals, zebrafish continuously replace their teeth throughout life and with each replacement cycle a new epithelial outgrowth is formed. This polyphyodont condition implies repeated initiation of tooth germs, which offers interesting possibilities of experimentation. Replacement teeth in zebrafish do not develop directly from the pharyngeal epithelium, as first-generation teeth do, but from a successional lamina issuing from the epithelial crypt surrounding the functional tooth. The formation of a first-generation tooth placode, the development of the successional lamina and the further development of the tooth all require substantial rearrangements of the cells.
The aim of this project was to test the hypothesis that remodelling of the different cell layers at the onset of zebrafish tooth development, and during ongoing morphogenesis and cytodifferentiation, is linked to specific changes in cell adhesion between the individual cells. To this end, I studied the expression and protein distribution, and to a limited extent also the role, of components of an important cell adhesion system, the adherens junctions, during zebrafish tooth development.

Inspired by a study of Jamora et al. (2003), I first questioned the possible involvement of **E-cadherin**, the main epithelial cadherin, in the formation of an epithelial bud. Jamora and colleagues (2003), working on hair follicle development in mouse, proposed that E-cadherin is down-regulated during the formation of an epithelial bud.

I tested this hypothesis by examining the expression and protein distribution of E-cadherin during the different phases of the development of first-generation as well as of replacement teeth (Section IV.1).

Given that the tooth is composed not only of an epithelial but also a mesenchymal component, I next focused on another classical cadherin, **N-cadherin**. N-cadherin is known to be expressed in neural crest-derived, mesenchymal tissues (as is the dental papilla) and to be a marker for differentiation. In addition to its role in cell adhesion, N-cadherin is also involved in multiple other processes such as migration and signal transduction. I wished to know where N-cadherin is distributed during zebrafish tooth development and replacement and whether N-cadherin has a possible role during tooth development is discussed in Section IV.2.

To collect information on the functionality of the cadherin-catenin complex, and on the presence and possible regulation of the desmosomes during the development of teeth, I next wished to examine the distribution of two cadherin-associated molecules, β-catenin and plakoglobin. **β-catenin** provides the link between the membrane-bound classical cadherin and the actin cytoskeleton but can also exist independently of the adherens junctions and act as a component of the Wnt signalling pathway. **Plakoglobin** is a close homologue of β-catenin and is predominantly found bound to a desmosomal cadherin at the desmosomes. However, because of their homology, plakoglobin competes with β-catenin for binding the cadherin tail. In addition, I also wished to collect evidence on the possible role of plakoglobin by examining morpholino-injected zebrafish (Section IV.3).

Finally, I wanted to explore one further component of the cadherin-catenin complex, not previously investigated in zebrafish, **p120ctn**. This catenin is known to stabilize classical cadherins at the plasma membrane and therefore to play a significant role in rendering the adhesion system functional (Section IV.4). To analyze zebrafish p120ctn, this gene had to be cloned and antibodies had to be generated to examine the expression profile during zebrafish tooth development.

I attempt to integrate the various findings on adhesion to understand how tooth initiation, morphogenesis and cytodifferentiation of zebrafish first-generation and replacement teeth may be established. I also wish to propose some future lines of investigation to approach the question of how morphogenesis of tooth tissues in the zebrafish can be accomplished (Section V).

SECTION III

MATERIAL & METHODS

1. Introduction

This section of the PhD thesis comprises two main elements. First, a chapter is dedicated to the requirement of zebrafish material necessary to conduct the experiments. It describes the fixation and subsequent storage of both larval and adult material. The following chapter includes the methods used to determine the antibodies, specific for zebrafish application and the staining used for histological examination. The second element of this section is a book chapter, currently in press. This book chapter provides a step-by-step protocol for whole mount immunohistochemistry and *in situ* hybridization of larval and adult zebrafish dental tissue. Not only does it contain a detailed description of the materials and methods for both techniques that I established for standard use in our lab. Furthermore, it lists a number of notes in which tips and tricks are given to obtain the adequate results. The further processing of the material in order to be able to make high quality sections is also described.

2. Material

Zebrafish (*Danio rerio*) obtained from a commercial source were mated and eggs were allowed to develop at 28.5°C standard temperature, in a 10-h dark / 14-h light cycle. The embryos were sacrificed according to the Belgian law on the protection of laboratory animals (KB d.d. 13 September 2004) by an overdose of MS222 (3-aminobenzoic acid ethyl ester) at specific times, starting at 40 hours post-fertilization (hpf), and then after every 4 hours until 4 days post-fertilization (dpf). From 4 dpf until 6 dpf, fish were sacrificed every 8 hours.

Fifth branchial arches were dissected from adult zebrafish under a Leica MZ Apo dissecting microscope using microscissors.

The embryos, larvae and dissected fifth branchial arches were fixed overnight at 4°C in 4% paraformaldehyde (PFA). Before storing the embryos and larvae in methanol (MeOH), they were depigmented in 0.5% KOH and 0.05% H_2O_2 in phosphate buffered saline (PBS) for 30 minutes to 1 hour at room temperature (RT). They were washed twice with 1x PBS, transferred through several MeOH steps (25%, 50%, 75%) and stored at –20°C in 100% MeOH. The dissected branchial arches were not depigmented but, immediately after fixation, they were transferred through a series of MeOH and stored in 100% MeOH at –20°C. In average, 20 specimens (controls not included) were used per developmental stage, per gene examined.

3. CONCISE OVERVIEW OF METHODS USED

A number of the adult PFA-fixed jaws was embedded in paraffin according to standard procedures and serially sectioned at 5 µm.

To validate the different antibodies used for whole mount and paraffin immunostaining procedures on zebrafish, western blots (WB) were performed. In this way, the appropriate commercially available antibodies that cross-reacted with zebrafish tissue could be selected for further use. When the WB yielded too high background or too low concentration of the protein, we additionally performed an immunoprecipitation (IP) according to the protocol described in Goossens et al. (2007). This procedure allows isolation and concentration of a particular protein from a sample containing thousands of copies of the protein. Additionally, we used this technique to extract intact protein complexes, a procedure called co-IP. Co-IP works by selecting an antibody that targets a known protein that is believed to be a member of a larger complex of proteins. By targeting this known member with an antibody, it becomes possible to extract the entire protein complex from the solution and thereby identify other members of the complex.

Many of the embryos, larvae and adult jaws served for *in situ* hybridization and immunohistochemistry (see section III and IV). After whole mount *in situ* hybridization or immunohistochemistry on zebrafish larvae and dissected adult tissues, the specimens were embedded in epoxy resin (Epon) to enable semi-thin serial sectioning yielding high quality histological detail (Fig. 17).

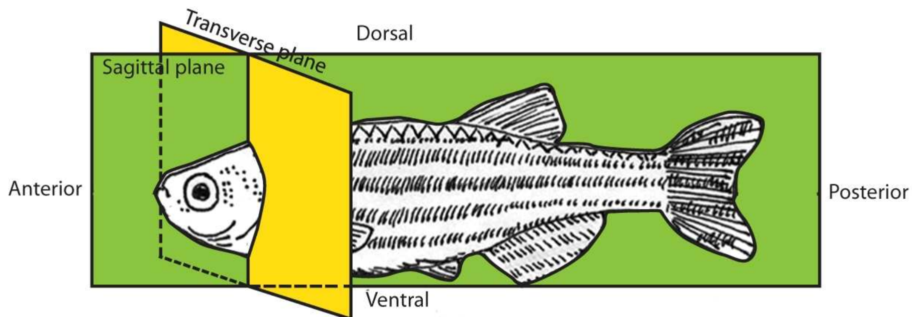

Figure 17. Orientation of plane of sectioning

The majority of sections are made in the transversal plane.

To visualize morphological structures such as in mutant or morphant embryos, specimens were similarly embedded in epoxy resin, serially sectioned at 1 or 2 µm, stained with toluidine blue (0.5% toluidine blue, 1% borax in bidistilled water) and mounted with depex.

All light microscopical observations (both in transmitted light and with differential interference contrast (DIC) light) were performed using a Zeiss Axio Imager microscope equipped with a Axiocam MRC camera.

4. Whole mount immunohistochemistry and in situ hybridization of larval and adult zebrafish dental tissues

Methods in Molecular Biology, Methods in Odontogenesis. Humana Press, USA, In press

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4.1. Summary

Tooth development is increasingly being studied in a variety of vertebrate model organisms, each contributing its own perspective to our understanding of dental diversity. *In situ* hybridization and immunohistochemistry are well established and frequently used techniques to study the presence of mRNA and protein. Here, we describe a protocol for whole mount immunohistochemistry and *in situ* hybridization that can be applied to all stages of zebrafish development and dissected bony parts. The description of these protocols is followed by the outline of a quick decalcification method and the procedure for embedding in epoxy resin to obtain serial sections with high histological quality.

4.2. Introduction

Over the past years, the zebrafish dentition which is restricted to the last pharyngeal arch has been increasingly used to study various aspects of tooth development and replacement, such as the involvement of stem cells (Huysseune and Thesleff, 2004), the role of transcription factors (Borday-Birraux et al., 2006), the involvement of various signalling pathways (Jackman et al., 2004; Jackman et al., 2010; Wise and Stock, 2010), or the distribution of adhesion molecules (Verstraeten et al., 2010a; Verstraeten et al., 2010b). It has also emerged as a model in regenerative dentistry (Yen and Yelick, 2011). Stock (2007) provides an excellent overview describing various features of the zebrafish pharyngeal dentition within a comparative context and illustrating how zebrafish can be used to explore the developmental genetic mechanisms of vertebrate dental evolution (Stock, 2007).

As is the case for mammals, studying gene expression and protein distribution constitutes a major tool to advance our knowledge on how teeth are formed, and, specifically in zebrafish, replaced continuously. Gene expression and protein distribution data can validate microarray results and RT-PCR data, and serve as a platform for functional studies. While in mammals, gene and protein expression data are routinely collected from sections of paraffin embedded tissue or on cryosections, the requirements for zebrafish teeth are different. This is largely because of the extreme small size of the first-generation (primary) teeth in zebrafish, which are only approximately 12 µm across at their broadest point (i.e., the thickness of one cryosection, or 1-2 paraffin sections) and contain less than a dozen odontoblasts in the pulp cavity. With such a small organ size, the level of histological detail that can be obtained quickly becomes critical, and losing one or a few sections rapidly turns the sample useless.

The zebrafish is widely used as a vertebrate model for developmental studies, one of its advantages being its fast embryonic development within the translucent chorion. Thus, not surprisingly, *in situ* hybridization and immunohistochemistry whole mount protocols have been developed in numerous variations. The advantage of whole mount methods is their speed, as results can be examined immediately following the completion of the protocol, using a stereomicroscope. This method is appropriate for early developmental stages (up to 5-6 days post-fertilization), and in the case of teeth it allows to identify the presence or absence of first-generation tooth germs, albeit on a rather coarse level. However, it is largely insufficient to identify the cells or cell layers that express a gene or protein. As the specimens become larger (larvae of 1-2 weeks old), and considering that the teeth develop close to the central axis of the body, whole mounts do not give any accurate information anymore, and sections need to be made. One way of achieving this is by confocal laser scanning microscopy. This approach yields optical sections and stacks, but has the downside that the resolution diminishes if the specimen becomes larger and the tissue of interest is situated deep inside the body, as is the case with the pharyngeal teeth. Moreover, it does not provide the histological detail required to localize transcripts or proteins to a certain cell type or tissue layer. For even older and/or larger fish (juveniles and adults), the whole mount protocol fails to work altogether.

Here, we present a whole mount protocol for immunohistochemistry and *in situ* hybridization on zebrafish embryos, larvae and dissected juvenile and adult tissue adapted from the protocol of Dr. P. Raymond. This protocol combines the ease of whole mount procedures with high-resolution histology to identify gene expression patterns and protein localization. The essential steps in the protocol include appropriate fixation, decalcification, whole mount *in situ* hybridization or immunohistochemistry, followed by embedding in epon (Luft,

1961) and sectioning at any desired thickness. We have tested the efficiency of this protocol using probes for signalling molecules, transcription factors and structural proteins, as well as for various antibodies.

The protocols described below are applicable for the study not just of teeth, but also of other organs or organ systems that develop late in zebrafish, such as the bony skeleton (Verreijdt et al., 2006). They allow us to combine expression data with high-resolution localization of transcripts and proteins.

4.3 Materials

4.3.1. Anaesthesia

1. 1% MS-222 stock solution: Dissolve 1 g of MS-222 powder in 100 mL distilled water. Store at 4°C.

4.3.2. Fixation of zebrafish embryos, larvae and adults

1. 1xPBS: Dissolve two PBS-tablets to 1 L distilled water. Store at RT for several months.
2. 4% paraformaldehyde (PFA): Dissolve 4 g paraformaldehyde powder in 100 mL 1xPBS (*see* **note 1**). Preferably, always prepare fresh.

4.3.3. Depigmentation

1. Depigmentation mix: Add to 8.5 mL distilled water 500 µL 30% H_2O_2 and 1 mL 5% KOH solution. Always prepare fresh.

4.3.4. Whole mount immunohistochemistry (no RNase-free solutions required)

1. 1xPBST: 0.1% Tween-20 in 1xPBS. Store at RT for several months.
2. Proteinase K stock solution: Dissolve 1 g proteinase K powder (Sigma, St. Louis, MO, USA) in 100 mL 1xPBS. This is a 1000x stock solution with 10 mg/mL concentration. Aliquot 0.5 mL in eppendorfs and store at -20°C for maximum one year.
3. Blocking solution: 1% Bovine serum albumin (BSA) and 1% Dimethylsulfoxide (DMSO) in 1xPBS (*see* **note 2**). Always prepare fresh.
4. StreptABComplex (Dako, Glostrup, Denmark): Add to 1 mL 1xPBS: 5 µL Streptavidin (A) and 5 µL horseradish peroxidase (B) (*see* **note 3**). Always prepare fresh.

5. Liquid DAB Substrate pack (Biogenex, Fremont, CA, USA): Add 2 drops of H_2O_2 solution, 4 drops of DAB chromogen solution and 500 µL 10x substrate buffer solution to 4.5 mL distilled water (*see* **note 4**). Always make fresh.

4.3.5. Whole mount *in situ* hybridization

All solutions are prepared using RNase-free 1x phosphate buffered saline (PBS) at room temperature (RT), unless stated otherwise.

Always work with RNase-free tips and eppendorfs.

4.3.5.1. Prehybridization

1. RNase-free DEPC-water: Add 1 mL DEPC to 1 L distilled water (*see* **note 5**). Leave overnight at RT or 1 hr at 37°C, shaking. Autoclave before use to inactivate. Store at RT for several months.
2. RNase-free 1xPBS: Dissolve two PBS-tablets to 1 L DEPC-water. Store at RT for several months.
3. 1xPBST: 0.1% Tween-20 in RNase-free 1xPBS. Store at RT for several months.
4. Methanol – xylene mixture: Add equal amounts 100% methanol (MeOH) and xylene and mix well (*see* **note 6**). Make fresh.
5. Methanol series: Always dilute with RNase-free 1xPBS. Store at RT.
6. Proteinase K stock solution: Dissolve 1 g proteinase K powder (Sigma, St. Louis, MO, USA) in 100 mL 1xPBS. This is a 1000x stock solution with 10 mg/mL concentration. Aliquot 0.5 mL in eppendorfs and store at -20°C for maximum one year.
7. 4% PFA: Dissolve 4 g paraformaldehyde powder in 100 mL 1xPBS (*see* **note 1**). Preferably make fresh.

8.0.1 M TEA (Triethanolamine): 6.7 M stock solution. Dilute 67 times by adding 750 µL stock solution to 50 mL DEPC-water. Store at RT for several months.

9.Acetic anhydride/TEA: Add 13 µL acetic anhydride (Sigma, St. Louis, MO, USA) to 10 mL 0.1 M TEA (*see* **note 7**). Always prepare fresh.

10. Hybridization solution (25 mL): 12.5 mL deionized formamid, 6.25 mL 20xSSC, 25 µL heparin (50 mg/mL), 25 µL Tween-20, 25 µL yeast tRNA (50 mg/mL) and 6.2 mL DEPC-water. Store at -20°C for months.

4.3.5.2. Posthybridization

1. 2xSSC: Starting from 20xSSC, add 10 mL 20xSSC to 90 mL distilled water. Store at RT.
2. 5xMAB buffer: Dissolve 11.6 g maleic acid disodium salt in 100 mL distilled water. Adjust pH to 7.5 with around 600 µL 1 N HCl. Add 53 mL 5 M NaCl and make up to 200 mL with distilled water. Autoclave and store at RT for several months.
3. Maleate blocking solution: 9.8 mL 1xMAB buffer (diluted from 5xMAB buffer with distilled water), 0.2 g Blocking Reagent (Roche), 0.2 g BSA powder, 200 µL sheep serum, 10 µL Tween-20 (*see* **note 8**). Always make fresh.

4.3.5.3. Color reaction

1. Genius buffer: 1 mL 1 M Tris-HCl (pH 9.5), 500 µL 2 M NaCl, 500 µL 1 M MgCl, 10 µL Tween-20 and 7.9 mL distilled water (*see* **note 9**). Always prepare fresh.

2. Nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP): dissolve 50 mg NBT powder in 700 µL N,N dimethylformamide (DMF) and 300 µL distilled water (store at -20°C). Dissolve 50 mg BCIP powder into 1 mL DMF anhydrous (store at -20°C). Add 45 µL NBT and 70 µL BCIP to 10 mL Genius buffer. Keep protected from light.

4.3.6. Epon embedding

1. 25% Decalc solution: Add 750 mL of distilled water to 250 mL Decalc (Histolab, Göteborg, Sweden). Store at RT.
2. Epon A: 6.2 mL epoxy embedding medium and 10 mL epoxy embedding medium, hardener DDSA (*see* **note 10**).
3. Epon B: 10 mL epoxy embedding medium and 8.9 mL Epoxy embedding medium, hardener MNA (*see* **note 10**).
4. Soft epon: 6 mL Epon A, 4 mL Epon B and 190 µL epoxy embedding medium, accelerator DMP30 (*see* **note 11**). Always make fresh.

4.4. Methods

All steps are performed at room temperature, unless stated otherwise.

Embryos and tissues are kept in eppendorfs throughout the protocol provided the eppendorfs are gently swirled after each change of solution.

4.4.1. Fixation of zebrafish embryos, larvae and dissected pharyngeal jaws of juveniles and adults

1. Euthanize the embryos or larvae by adding 1 mL 1%MS-222 in 5 mL aquarium water or 10 mL 1% MS-222 in 50 mL aquarium water for adult zebrafish.
2. If necessary, dechorionate the zebrafish embryos (*see* **note 12**).
3. Dissect the pharyngeal jaws from juvenile or adult zebrafish using forceps and micro-scissors.
4. Transfer the embryos, larvae or dissected tissue into an eppendorf containing 4% PFA and fix overnight at 4°C.

4.4.2. Depigmentation (required for all developmental stages, except for dissected pharyngeal jaws)

1. Remove the 4% PFA and rinse the embryos twice with 1xPBS.
2. Add 1 mL of depigmentation mix to the embryos or larvae and expose to a spotlight for 10 – 15 minutes (*see* **note 13**).
3. Once pigments are lost, wash the embryos two times for 5 minutes with 1xPBS.
4. Finally, transfer them through an ascending MeOH series diluted in 1xPBS (25%, 50%, 75% and 100%, each step for 5 minutes). They can now be stored at -20°C for months.

4.4.3.Whole mount immunohistochemistry (Fig. 18A, C)

Day 1:

1. Rehydrate the embryos or tissue by transferring them through a descending MeOH series diluted in 1xPBS (75%, 50%, 25%, each step for 5 minutes).
2. Wash two times for 5 minutes with 1xPBST.
3. Permeabilize the embryos, larvae or adult tissue with proteinase K in an age-dependent concentration according to Table 1. This is the most critical step of the protocol and has to be performed at the concentration and time as described. A concentration or incubation time that is too low will compromise the penetration of the antibody. A concentration or incubation time that is too high will lead to the destruction of the tissue and the deterioration of the embryos (*see* **note 14**).
4. Remove the proteinase K by washing two times for 5 minutes with 1xPBST.

5. Block aspecific binding by incubating in blocking solution for 2 hours (*see* **note 15**).
6. Dilute the primary antibody in the blocking solution (*see* **note 16**).
7. Incubate the embryos, larvae or tissue with primary antibody overnight at 4°C. Meanwhile the corresponding negative control remains in blocking solution also overnight at 4°C.

Age	**PK concentration Diluted from 1000x stock solution**	**Time**
36 – 48 hpf	1 x PK	40 min
2.5 dpf	1.5 x PK	40 min
3 dpf	2 x PK	40 min
4 dpf	3 x PK	40 min
5 dpf	4 x PK	40 min
6 dpf	5 x PK	40 min
7 dpf	6 x PK	40 min
Pharyngeal jaw	7 x PK	40 min

Table 1. Overview of concentration and duration of proteinase K (PK) treatment in relation to larval age (in hours or days post-fertilization, hpf and dpf, resp.) and dissected pharyngeal jaws of juvenile and adult zebrafish

Day 2:

8. Wash four times for 30 minutes with blocking solution (*see* **note 17**).
9. Dilute the secondary antibody in blocking solution (*see* **note 16**).
10. Incubate all stages (including negative controls) with the secondary antibody overnight at 4°C.

Day 3:

11. Wash three times for 30 minutes with blocking solution (*see* **note 17**).
12. Make StreptABComplex and let it rest for 30 minutes at RT before use.
13. Wash one more time for 30 minutes with blocking solution (*see* **note 17**).
14. Incubate in StreptABComplex for 45 minutes, never more than 1 hour.
15. Wash three times for 30 minutes with blocking solution (*see* **note 17**).
16. Wash one time for 30 minutes with 1xPBST.
17. Incubate in freshly prepared liquid DAB solution (*see* **note 4**). For a given primary antibody, always start with determining the optimal incubation time for one developmental stage by checking at regular intervals using a stereomicroscope. Use the same incubation time for all stages in order to allow comparisons.
18. To stop the color development and rinse away the DAB, wash three times for 5 minutes with 1xPBS. Hereafter, the samples are ready to be embedded in epon (see 4.4.5).

4.4.4. Whole mount *in situ* hybridization (Fig. 18B, D)

Day 1: Prehybridization

1. Rinse in 100% MeOH for 5 minutes.
2. Rinse in 1:1 100% MeOH and xylene for 5 minutes.
3. Rinse in 100% xylene for 30 minutes.
4. Rinse in 100% MeOH for 30 minutes.
5. Rehydrate tissue in a descending methanol series in 1xPBST (90%, 70%, 50%; each step for 5 minutes).
6. Rinse in 1xPBST two times, 15 minutes each.
7. Make an appropriate concentration of proteinase K (PK) in PBST starting from the 1000x stock solution. This treatment is age-

dependent and should be performed according to Table 1 (*see* **note 14**).

8. Rinse embryos or tissue for 10-15 seconds in 1xPBST.
9. Re-fixation: add 4% PFA in PBS for 20 minutes.
10. Wash with 1xPBST twice for 5 minutes.
11. Rinse in 0.1 M TEA for 3 minutes.
12. Incubate tissue in acetic anhydride/TEA solution for 10 minutes.
13. Rinse for 30 minutes in 1xPBST.
14. Add 500 µL of prewarmed hybridization solution to each eppendorf, prehybridize in this solution for 1-2 hours at 65°C.
15. Prepare DIG-labeled probes (both sense and anti-sense probes): Dilute to known working concentration in hybridization solution (*see* **note 18**).
16. Heat the diluted probe at 80°C for 10 minutes and put on ice to cool down quickly.
17. Remove prehybridization solution from eppendorfs and add hybridization solution containing diluted probe.
18. Hybridize overnight at 65°C.

Day 2: Posthybridization

19. Prewarm the following solutions: 1:1 formamide/ 2xSSC at 65°C and 10 mL 2xSSC at 37°C.
20. Rinse briefly in 2xSSC at RT.
21. Wash 1 hour in 1:1 formamide/ 2xSSC at 65°C.
22. Wash two times for 15 minutes each in 2xSSC at 37°C (*see* **note 19**).
23. Wash in 1xPBST for 15 minutes at RT.
24. Block with maleate blocking solution for 2 hours at RT.
25. Dilute α-DIG-alkaline phosphatase-coupled antibody in maleate blocking solution (*see* **note 16**), and incubate overnight at 4°C.

Day 3: Color reaction

26. Start with a maleate blocking solution wash, three times for 10 minutes each.
27. Incubate two times for 5 minutes each in Genius buffer.
28. Transfer embryos or tissue to a 24 or 96-well plate.
29. Prepare NBT/BCIP solution and add 200 µL per well.
    Incubate at RT in the dark (wrap the well plate in aluminum foil).
    Monitor reaction frequently using a stereomicroscope.
30. Once the signal is sufficiently strong, wash in 1xPBST two times for 15 minutes at RT to stop the reaction.
31. Fix in 4% PFA for 30 minutes at RT or overnight at 4°C.
32. Rinse 5 minutes in PBS to remove the PFA. Hereafter, the samples are ready to be embedded in epon (see 4.4.5).

4.4.5. Epon embedding

Once the whole mount immunostaining or *in situ* hybridization is finished, the embryos, larvae or tissues can be embedded in plastic to make histological sections. For zebrafish up to 7 days post-fertilization (dpf), a decalcification step is not needed and the embedding protocol can be started at the dehydration step (step 3). Older specimens and dissected pharyngeal jaws need to be decalcified before embedding to enable sectioning.

1. Add 1 mL of 25% Decalc to the tissue in an eppendorf and leave for one week at RT, changing the Decalc solution on a daily basis.

2. Wash larvae in several steps of 1xPBS; large zebrafish or dissected pharyngeal jaws can also be washed overnight in running tap water in a plastic biopsy cassette.

3. 30% EtOH for 15 minutes.

4. Remove half of the volume and add an equal amount of 50% EtOH so you obtain 1:1 30% / 50% EtOH. Leave the sample in this solution for 15 minutes.
5. 50% EtOH for 20 minutes.
6. 50% / 70% EtOH for 20 minutes.
7. 70% EtOH for 30 minutes.
8. 70% / 95% EtOH for 30 minutes.
9. 95% EtOH for 30 minutes.
10. 95% / 100% EtOH for 30 minutes.
11. 100% EtOH for two times one hour.

To be able to observe the colored precipitate, sections are preferably made at 4 µm. Therefore so-called soft epon is used to embed the specimens.

1. Prepare soft epon (*see* **note 10, 11**).
2. Add equal amounts of soft epon and 100% EtOH to a glass jar.
3. Gently stir the mixture (to avoid air bubbles) until epon and ethanol are mixed well.
4. Add the dehydrated samples to the epon-ethanol mixture and close the jar from the air for 48 hours, keep at RT.
5. After 48 hours, open the jar under the fumehood overnight so that the ethanol can evaporate.
6. Next day, prepare fresh soft epon.
7. Transfer the embryos, larvae or tissue into a rubber mould containing freshly made soft epon.
8. Place the mould in a vacuum oven at 37°C under a partial vacuum of 400 mmHg for 4 hours. In this way, trapped air bubbles can escape from the epon.

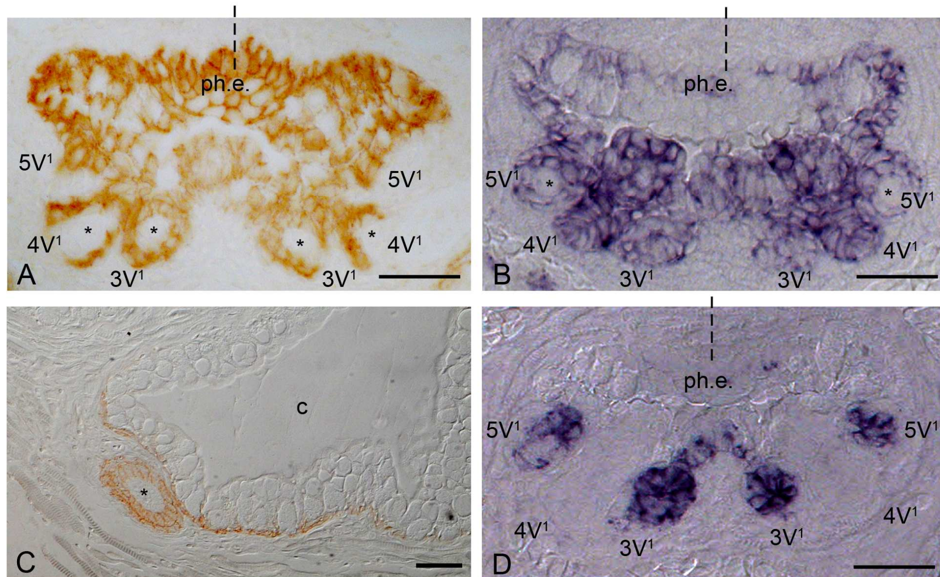

Figure 18. Whole mount immunohistochemistry and whole mount *in situ* hybridization results obtained using the protocols described in this chapter

Four µm cross sections of the pharyngeal region of larval zebrafish (A, B, D) and dissected pharyngeal jaws of adult zebrafish (C) after whole mount immunostaining ((A, C) or *in situ* hybridization ((B, D). A: E-cadherin distribution in a 80 hours post-fertilization (hpf) old zebrafish larva showing E-cadherin present in the enamel organ of each first-generation tooth ($3V^1$, $4V^1$, $5V^1$; tooth coding as described in (Van der heyden and Huysseune, 2000); B: whole mount *in situ* hybridization of a 80 hpf zebrafish larva showing E-cadherin mRNA expression; C: E-cadherin distribution in morphogenesis stage of a later generation tooth obtained from immunohistochemistry on a dissected adult pharyngeal jaw; D: sonic hedgehog (*shh*) expression in first-generation teeth at 72 hpf; note absence of expression in late cytodifferentiation stage of $4V^1$. Abbreviations: *: dental papilla, c:crypt, ph.e.: pharyngeal epithelium. Orientation: A, B, D: dorsal to the top, ventral to the bottom of each figure; dashed line indicates mediosagittal plane. C: dorsal to the top, ventral to the bottom, medial to the right and lateral to the left of the figure. Scale bars= 25µm.

9. For the final embedding, transfer the larvae to the definite mould with soft epon and position them as you like. Put the mould in the oven at 60°C in order to polymerize the epoxy resin. It will take two days to fully harden.

10. Take the blocks from the mould. They are ready to be sectioned with a glass or diamond knife on a standard microtome.

4.5. Notes

1. To dissolve the powder, gently stir with a magnetic stirrer while heating up to 70°C. Never boil the solution. It is advisable to use finest grain powder as it will dissolve without adding NaOH. If the powder does not dissolve well, add 1-2 drops 1N NaOH to change the pH and enhance the dissolution. To fix specimens for *in situ* hybridization, always dissolve PFA-powder in RNase-free 1xPBS (see 4.3.5).
2. Make this blocking solution in a falcon approximately 15 minutes before use. By using a falcon, it is possible to shake and vortex the solution, in order to dissolve the BSA flakes quicker. Prepare this blocking solution fresh on the first day of the protocol. It will be used to dilute the primary and secondary antibody and can be stored at 4°C during the time span of the protocol.
3. Prepare this mixture 30 minutes before use to allow appropriate complex formation. This kit combines streptavidin and horseradish peroxidase (HRP). Streptavidin has a high affinity for the biotin-coupled secondary antibody. At the sites where the primary antibody is bound to the targeted protein, HRP will convert the chromogen DAB into a brown precipitate.
4. Always wear protective clothing while using DAB: it is very carcinogenic. If spilled, clean with bleach to deactivate. After use, dispose into the appropriate disposal bin.
5. DEPC is carcinogenic. Always manipulate under the fumehood. Avoid contact with skin by wearing protective clothing. Once dissolved in water and autoclaved, these precautions are no longer necessary.

6. Be aware that xylene can dissolve certain plastics. Therefore, always use eppendorfs or falcons which are xylene-resistant. You can always use glassware but make sure it is RNase-free. Always make this mixture fresh.
7. Manipulate under the fumehood as acetic anhydrid is irritant and the vapour is harmful.
8. Mix the Blocking Reagent with 1xMAB and stir with a magnetic stirrer on a hot plate (60°C) until the powder has dissolved. Let it cool down at RT and add the Tween-20, BSA powder and sheep serum.
9. Genius buffer is always made fresh and diluted with distilled water. When diluted with another buffer, it will precipitate.
10. Mix the components well by stirring gently with a magnetic stirrer. Avoid making air bubbles. Components can be stored separate at -20°C in a tightly closed plastic container, sealed with parafilm. Gently thaw the separate components at room temperature before opening the vial to avoid any condensation water entering the vials. It is absolutely crucial that the epon as well as the ovens used for vacuum and polymerization are never in contact with water. Any moist in the epon mixture will prevent appropriate polymerization.
11. Mix the components well by stirring gently with a magnetic stirrer. Avoid making air bubbles. Always make fresh, starting from thawed or from freshly made Epon A and Epon B.
12. When raising the embryos at standard water temperature of 28.5°C, the embryos hatch at approximately 48 hpf. If they did not hatch at the time of fixation, they need to be dechorionated to make sure the probe or antibody can reach the tissue. Dechorionation of zebrafish embryos can be done manually with forceps and scalpel or by enzymatic pronase treatment. We prefer manual dechorionation.

13. If you do this depigmentation step in an eppendorf, make sure to make a little hole in the cap of the eppendorf as the combination of KOH and H_2O_2 produces a small amount of gas. Without a hole in the cap, the eppendorf may explode and the material will be lost. It is also possible to work in a well plate. As the embryos lose their pigments, they become very hard to see. Putting the well plate on a black background will facilitate the observation of the colorless samples.
14. To determine the concentration of proteinase K, always use the stage closest to the stage of the embryo or larva you are using. For example, larvae of 68 hpf will be treated with 2x proteinase K for 40 minutes.
15. There is no separate step in which endogenous peroxidase is blocked. This is because during depigmentation, the embryos are already treated with H_2O_2 and therefore endogenous peroxidase is already blocked.
16. The working concentration needs to be determined for each primary or secondary antibody separately. Always check the corresponding data sheet of the antibody, as it will contain more information about the optimal concentration of the antibody. Generally, a good starting point is 1:300.
17. Washing steps are performed on a rocking plate, medium speed. The eppendorfs are placed flat on the rocking plate so that washing can be more intense and thus reduce aspecific binding of the primary or secondary antibody.
18. The ideal concentration depends mainly on the probe but also on the tissue. Concentrations are commonly between 0.4 – 2 µg/mL hybridization solution.
19. To reduce background an additional RNase treatment can be performed after this washing step. Wash 30 minutes with RNAse A

(20µg/ml) in 2xSSC. Wash with 2xSSC for 10 minutes at 37°C and wash with 2xSSC for 30 minutes at 65°C. Then, continue the protocol with step 23.

4.6. Acknowledgements

We thank Tommy D'heuvaert for technical assistance.
This work was supported by a GOA research grant (BOF08/GOA/019) to ES and AH. BV acknowledges a grant of the IWT.

SECTION IV

RESULTS

The following chapters comprise the results of this PhD thesis. Each chapter is formatted as a manuscript and deals with a specific component of the cadherin-catenin complex.

The **first chapter** is a published manuscript in which both the expression pattern and distribution of the main epithelial cadherin, **E-cadherin** during the successive stages of tooth development in both first-generation and later-generation teeth are described and discussed.

Chapter 2 shows the remarkable distribution of a second cadherin, **N-cadherin**, during tooth development and replacement in the zebrafish. Our data indicate that N-cadherin expression might be related to the start of differentiation of ameloblasts and odontoblasts.

The distribution of the cadherin-associated proteins, **β-catenin and plakoglobin**, is analyzed in **chapter 3**. Both proteins are visualized by immunohistochemistry which shows the specific distribution for both proteins. Moreover, plakoglobin morpholino-injected zebrafish were examined to investigate if there is a role for this protein during zebrafish tooth development.

The final chapter, chapter 4, contains the data collected on a catenin that so-far has not been studied in zebrafish, **p120ctn**. Here, the mRNA expression is visualized in all stages of first-generation teeth. The accompanying appendix shows the molecular cloning and possible isoforms of this zebrafish p120ctn.

1. Zebrafish teeth as a model for repetitive epithelial morphogenesis: dynamics of E-cadherin expression

BMC Dev Biol. 2010 Jun 1;10:58.

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1.1. Abstract

Background

The development of teeth is the result of interactions between competent mesenchyme and epithelium, both of which undergo extensive morphogenesis. The importance of cell adhesion molecules in morphogenesis has long been acknowledged but remarkably few studies have focused on the distribution and function of these molecules in tooth development.

Results

We analyzed the expression pattern of an important epithelial cadherin, E-cadherin, during the formation of first-generation teeth as well as replacement teeth in the zebrafish, using *in situ* hybridization and whole mount immunostaining to reveal mRNA expression and protein distribution. E-cadherin was detected in every layer of the enamel organ during the different stages of tooth development, but there were slight differences between first-generation and replacement teeth in the strength and distribution of the signal. The dental papilla, which is derived from the mesenchyme, did not show any expression. Remarkably, the crypts surrounding the functional teeth showed an uneven distribution of E-cadherin throughout the pharyngeal region.

Conclusions

The slight differences between E-cadherin expression in zebrafish teeth and developing mouse and human teeth are discussed in the light of fundamental differences in structural and developmental features of the dentition between zebrafish and mammals. Importantly, the uninterrupted expression of E-cadherin indicates that down-regulation of E-cadherin is not required for formation of an epithelial tooth bud. Further research is needed to understand the role of other cell adhesion systems during the development of teeth and the formation of replacement teeth.

1.2. Background

The dentition of vertebrates consists of repetitive units, teeth, which have an epithelial as well as a mesenchymal component. Some features of tooth development are shared (e.g. formation of an epithelial bud, condensation of mesenchyme, polarized deposition of mineralizing matrices) whereas others are unique to certain taxa (e.g. different ways of attachment, different ways of eruption, whether enamel or enameloid is produced) (Borday-Birraux et al., 2006; Huysseune and Sire, 1998; Stock, 2007).

Like many other vertebrate organs, teeth arise by epithelio-mesenchymal interactions. Their development starts in the same way in all vertebrates by the formation of an epithelial thickening (placode) that invaginates into the underlying mesenchyme and forms a bud surrounding the condensed mesenchyme (dental papilla) (Huysseune and Sire, 1998).

The zebrafish, a widely used model in genetic, molecular and developmental research, has no oral teeth, but teeth attached to the fifth branchial arch only (Stock, 2007). The complete dentition of the zebrafish consists of three rows of teeth on each side, all of which are replaced throughout life: a ventral row (V) of five teeth, a mediodorsal row (MD) of four teeth and a dorsal row (D) of two teeth. The teeth in the ventral row are named 1V to 5V, rostral to caudal (Van der heyden and Huysseune, 2000). The first tooth bud starts to develop after 2 dpf (days post-fertilization) at position 4V and is quickly followed by the development of the tooth germs at positions 3V and 5V. The teeth in the two rostral positions, 2V and 1V, develop at 12 and 16 dpf, respectively (Van der heyden and Huysseune, 2000). Replacement of zebrafish first-generation teeth starts early, between 3 and 4 dpf for the first tooth (position 4V).

In the zebrafish, buds of first-generation teeth develop directly from the pharyngeal epithelium. In contrast, buds that will form replacement teeth develop from an epithelial outgrowth at the base of the epithelial crypt surrounding the tip of the erupted functional tooth. This outgrowth is called the successional lamina (Huysseune, 2006).

Formation of the successional lamina and of the epithelial tooth bud, as well as condensation of the mesenchyme to form the dental papilla, requires substantial rearrangements of cells. It is very likely that cell adhesion molecules play an important role in these rearrangements. Although the importance of cell-cell adhesion during morphogenesis has been known for quite some time (Halbleib and Nelson, 2006), there are remarkably few studies on the distribution and function of cell adhesion molecules in tooth development (Heymann et al., 2002; Obara and Lesot, 2004; Obara et al., 1998; Palacios et al., 1995). This poor knowledge stands in sharp contrast with the amount of data collected over the previous years on regulatory mechanisms in tooth development, which involves transcription factors, growth factors, signalling molecules and receptors of the extracellular matrix (Pispa and Thesleff, 2003).

The few studies about cell adhesion molecules in tooth development have focused on mouse or human teeth or on one type of cell only (Heymann et al., 2002; Obara et al., 1998; Palacios et al., 1995). Moreover, the focus has been mainly on embryonic tooth development (formation of first-generation teeth) and little or no attention has been paid to the process that underlies the renewal of teeth (development of replacement teeth).

Cadherins constitute a large family of Ca^{2+}-dependent adhesion molecules. The family is divided into several subfamilies, of which the classical cadherins are the most studied (Halbleib and Nelson, 2006). E-cadherin belongs to the subfamily of classical cadherins and is an epithelial cadherin responsible for the maintenance of pithelial cell layers (van Roy and Berx, 2008).

Other classical cadherins are typical for different tissues: e.g. N-cadherin is expressed in muscle and neural tissue, R-cadherin in forebrain and bone, and VE-cadherin in endothelial cells (Ivanov et al., 2001).
Cadherins play important roles in cell adhesion through their link to the actin cytoskeleton. Newly synthesized cadherins are transported to the cell membrane while coupled to β-catenin. At the cell membrane p120 catenin binds to the juxtamembrane domain of the intracellular part of the cadherin, thus stabilizing it at the membrane (Xiao et al., 2007). β-catenin and plakoglobin compete for binding to the C-terminal domain of E-cadherin, and are each also linked to α-catenin. The latter can also bind the actin cytoskeleton. Together, these molecules form the cadherin-catenin complex. When the expression of E-cadherin is downregulated (e.g., in tumor development), cell adhesion decreases and cell migration increases (Jeanes et al., 2008; Margineanu et al., 2008; van Roy and Berx, 2008). Whereas different intracellular signalling pathways can modulate the formation of the complex and the strength of the adhesion, cadherin-mediated adhesion itself can also influence intracellular signalling via Rho-GTPases or indirectly via several growth factors (Braga, 2002; Nelson, 2008). Cadherin-dependent signalling influences cellular processes such as proliferation, survival, differentiation, morphogenesis and migration. These processes are important during embryogenesis and organogenesis (Bar-Sagi and Hall, 2000; Kuroda et al., 1997; Mackay and Hall, 1998).
Jamora et al. (2003), using mouse hair follicle as a model, proposed that E-cadherin is downregulated at the start of the formation of an epithelial bud as a result of activation of the Wnt signalling pathway, concomitantly with an inhibition of BMP signalling. Such down-regulation would allow rearrangement of the epithelial cells to enable formation of a bud, such as during the formation of glands or hair follicles.

Our study was inspired by the lack of data on the distribution and role of E-cadherin during the formation of tooth buds and by the hypothesized role of E-cadherin down-regulation in epithelial bud formation. We report on the dynamics of E-cadherin expression, both at the mRNA and protein level, in first-generation teeth and their successors in an animal displaying continuous tooth renewal, the zebrafish. This is part of a larger study aiming at elucidating the role of specific cell adhesion molecules in the development and renewal of teeth.

1.3. Results

E-cadherin mRNA expression and protein localization coincided at all material examined. Therefore, we do not distinguish between them in the following sections.

Outside the prospective dental placode E-cadherin expression was also detected in the pharyngeal epithelium as well as in the presumptive gill slits. E-cadherin was detected in the pharyngeal epithelium, both before (48 hours post-fertilization (hpf)) and after (56 hpf) the formation of the pharyngeal cavity. All layers of the pharyngeal epithelium expressed E-cadherin to the same extent in 40 hpf until 6 days post-fertilization (dpf) old fish. Also the epithelial lining of the presumptive gill slits and the cells within the multiple layers of the presumptive keratinized pad, facing the developing pharyngeal jaws, were E-cadherin-positive. However, at about 4 dpf, the most apical layer of the keratinized pad, i.e. the layer that is shed, lost its E-cadherin expression.

1.3.1. Distribution of E-cadherin during the development of first-generation teeth

Below we will describe the expression of E-cadherin in tooth $4V^1$, the first tooth to develop, as representative of the pattern observed in first-generation teeth. We found no difference in expression pattern or cellular distribution of E-cadherin during development when we compared teeth $3V^1$ and $5V^1$ with $4V^1$.

Initiation and morphogenesis

Initiation of tooth development is characterized morphologically by formation of an epithelial thickening, the placode. In this area, the cells of the basal layer of the epithelium are columnar and polarized. These cells start to invaginate into the underlying mesenchyme during morphogenesis.

Cells that form the placode show strong expression of E-cadherin. No difference in strength of the signal could be detected between placodal cells and the non-differentiated parts of the pharyngeal epithelium (Fig. 19A,B,C; Table 2).

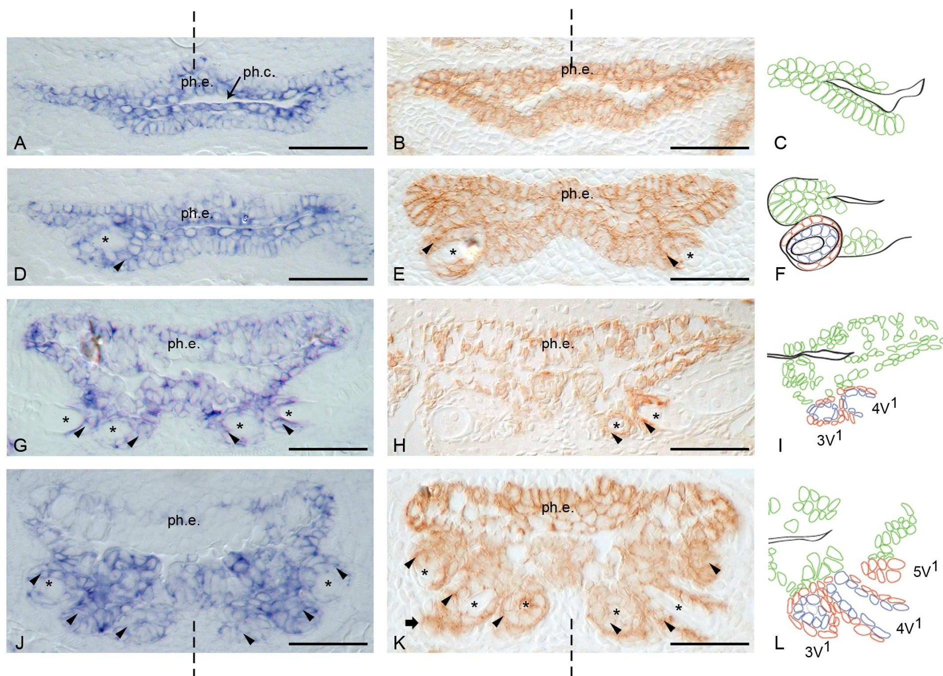

Figure 19. Expression pattern of E-cadherin in first-generation teeth

Left panels: mRNA expression; middle panels: protein expression; right panels: diagram of different cell layers of the tooth.

A, B, C: Initiation phase. D, E, F: Morphogenesis phase; G, H, I: Early cytodifferentiation and attachment phase; J, K, L: Late cytodifferentiation phase and initiation of successor. ph.c.: pharyngeal cavity; ph.e.: pharyneal epithelium; *: dental papilla; arrowhead: enamel organ; block arrow: initiation replacement tooth. Diagrams: green cells: pharyngeal epithelium; red cells: outer dental epithelium; blue cells: inner dental epithelium. Orientation: dorsal to the top, ventral to the bottom of each figure; dashed line indicates mediosagittal plane. Scale bars=25 µm.

During morphogenesis the epithelium starts to protrude into the mesenchymal layer underneath. Subsequently, the epithelial cells start to differentiate into two

layers of cells: the inner dental epithelium (IDE) and the outer dental epithelium (ODE). These two layers together form the enamel organ of the developing tooth. Opposite the epithelial cells, mesenchymal cells condense to form the dental papilla of the tooth.

During morphogenesis stage E-cadherin was expressed in the cells of both the IDE and the ODE, but no signal was present in the mesenchymal part of the tooth, the dental papilla (Fig. 19D,E,F; Table 2).

Early cytodifferentiation

While the free margin of the enamel organ, called the cervical loop, grows deeper into the mesenchyme, the IDE cells differentiate into ameloblasts and the cells of the dental papilla into odontoblasts. The ameloblasts start to produce enameloid. The odontoblasts, on the other hand, start to produce predentin which will later mineralize into dentine.

During early cytodifferentiation, the ODE and ameloblasts maintain strong expression of E-cadherin, at both the mRNA and the protein level. Again, all the cells that form the dental papilla show no sign of expression of this cell adhesion molecule (Fig. 19G,H,I, tooth $3V^1$; Table 2).

	epithelium		mesenchyme	
	IDE	**ODE**	**odontoblasts**	**dental papilla**
initiation	++	++	-	-
morphogenesis	++	++	-	-
early cytodifferentiatio	++	++	-	-
late cytodifferentiatior	++	++	-	-
attachment	++	++	-	-

Table 2. Overview of expression of E-cadherin in the different cell layers of a first-generation tooth on the basis of mRNA and protein distribution

The epithelial part of the tooth remains positive for E-cadherin throughout all stages of tooth development. Odontoblasts and dental papilla show no E-cadherin expression at any developmental stage.

Late cytodifferentiation

As cytodifferentiation proceeds, the matrix becomes clearly visible on the sections, but enameloid and dentine are hardly distinguishable. The cervical loop is now lying close to the cartilage of the branchial arch but no attachment bone has been deposited yet.

During late cytodifferentiation stage, the expression pattern has not changed. Both the ODE and ameloblasts remain positive while the odontoblasts remain negative for E-cadherin (Fig. 19J,K,L; Table 2).

Attachment and eruption

Finally, the attachment bone is formed in the prolongation of the tooth base and the tooth is attached to the perichondral bone surrounding the cartilaginous branchial arch.

The epithelial layers overlying the tooth tip then fold back, thus exposing the tooth tip into the pharyngeal lumen (Huysseune and Sire, 2004). The tooth is now erupted and becomes functional.

The expression pattern of E-cadherin in a functional tooth is identical to that in a developing tooth. Both epithelial layers, ODE and IDE, which form the reduced enamel organ at this stage, are strongly positive for both E-cadherin mRNA and protein. This is in sharp contrast to the dental papilla, which does not express E-cadherin at all (Fig. 19H, tooth $4V^1$; Table 2).

1.3.2. Distribution of E-cadherin during the development of replacement teeth

To analyze E-cadherin distribution during tooth replacement, whole mount immunohistochemistry was performed on specimens of different ontogenetic stages up to 6 dpf and on dissected jaws of an adult zebrafish. Additionally, *in situ* hybridization was conducted on specimens of similar stages and on dissected jaws of an adult fish to clarify the expression pattern of this cell adhesion molecule. The examination of adult jaws provided better insight into the mRNA expression and distribution of E-cadherin protein in teeth of later generations. In adults, all eleven tooth positions are established and replacement teeth are in different stages of development. Moreover, the teeth are larger than first-generation teeth and thus the tissues consist of many more cells. This enables detailed observations of the expression at the cellular level. In the description below, we focus on the results obtained from the analysis of whole mount immunostaining of dissected adult jaws.

The branchial arches express E-cadherin in the epithelial lining of the gills and of the pharyngeal cavity. We observed expression in the rostral part of the keratinized pad opposite the pharyngeal jaws, but not in the caudal part.

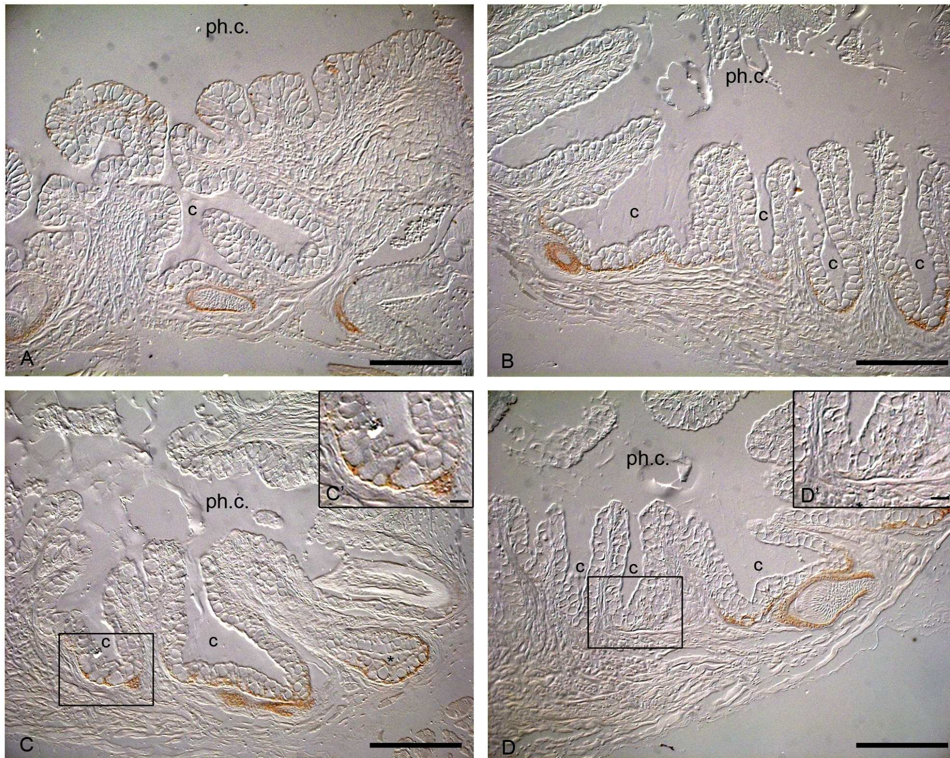

Figure 20. Uneven distribution of E-cadherin in the crypt epithelium

Sections of whole mount immunocytochemical examination of dissected adult jaw.
The rostral crypts in the pharyngeal region of adult zebrafish express E-cadherin throughout the epithelium (A).This contrasts to the more caudal crypt epithelium, which loses E-cadherin expression, except along the crypt bases (B). Cells placed rostrally at the base of the crypt always have stronger E-cadherin expression (C, C') than cells placed more caudally within the same crypt (D, D'). c: crypt; ph.c.: pharyngeal cavity. Orientation: dorsal to the top, ventral to the bottom, medial to the left in A, C, D and to the right in B. Scale bars=50 µm, scale bar C', D'= 10 µm.

An E-cadherin signal is evident in the crypt epithelium surrounding the tip of each functional tooth, but it is distributed unevenly. The epithelium of the rostral crypts consists of cells all of which express E-cadherin (Fig. 20A). The E-cadherin signal decreases in crypts more caudally, and finally disappears in the most caudal crypts, except at their base (Fig. 20B).

In each crypt belonging to a specific functional tooth, whether positioned more rostral or more caudal, the rostral cells at the base of the crypt always have stronger E-cadherin expression than the cells that constitute the caudal side of the same crypt (Fig. 20C,C',D,D').

We observed a dynamic pattern of E-cadherin expression during the formation of a replacement tooth early in post-embryonic development and in adults.

The stages of the odontogenesis distinguished during development of replacement teeth resemble those described for first-generation teeth.

Initiation

Unlike first-generation teeth, replacement teeth do not arise directly from the pharyngeal epithelium. They are formed medial to their predecessors from an epithelial protrusion, called the successional lamina (Fig. 21A), which develops close to the base of the crypt surrounding the tip of the predecessor. During the first replacement cycles the successional lamina is inconspicuous because the teeth are small. It becomes prominent in later ontogenetic stages, when teeth are larger and have gone through several replacement cycles (Huysseune, 2006). As replacement teeth develop, they assume a horizontal position, with their dorsal side closely adjoining the crypt epithelium (Fig. 21B-D).

The successional lamina expresses E-cadherin, except for the centre of the lamina (Fig. 21A'). This pattern was observed consistently for all four replacement teeth in the ventral row observed in this developmental stage. The signal in the cells of the successional lamina is extremely strong. During this stage corresponding to the

initiation stage of the replacement tooth, the base of the crypt of the functional tooth expresses E-cadherin as well, particularly on the medial side of the crypt (Fig. 21A; Table 3). It should be noted that the successional lamina displays strong, membrane-bound, immunolocalization of β-catenin (unpublished results and Fig. 22).

Morphogenesis

As the distal part of the successional lamina develops into the enamel organ of the new tooth bud, a strong E-cadherin signal is maintained. Likewise, the base of the crypt maintains the same expression pattern seen in the initiation phase, i.e. the medial side of the crypt is more intensely stained than the lateral side (Fig. 21B; Table 3).

Early cytodifferentiation

The cells of the enamel organ of the new tooth, while growing obliquely into the underlying mesenchyme, differentiate into the IDE and ODE, which maintain E-cadherin expression. E-cadherin expression is remarkably stronger on the ventral side of the developing tooth than on its dorsal side. At the same time, the layer of mesenchymal cells opposite the IDE differentiates into odontoblasts. The mesenchymal cells express no E-cadherin (Fig. 21C; Table 3).

From this stage onwards, the strength of the E-cadherin signal in the different cell layers of the teeth decreases. Moreover, its expression at the base of the crypt also diminishes. Yet, the medial side of the crypt still expresses E-cadherin more strongly than the lateral side.

Late cytodifferentiation

Late cytodifferentiation is marked by the presence of a noticeable amount of matrix (enameloid first, followed by dentine).

During this stage, the IDE and ODE continue to express E-cadherin, albeit at a lower level, while the dental papilla remains negative (Fig. 21D; Table 3). The strongest expression is in the cells on the ventral side of the tooth. In the crypt of the corresponding functional tooth, the expression of E-cadherin becomes barely noticeable.

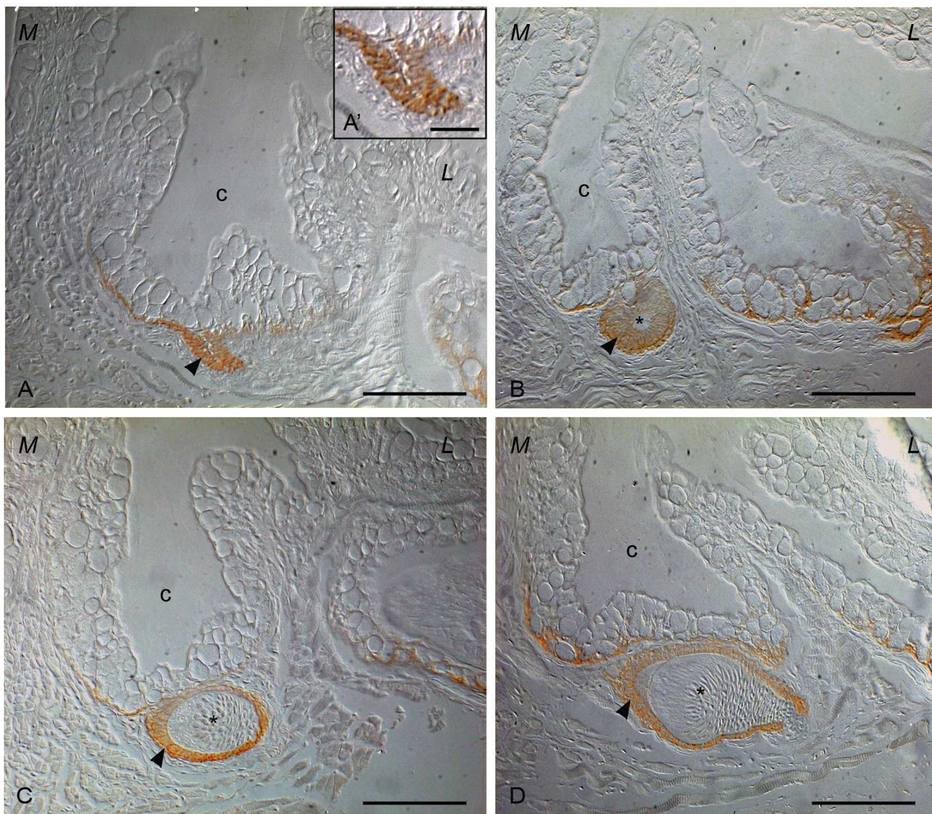

Figure 21. E-cadherin expression pattern during tooth replacement

Sections of whole mount immunocytochemical examination of dissected adult jaw.
A, A’: Successional dental lamina is positive for E-cadherin; B: Morphogenesis phase; C: Early cytodifferentiation, IDE and ODE express E-cadherin; D: Late cytodifferentiation.
*: dental papilla; arrowhead: enamel organ; c: crypt slightly posterior to the tip of the functional predecessor; L: lateral; M: medial. Orientation: dorsal to the top, ventral to the bottom, medial to the left and lateral to the right of the figure. Scale bars=50 µm, scale bar A’= 25 µm.

	epithelium		mesenchyme	
	IDE	**ODE**	**odontoblasts**	**dental papilla**
initiation	++	++	-	-
morphogenesis	++	++	-	-
early cytodifferentiation	++ *	++ *	-	-
late cytodifferentiation	++ *	++ *	-	-
attachment				

Table 3. Overview of expression of E-cadherin in the different cell layers of a replacement tooth

The two layers of the enamel organ, IDE and ODE, maintain the expression of E-cadherin. The mesenchyme-derived dental papilla does not express this cell adhesion molecule during development. *: stronger expression of E-cadherin on the ventral side of the developing tooth.

1.4. Discussion

We examined mRNA expression and protein localization of the cell adhesion molecule E-cadherin during the development stages of first-generation teeth and replacement teeth in zebrafish. We observed no difference in the localization of E-cadherin transcripts or protein, which indicates that transcription leads to translation. In both first-generation and replacement teeth, the two layers that constitute the enamel organ express E-cadherin during the different stages of tooth formation. The dental papilla, on the other hand, is consistently negative for E-cadherin. Despite this overall pattern, there are some differences in the distribution of the E-cadherin signal between first-generation and replacement teeth. First, unlike first-generation teeth, developing replacement teeth display a decrease of E-cadherin expression beyond morphogenesis stage. Likewise, the crypt surrounding the tip of the functional tooth maintains E-cadherin expression along the medial side of the crypt base longer than along the lateral side. This regional difference could be related to the position of the developing replacement tooth with respect to the crypt base. The cells at the base of the crypts have E-cadherin expression at the position from which the successor arises from the outer dental epithelium of the predecessor.

Remarkably, crypts displaying an E-cadherin signal were unevenly distributed throughout the pharyngeal region. Anteriorly, all crypts showed a signal, whilst the signal diminished more posteriorly within the pharyngeal cavity, until no expression was left in the most posterior crypts, except at their base. An even more puzzling finding was that only the crypts of the ventral teeth seem to express E-cadherin. No expression was found at the base of the crypts of the medio-dorsal or dorsal teeth. We cannot exclude the possibility that a cadherin-switch could be responsible for this differential expression pattern as described during morphogenesis and tumor progression (Sakamoto et al., 2008; Wheelock et al., 2008).

Previous studies on cell adhesion molecules in teeth focused on mouse and human teeth (Heymann et al., 2002; Palacios et al., 1995). Moreover, these studies examined the expression pattern during development of first-generation teeth or focused on specific cell types (Obara et al., 1998). During the development of teeth in the mouse, E-cadherin is expressed in all epithelial cells during the initiation phase. Distinct E-cadherin expression is also observed during morphogenesis in the dental lamina, stellate reticulum, stratum intermedium, ODE and the cervical loop in mouse tooth development. During cytodifferentiation, the polarizing ameloblasts are also positive. The expression pattern of E-cadherin in mouse and zebrafish teeth is similar, the major difference being the absence of E-cadherin expression in the IDE during morphogenesis in the mouse. The patterns of E-cadherin and P-cadherin expression in IDE cells in the mouse change during development. During morphogenesis of the developing mouse molar, the IDE expresses P-cadherin but not E-cadherin (Obara et al., 1998). Expression of P-cadherin has been associated with the proliferation of the cells and with their ability to induce condensation of the surrounding mesenchyme (Palacios et al., 1995). Moreover, the differential expression of E- and P-cadherin in IDE cells is also related to their differentiation stage. Undifferentiated IDE cells are positive for P-cadherin, whereas polarized and differentiated ameloblasts in mouse express E-cadherin (Obara et al., 1998). Heymann et al. (2002) did not detect any expression of E-cadherin during the initiation phase of human deciduous teeth but observed that the amount of protein in the enamel organ increased during morphogenesis. During later developmental stages, E-cadherin expression diminishes in the apical to coronal direction, while N-cadherin is up-regulated. This expression pattern is explained by the suggestion that increased N-cadherin expression might be important for ameloblast transformation and polarization required for enamel matrix secretion (Heymann et al., 2002).

It is important to keep in mind several features that distinguish zebrafish from mammalian (mouse and human) teeth.

First, the mouse is monophyodont and all studies on murine tooth development therefore concern first-generation teeth. Second, the structure of the enamel organ differs considerably from that of zebrafish. The enamel organ of zebrafish teeth possesses no stellate reticulum or stratum intermedium, and IDE and ODE are directly apposed. Ameloblasts in mammalian teeth are involved in deposition of enamel matrix, its subsequent mineralization and removal of organic matrix during enamel maturation; therefore, ameloblasts cycle through multiple functional stages (Kallenbach, 1974; Zeichner-David et al., 1995). In contrast, ameloblasts in zebrafish are involved in the deposition of enameloid, which is both structurally and developmentally different from mammalian enamel (Huysseune and Sire, 1998; Sasagawa, 1995; Sasagawa, 1997). Moreover, tooth tissues of zebrafish consist of substantially fewer cells than their murine counterparts. All these factors could contribute to the slight differences in E-cadherin expression pattern between zebrafish and mouse teeth.

Jamora et al. (2003), using data from the mouse, hypothesized that formation of an epithelial bud is facilitated by down-regulation of E-cadherin in response to Wnt signalling. Consistent with their hypothesis, they found that overexpression of E-cadherin inhibits invagination and production of hair follicles in mouse skin (Jamora et al., 2003). Morphogenesis of a tooth bud and a hair bud share many features (Mikkola, 2007; Pispa and Thesleff, 2003; Thesleff et al., 1995). Thus, we focused specifically on whether we can observe down-regulation of E-cadherin during formation of tooth buds of first-generation or replacement teeth. Contrary to what could be predicted based on the suggestion made by Jamora et al. (2003), we observed uninterrupted and very strong expression of E-cadherin during formation of the placode (in first-generation teeth) or the successional lamina (in replacement teeth) (Huysseune and Thesleff, 2004; ten Berge et al., 2008). E-cadherin mutants of zebrafish, as well as morphants (induced by morpholino injections) do not survive gastrulation (Babb and Marrs, 2004; Shimizu et al., 2005).

As teeth start to develop at 2 dpf, loss-of-function experiments to unravel the function of E-cadherin during tooth development were therefore not possible to conduct.

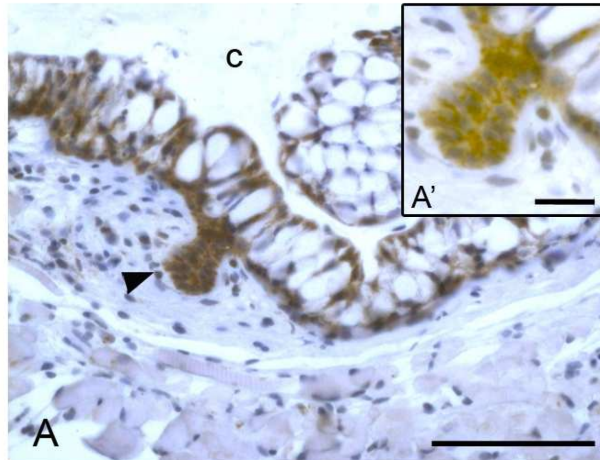

Figure 22. Beta-catenin immunolocalization in the successional lamina

The onset of formation of a replacement tooth, the successional lamina, has strong β-catenin expression along the membrane of every cell (A, A'). This β-catenin expression co-localizes with the expression of E-cadherin suggesting that the cadherin-catenin complex is functional as adhesive force. Orientation: dorsal to the top, ventral to the bottom, medial to the left and lateral to the right of the figure. arrowhead: successional lamina; c: crypt surrounding the tip of the functional predecessor. Scale bar=50 µm, scale bar A'= 15 µm.

Many tissues (not just hair follicles, but also glands such as mammary and salivary glands) share the same developmental dynamics of invagination of an epithelial layer into the underlying mesenchyme to form an epithelial bud. Studies describing the regulation of different cell adhesion molecules during this process in hair follicles, mammary glands and salivary (submandibular) glands show that expression of different cell adhesion systems is dynamic early during morphogenesis.

In these tissues, desmosomal and hemidesmosomal components are downregulated during formation of a placode and there is complete loss of these structures in the epithelial bud (Hieda et al., 1996; Hieda and Nakanishi, 1997; Inamatsu et al., 2006; Nanba et al., 2001).

Recent studies on cancer and tumor development consider the possibility that E-cadherin can remain at the cell membrane, yet not function any longer in adhesion. This contrasts to the long standing idea that disruption of the cadherin-catenin complex would lead to internalization and degradation of E-cadherin. Rather, it has been suggested that it might be the loss of β-catenin that makes the cadherin-catenin complex incompetent, thereby reducing adhesion strength (Da Silva et al., 2008; Sundfeldt, 2003). This emerging hypothesis can explain how a straight layer of cells can change cell shape and position without loss of E-cadherin expression. The distinct membrane-bound presence of β-catenin in the successional lamina of zebrafish replacement teeth, however, argues against this possibility.

In conclusion, our present data, together with data published on other organ systems, lead us to suggest that formation of an epithelial bud is not necessarily associated with down-regulation of E-cadherin. Other cell adhesion systems can play an important role in the first stages of development in tissues that arise through epithelio-mesenchymal interactions.

1.5. Conclusions

In this study, we present the expression pattern and protein distribution of the epithelial cell adhesion molecule E-cadherin during the different stages of tooth development in the zebrafish. We have shown that E-cadherin is present in the epithelial-derived part of the developing tooth, both in the inner as well as in the outer dental epithelium. The mesenchymal-derived dental papilla remains negative for E-cadherin throughout development. The same expression pattern is found in replacement teeth.

Zebrafish replace their teeth continuously throughout life and therefore their teeth represent an attractive model for studying repetitively renewing structures that start their formation with an epithelial bud. Our data do not show a down-regulation of E-cadherin at the onset of formation of an epithelial bud such as proposed in hair follicles by Jamora et al. (2003).

Further research is needed to understand the role of other cell adhesion systems during the development of teeth and the formation of replacement teeth.

1.6. Methods

1.6.1. Fish strains

Zebrafish (*Danio rerio*) were mated and eggs were allowed to develop at 28.5°C in a 10-h dark / 14-h light cycle. The embryos were sacrificed according to the Belgian law on the protection of laboratory animals (KB d.d. 13 September 2004) by an overdose of MS222 (3-aminobenzoic acid ethyl ester) at specific times. The first embryos were sacrificed at 40 hpf and then after every 4 hours until 4 dpf. From 4 dpf until 6 dpf, fish were sacrificed every 8 hours.

Fifth branchial arches were dissected from adult zebrafish under a Leica MZ Apo dissecting microscope using microscissors.

1.6.2. Tissue processing

The embryos, larvae and dissected fifth branchial arches were fixed overnight at 4°C in 4% paraformaldehyde (PFA). Before storing the embryos and larvae in methanol (MeOH), they were depigmented in 0.5% KOH and 0.05% H_2O_2 in phosphate buffered saline (PBS) for 30 minutes to 1 hour at room temperature (RT). They were washed twice with 1x PBS put through several MeOH steps (25%, 50%, 75%) and stored at –20°C in 100% MeOH. The dissected branchial arches were not depigmented but, immediately after fixation, they were put through a series of MeOH and stored in 100% MeOH at –20°C.

Adult PFA-fixed jaws selected for immunolocalization of β-catenin were paraffin-embedded according to routine procedures and serially sectioned at 5 µm.

1.6.3. Immunohistochemistry

The embryos and larvae selected for whole mount immunohistochemistry were rehydrated through a descending MeOH series. They were next treated with proteinase K for 40 minutes in a concentration depending on the age of the animal. This pretreatment with proteinase K does not affect the antigens as evidenced by strong positive staining of typical epithelial tissues. The primary antibody used was the E-cadherin antibody clone 36 against the C-terminal domain of human E-cadherin, BD Transduction Laboratories, tested for specificity on a western blot using a zebrafish lysate. This antibody was successfully applied to zebrafish (Kiener et al., 2008; Mich et al., 2009). The specimens were incubated with the primary antibody overnight at 4°C (dilution 1:300 in blocking solution) and incubated for 20–24 h at 4°C with the secondary antibody (Polyclonal goat anti-mouse immunoglobulin biotinylated, Dako, diluted 1:300 in blocking solution). They were next treated with the streptABComplex-horseradish peroxidase (Dako) and the location of the antibodies was detected with DAB (Biogenex). Control embryos were treated with secondary antibody only. To analyze the signal in detail, the specimens were subsequently dehydrated, embedded in epon, serially sectioned at 4 µm, and the sections were mounted with Depex.

Immunolocalization on paraffin sections was performed according to standard procedures (Sklyarova et al., 2008) using β-catenin primary antibody (C7207, Sigma, dilution 1:1000) and polyclonal goat anti-mouse immunoglobulin biotin-coupled secondary antibody (Dako, dilution 1:500).

1.6.4. Probe synthesis and *in situ* hybridization

Digoxigenin-labeled probes were synthesized from template cDNA of *cdh1* clone 26 in plasmid BlueScript (plasmid kindly provided by Dr. James Marrs, Indiana University Medical Center, IN). The plasmid was linearized with *Xho*I and

transcribed with T3 RNA polymerase to generate the sense probe and linearized with *Eco*RI and transcribed with T7 RNA polymerase to obtain the anti-sense probe.

In situ hybridization with sense and antisense probes was performed following the protocol established by Pamela Raymond (University of Michigan, MI) (http://www.mcdb.lsa.umich.edu/labs/praymond). The probes were detected with NBT/BCIP. After staining, the specimens were fixed overnight at 4°C in 4% PFA. They were next dehydrated, embedded in epon, and serially sectioned at 4 µm. The sections were mounted with Depex.

All sections were examined under a Zeiss Axio Imager Microscope equipped for DIC and photographed using an Axiocam MRC videocamera.

1.7. Authors' contributions

BV contributed to conception and design of the project, the acquisition of all data, she analysed the data and was involved in drafting the manuscript. ES significantly contributed to the establishment of ISH protocols. JVH contributed to design, extensively discussed results and revised the manuscript for intellectual content. AH contributed to conception of the project, interpreted results, and helped to draft the manuscript. All authors read and approved the final manuscript.

1.8. Acknowledgements

We thank Prof. Dr. James Marrs for providing the plasmid containing partial cDNA of zebrafish *Cadherin-1*, Tommy D'heuvaert for technical assistance, Dr. Maxime Willems for the help with the figures and Prof. Dr. Frans van Roy for revising the manuscript critically for intellectual content.

This work was supported by a GOA research grant (BOF08/GOA/019) to ES, JVH and AH. BV acknowledges a grant of the IWT.

2. N-CADHERIN EXPRESSION AND DISTRIBUTION DURING TOOTH DEVELOPMENT AND TOOTH REPLACEMENT IN THE ZEBRAFISH

Manuscript to be submitted

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2.1. Abstract

N-cadherin is a well-studied classical cadherin known to provide strong cell-cell adhesion between neighbouring cells. Moreover, it has been proven that N-cadherin also has a signalling function and is known to be involved in multiple processes. In zebrafish, N-cadherin is known to influence many important processes, such as neurulation and cardiac development.

Zebrafish tooth development can serve as a model for repetitive morphogenesis as zebrafish continuously replace teeth throughout life. Each tooth is initiated as an epithelial placode of which the cells will invaginate into the underlying mesenchyme. Although a lot is already known on how cell adhesion molecules are regulated during morphogenesis, there are few studies concerning the importance of cell adhesion during tooth development.

We describe both the expression and distribution of N-cadherin during the multiple stages of tooth development and tooth replacement. N-cadherin is absent during the initiation of a new tooth placode or successional lamina. Also during morphogenesis stage, there is no N-cadherin detected, neither in the enamel organ nor in the condensed mesenchyme. We found that N-cadherin expression starts simultaneously with the onset of the differentiation of cells of the inner dental epithelium and the dental papilla in ameloblasts and odontoblasts, respectively. In *parachute* zebrafish mutants, deficient for N-cadherin, only teeth during morphogenesis stage were detected. We suggest there might be a role for N-cadherin during both ameloblast and odontoblast differentiation and therefore during the development of teeth.

2.2. Introduction

Cell adhesion plays an important role in many morphogenetic processes such as cell proliferation, migration and differentiation. The regulation of the adhesion molecules involved makes these essential movements possible.

Cell adhesion can be provided by different types of junctions (adherens junctions, tight junctions, desmosomes, gap junctions) which all have their own function, area of occurrence and binding partners. These junctions operate through a wide range of cell adhesion proteins. A very important and well-studied group is the cadherin superfamily. It comprises a diversity of molecules with structural differences which enable them to have different functions at different time points during development (Halbleib and Nelson, 2006). The largest group are the classical cadherins comprising, amongst others, E-cadherin, N-cadherin, R-cadherin and cadherin-11. Classical cadherins have an extracellular part that consists of five extracellular domains, a short transmembrane domain and an intracellular part which includes the juxtamembrane and C-terminal domain. These molecules achieve strong cell-cell adhesion force because of the presence of a conserved HAV tripeptide motif in the most distal extracellular domain. They can interact both *cis* and *trans* with other cadherins establishing a close interaction between neighbouring cells. The intracellular tail is able to bind other molecules such as p120 catenin (at the juxtamembrane domain), β-catenin and plakoglobin (at the C-terminal domain) (Davis et al., 2003). β-catenin and plakoglobin compete for binding the cadherin and create a link, via α-catenin, with the actin cytoskeleton forming the cadherin-catenin complex (Zhurinsky et al., 2000).

N-cadherin, also called cadherin-2, was first discovered in neural tissue and muscles (Grunwald et al., 1982).

N-cadherin tends to be expressed in mesenchymal cell types but is reported to be expressed in epithelial cells as well (Kaarteenaho et al., 2010; Niessen et al., 2011). N-cadherin is known to stimulate migration and invasion of cells but this motile phenotype can be inhibited, by antibody blocking, while cell adhesion remains (Derycke and Bracke, 2004). Moreover, N-cadherin plays an important role in precartilage condensation and skeletal muscle differentiation (Chimal-Monroy and Diaz de Leon, 1999; George-Weinstein et al., 1997). N-cadherin has been extensively studied during zebrafish, *Danio rerio,* development and appears to be involved in numerous processes. N-cadherin is required for neurulation (Hong and Brewster, 2006; Lele et al., 2002), cardiovascular development (Bagatto et al., 2006), development of the pectoral limb bud, and for the development of visual structures (Erdmann et al., 2003; Liu et al., 2003; Liu et al., 2002).

One developmental process in zebrafish that involves important cell movements and has only recently received interest in terms of cell adhesion, is tooth development and continuous replacement (Verstraeten et al., 2010b). During tooth development, a local thickening (placode) of the epithelial layer first invaginates into the underlying mesenchyme to form an epithelial bud. This tooth bud will next pass through different stages of development (initiation, morphogenesis, early and late cytodifferentiation, see Huysseune et al. (1998); Laurenti et al. (2004)) leading to an attached and functional tooth. In the past, tooth development has been studied mainly in human and mouse (Heymann et al., 2002; Obara et al., 1998; Pispa and Thesleff, 2003). Each of these species has its own limitations: human material is rare and difficult to obtain. Moreover, functional studies can only be performed on cell cultures.

For mice, many molecular tools are available but their dentition is highly derived, with incisors that are continuously growing which is based on the presence of a stem cell niche (Wang et al., 2007), and with non-replacing

molars. Therefore, mice do not provide an adequate model to study tooth replacement. Zebrafish, on the other hand, have teeth that replace continuously throughout life. Zebrafish teeth develop on the fifth branchial arch and are positioned into three rows: a ventral row (V) of five teeth (1V to 5V), a mediodorsal row (MD) of four teeth (1MD to 4MD) and a dorsal row (D) of two teeth (1D and 2D) (Van der heyden and Huysseune, 2000). Rapidly after the initiation of development of the first tooth, 4V, at 48 hours post-fertilization (hpf), the two neighbouring tooth placodes (3V and 5V) develop from the pharyngeal epithelium. Tooth 2V and 1V start to develop at 12 and 16 days post-fertilization (dpf), respectively, while the first replacement tooth already develops between 3 and 4 dpf at the tooth position 4V. The formation of a replacement tooth starts with the development of a successional lamina. This successional lamina does not develop as an outgrowth of the pharyngeal epithelium (i.e., from a placode) but from the crypt epithelium surrounding the functional tooth (Huysseune, 2006). The formation of a placode (for first-generation teeth), the development of a successional lamina (for replacement teeth), and the development of the tooth itself, require the epithelial and mesenchymal cells to rearrange constantly. We hypothesize that all these morphogenetic events are accompanied, and possibly preceded, by changes in cell adhesion.

Verstraeten et al. (2010b) described the dynamic changes in expression pattern of the classical epithelial cadherin, E-cadherin, during the development of first-generation and replacement. Briefly, they found that E-cadherin was expressed in the epithelial-derived part of the tooth throughout development. This in contrast to the mesenchyme which remained negative for E-cadherin at all stages. They did not observe a down-regulation of E-cadherin at the start of the formation of a new tooth bud. N-cadherin on the other hand, is known to be expressed in neural crest-derived, mesenchymal tissues and to be a marker for

differentiation, both applicable on tooth development. Not only has N-cadherin an important role as a cell adhesion molecule, it is also involved in multiple other processes such as embryogenesis, migration and signal transduction (Derycke and Bracke, 2004; Niessen et al., 2011). Up till now, it is unknown if and where N-cadherin is expressed during development of first-generation teeth and during tooth replacement in a dentition characterized by continuously replacing teeth. Moreover, the function of this important cell adhesion molecule during tooth development is yet to be demonstrated.

Here, we examine the distribution of N-cadherin during tooth development and tooth replacement in zebrafish and explore if there is a possible role for N-cadherin during these morphogenetic processes. To this end, we examine *parachute* (*pac*) mutants, which are deficient for N-cadherin. This *pac* mutant (allele *tm101b*) has first been reported by Jiang and colleagues (1996) and later two extra alleles (pac^{fr7} and pac^{paR210}), resulting in a similar phenotype, have been discovered by Lele et al. (2002) (Jiang et al., 1996; Lele et al., 2002). *Pac* mutant embryos lack a morphologically distinct midbrain-hindbrain boundary combined with a generally disorganized midbrain and hindbrain itself. In the tail, the most caudal part of the dorsal finfold is reduced, less erect and often split along the midline. Sequencing has revealed that point mutations in the cDNA of allele pac^{tm101b} *and* pac^{fr7} give rise to premature stop codons leading to a defective N-cadherin protein. The three *pac* alleles produce null phenotypes (Lele et al., 2002).

In this study, we analyze the distribution of the cell adhesion molecule N-cadherin throughout the different stages of zebrafish tooth development. Additionally, N-cadherin defective larvae are screened for abnormal tooth development. These data shed a light on if and how N-cadherin might be involved during zebrafish tooth development.

2.3. Results

2.3.1. N-cadherin expression during tooth development and replacement

Initiation

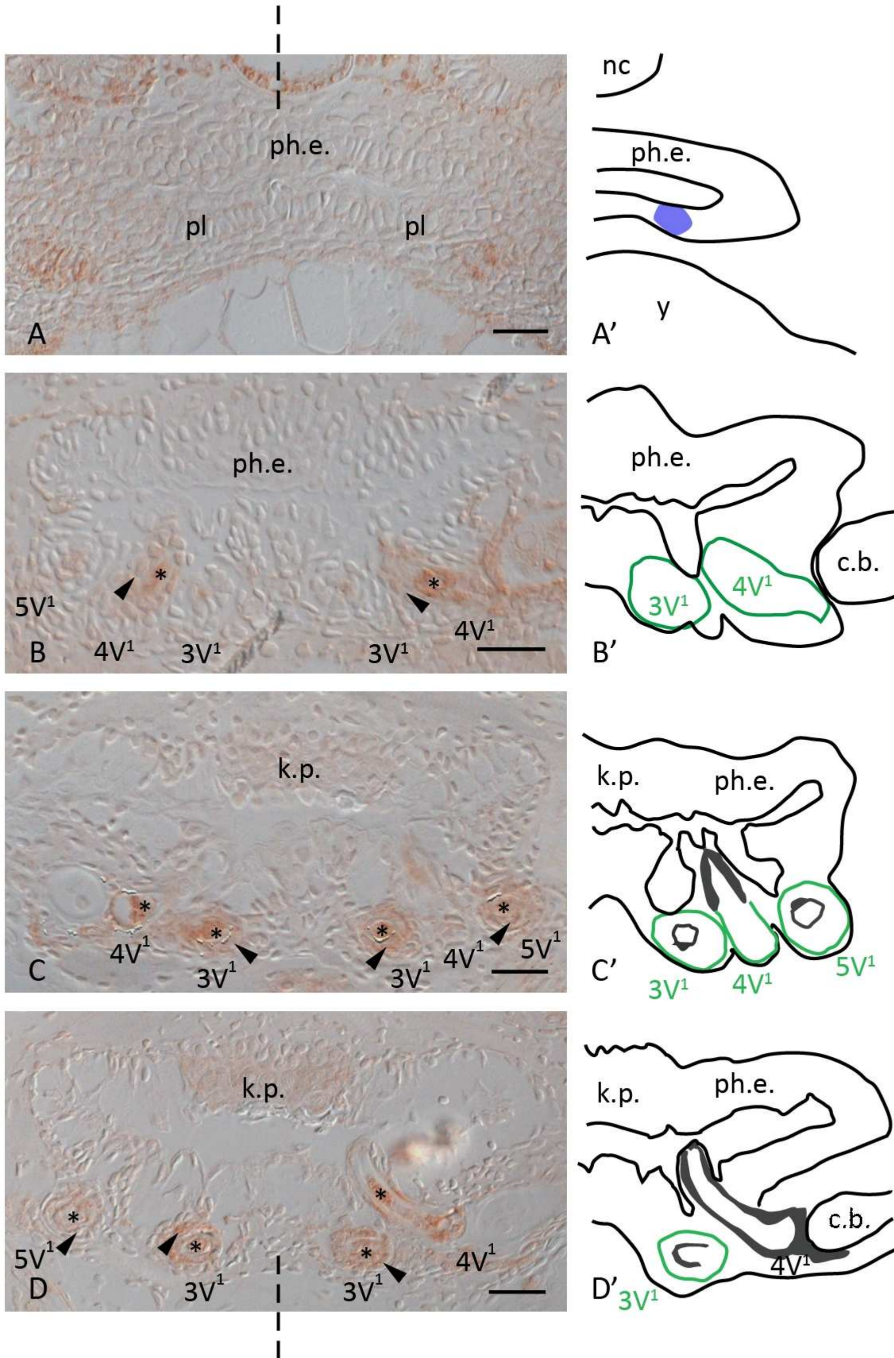
First-generation teeth arise directly from the pharyngeal epithelium, lining the floor of the pharyngeal cavity. Tooth initiation starts with the formation of an epithelial placode. The cells of this placode do not show N-cadherin expression (Fig. 23A,A').

The so-called successional lamina, onset of a replacement tooth, forms from the base of the crypt and protrudes into the underlying mesenchyme. Both the successional lamina and the surrounding mesenchyme do not express N-cadherin (Fig. 24A,A').

Morphogenesis

Morphogenesis stage is characterized by the protrusion of the epithelial cells in the underlying mesenchyme resulting in the typical bell stage of the developing tooth. N-cadherin protein is not detectable in the enamel organ and mesenchyme of first-generation teeth (Fig. 23B,B').

The successional lamina of the developing replacement tooth eventually gives rise to a bell-shaped structure. There is no N-cadherin detectable in the epithelium or mesenchyme during this stage of replacement tooth development (Fig. 24B).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D
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c.b.
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Figure 23. N-cadherin protein distribution during development of first-generation teeth (left page)

A, A’: 48 hpf, during initiation and morphogenesis stage, no N-cadherin protein is detected in either the pharyngeal epithelium or in the epithelial placode. B, B’: At 56 hpf, tooth $4V^1$ is in early differentiation stage and N-cadherin is present while simultaneously tooth $3V^1$ and $5V^1$ have just been initiated, yet do not express N-cadherin. C, C’: At 72 phf, when tooth $3V^1$ and $5V^1$ reach early cytodifferentiation stage, tooth $4V^1$ is in late cytodifferentiation stage. N-cadherin is now present in all tooth germs present. D, D’: At 80 hpf tooth $4V^1$ is attached and functional and shows N-cadherin in the odontoblasts but not in the reduced enamel organ. Tooth $3V^1$ and $5V^1$ are also showing the presence of N-cadherin in the differentiating odontoblasts and ameloblasts. Note the absence of N-cadherin expression in the keratinized pad throughout development. Diagrams: blue patch: placode; green line: border of the tooth; dark grey: tooth matrix. Orientation: dorsal to the top, ventral to the bottom of each figure; dashed line indicates mediosagittal plane. c.b.: ceratobranchial V^{th}; k.p.: keratinized pad; nc: notochord; ph.e.: pharyneal epithelium; pl: placode; *: dental papilla; arrowhead: enamel organ. Scale bars=20 µm.

Replacement teeth display the same expression pattern as in first-generation teeth during early cytodifferentiation stage. N-cadherin starts to be expressed both in the IDE and in some cells of the dental papilla. (Fig. 24C). The cytodifferentiation of the cells coincides with the expression of matrix metallopeptidase-13a (MMP-13a). MMP-13a is expressed in the differentiating ameloblasts and odontoblasts (Fig. 25A,B).

The expression pattern is maintained during late cytodifferentiation stage, which is marked by growth of the tooth and the presence of a large amount of matrix deposited.

Both in first-generation teeth and in replacement teeth the enamel organ still shows expression of N-cadherin but a much stronger expression, compared to the expression in the enamel organ, is observed in the differentiated odontoblasts adjacent to the matrix. The other cells of the dental papilla show no N-cadherin expression (Fig. 23D,D’; Fig. 24D,D’).

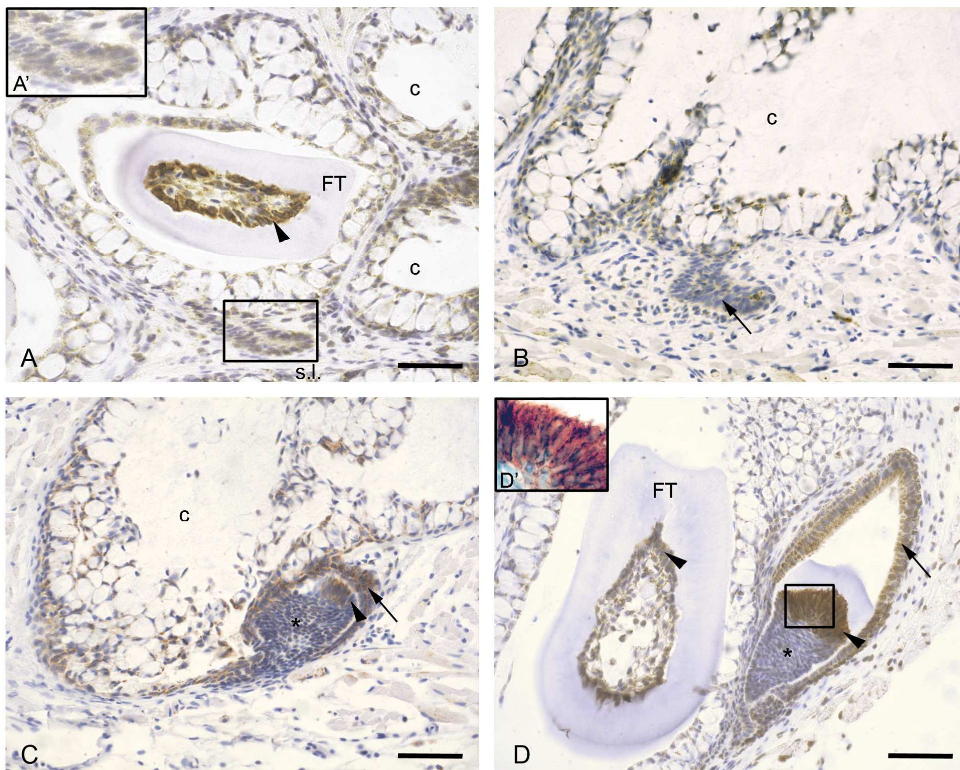

Figure 24. N-cadherin protein distribution during tooth replacement in an adult zebrafish

A,A’: At initiation of a new replacement tooth, the successional lamina does not show any expression of N-cadherin. The surrounding mesenchyme is also negative for N-cadherin. Note strong expression in the polarized odontoblasts of the young functional predecessor (arrowhead). B: Neither the epithelial enamel organ nor the mesenchymal, condensed cells express N-cadherin during morphogenesis stage. C: At early cytodifferentiation stage, the differentiating ameloblasts (arrow) and odontoblasts (arrowhead) start to express N-cadherin. D,D’: During late cytodifferentiation stage, N-cadherin is most strongly expressed in the odontoblasts adjacent to the deposited matrix. The other cells of the dental papilla do not express N-cadherin. In the now more mature functional predecessor, the odontoblasts have lost their polarized appearance and N-cadherin immunoreactivity has diminished compared to that in the polarized odontoblasts of young functional teeth (compare with Fig. 24A). Orientation: A, B: dorsal to the top, ventral to the bottom, medial to the right and lateral to the left of the figure; C, D: dorsal to the left, ventral to the right, medial to the top and lateral to the bottom of the figure. c: crypt slightly posterior to the tip of the functional predecessor; FT: functional tooth; s.l.: successional lamina; *: dental papilla. Scale bars=50 µm.

Attachment

A functional tooth is attached to the fifth branchial arch by means of a collar of attachment bone and the tooth tip is exposed into the pharyngeal cavity. A functional first-generation tooth continues to express N-cadherin in the odontoblasts. The reduced enamel organ, in contrast, has lost its N-cadherin expression (Fig. 23D,D').

Functional teeth belonging to replacement generations can be identified as young (newly attached) or as more mature, according to the aspect of the pulp cavity and the usual correlation with the age of the successor tooth (cf. Huysseune (2006)). Thus, young functional teeth associated with a replacement tooth in successional lamina stage still possess highly polarized, actively secreting odontoblasts. These odontoblasts maintain strong N-cadherin immunoreactivity (Fig. 24A). In contrast, more mature functional teeth with a replacement tooth in more advanced stage have odontoblasts that have lost their polarized appearance. These cells have lost most of their N-cad immunoreactivity (Fig. 24D).

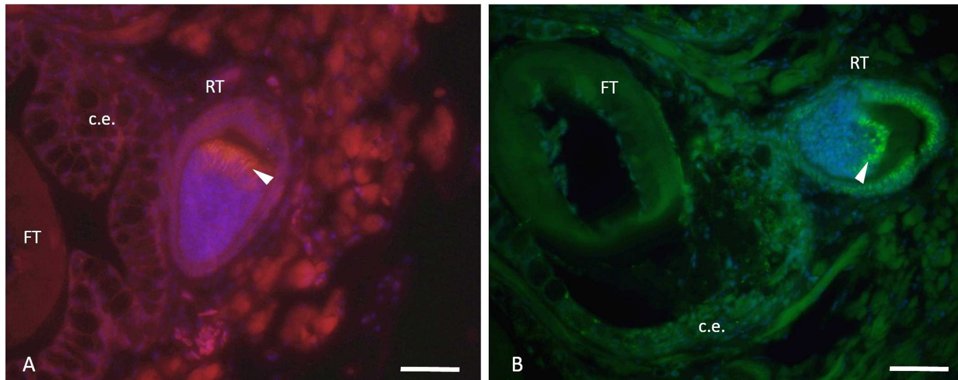

Figure 25. N-cadherin and MMP-13 are both expressed in differentiating odontoblasts

A: N-cadherin distribution during cytodifferentiation stage. The differentiating odontoblasts show N-cadherin (red). B: MMP-13 expression (green) is expressed in the differentiating odontoblasts and ameloblasts. Differentiating odontoblasts co-express N-cadherin and MMP-13. Nuclear counterstaining with Dapi (blue). c.e.: crypt epithelium; FT: functional tooth; RT: replacement tooth; arrowhead: differentiating odontoblasts. Scale bars=50 µm.

2.3.2. Tooth development in N-cadherin deficient *Parachute* zebrafish mutants

To determine if there is a possible role for N-cadherin during tooth development and replacement in the zebrafish dentition, we examined the development of teeth in the pac^{tm101b} mutant. The *pac*^{tm101b} allele has been shown to result in a null phenotype.

In general, we could confirm the specific characteristics described in previous papers with as the most striking feature, the abnormal tail morphogenesis. It was also clear that even though the embryos were raised at 28.5°C and at standard light/dark cycle, their development appeared to be generally delayed with early characteristics such as the closed pharyngeal opening, the small brainvolume and no differentiating pharyngeal epithelium cells were detectable. We were able to detect the first developing tooth only, tooth 4V^1, at 104 hpf.

The tooth was in morphogenesis stage and appeared to contain all layers of a normal developing tooth in morphogenesis stage in a wild-type embryo. We could not find tooth $4V^1$ at later developmental stages. We were also unable to detect tooth $3V^1$ and $5V^1$ flanking tooth $4V^1$. These two teeth normally initiate development when $4V^1$ reaches early cytodifferentiation stage (Fig. 26A,B).

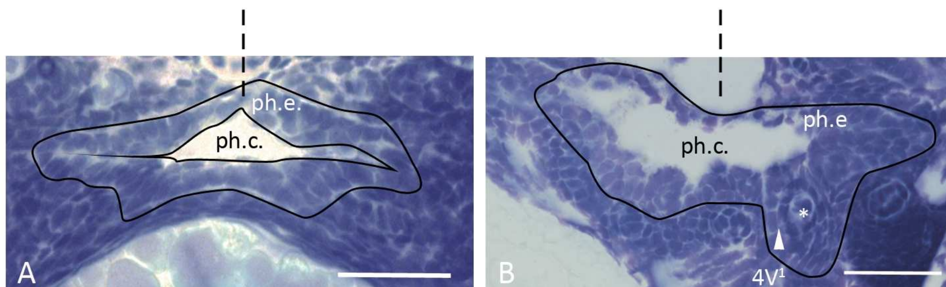

Figure 26. Semithin toluidine blue stained section of the pharyngeal region of *pac*^{tm101b} mutant zebrafish

A: Pharyngeal region of a *pac* mutant zebrafish at 64 hpf showing delayed development of the pharyngeal epithelium, as evidenced by the small epithelial opening and lack of differentiated epithelial cells. Tooth germs are lacking. B: At 104 hpf, tooth $4V^1$ is present in its normal position and has developed up to morphogenesis stage. The tooth shows the development of the enamel organ in two distinct layers (inner and outer dental epithelium) enveloping the condensed mesenchyme. Orientation: dorsal to the top, ventral to the bottom of each figure; dashed line indicates mediosagittal plane. ph.c.: pharyngeal cavity; ph.e.: pharyneal epithelium; *: dental papilla; arrowhead: enamel organ. Scale bars=25 µm.

2.4. Discussion

The development of zebrafish teeth shows a dynamic expression of the cell adhesion-related cadherins. Such dynamic changes are often observed in developing structures and organs. Different cadherins are expressed at different time points or in different tissues or cells reflecting either their origin or their need for a specific type of cadherin to fulfil a certain process. We show that there is no N-cadherin present (neither at transcript nor at protein level) during the initiation of both first-generation and replacement teeth. Also during morphogenesis stage, all cells of the developing tooth are negative for N-cadherin. Only when cytodifferentiation begins, does N-cadherin start to be expressed in the differentiating cells of the IDE and the dental papilla, i.e., the ameloblasts and the odontoblasts, respectively. Once the tooth becomes attached to the fifth branchial arch and therefore functional, N-cadherin expression persists in the odontoblasts until they lose their polarized aspect, as evidenced from larger (adult) teeth.

This expression pattern of N-cadherin observed in the zebrafish dentition is similar to the expression of this classical cadherin during human tooth development (Heymann et al., 2002). The dental bud (similar to early morphogenesis stage, cf. Borday-Birraux et al. (2006)) shows no N-cadherin expression. Heymann et al. (2002) observed a strong staining for E-cadherin in the cells that constitute the IDE and ODE, combined with a weak N-cadherin staining. As development proceeds, N-cadherin expression increases in cells that have acquired a higher degree of differentiation such as the preameloblasts and ameloblasts. A strong N-cadherin immunoreactivity was also detected in differentiating and functional odontoblasts (Heymann et al., 2002).

The study of the expression of different cadherins during mouse tooth development is limited to E-cadherin and P-cadherin whereas N-cadherin expression in mice teeth has not yet been studied (Obara et al., 1998; Palacios et al., 1995).

Based on our results and the similar results during human tooth development, it is clear that the start of N-cadherin expression is concurrent with the start of differentiation of certain cell types. Only the cells that terminally differentiate, the ameloblasts and the odontoblasts, express N-cadherin. In contrast, the other cells of the dental papilla, mainly odontoblasts precursors and later blood vessels and nerve associated cells, are negative for N-cadherin. During this cytodifferentiation, cells undergo extensive polarization and cytological changes which are related to their secretory function as differentiated cells. Thus, we can suggest that N-cadherin is necessary for the transition of ameloblasts and odontoblast prior to their differentiation, required for the deposition of enameloid and dentine. This idea is supported by the fact that N-cadherin has been described as a marker of mesenchymal differentiation and has shown to enhance cellular differentiation in certain tissues such as corneal endothelium and cartilage (Delise and Tuan, 2002; Derycke and Bracke, 2004; Koh et al., 2008; Larue et al., 1996).

Although mammalian skin appendages such as hair, teeth and ectodermal glands do not share much morphological resemblance, they share a number of morphogenetic movements such as the formation of a placode, and an epithelial bud (Mikkola, 2007; Sharpe, 2001). During hair follicle development, no N-cadherin expression is detected; only the presence of cadherin-11 was shown (Nanba et al., 2003). Menko et al. (2002) observed N-cadherin expression during cytodifferentiation of the immature salivary cells in the mouse submandibular gland. N-cadherin was specifically detected in cells that were being organized into salivary tissue-specific structures.

Verstraeten et al. (2010) described the expression and distribution of the cadherin typical for epithelial tissues, E-cadherin, during tooth development and continuous replacement in the zebrafish. E-cadherin expression was shown to have a space-restricted expression. Only the epithelial-derived part of the tooth expressed E-cadherin whereas the mesenchymal cells showed no expression in any developmental stage (Verstraeten et al., 2010a; Verstraeten et al., 2010b). The distribution of N-cadherin shown in this study supplements the E-cadherin distribution since N-cadherin is strongly expressed in the differentiated, mesenchymal-derived cells of the developing tooth. The cells of the IDE express both E-cadherin and N-cadherin. The co-expression of different cadherins in the same cell has been described before: co-expression of E-cadherin and P-cadherin in the cells of the cervical loop (Palacios et al., 1995) and in the IDE and outer dental epithelium (ODE) at cap stage (Obara et al., 1998) during mouse tooth development. Different cadherins can be expressed in the same cell, as they can play distinct roles (Halbleib and Nelson, 2006; Nandadasa et al., 2009; Takeichi, 1990). It has also been shown that heterophilic adhesion can be as strong as homophilic adhesion. Homodimerization affinity between two E-cadherin molecules is weaker than the heterophilic binding of E-cadherin with N-cadherin. Interestingly, homophilic interactions of two N-cadherin molecules appeared to be stronger than the E-cadherin/N-cadherin binding (Katsamba et al., 2009; Prakasam et al., 2006; Takeichi, 2011).

During tooth development in zebrafish, there is no down-regulation of E-cadherin, whereas N-cadherin is upregulated at cytodifferentiation stage. This implies that cell rearrangements essential for morphogenetic movements can occur without the loss of cadherin-based cell adhesion.

One possible mechanism for epithelial morphogenesis combined with expression of different cadherins involves that E- and N-cadherin have primary function in the control of assembly of the actin cytoskeleton. The differential expression of cadherin molecules results in different types of actin assembly and therefore different types of tissue movement. The difference in availability of actin assembly is essential for the specific morphogenetic movements (Nandadasa et al., 2009). How actin assembly is regulated during tooth development is an interesting topic yet to be examined. Epithelial morphogenesis can take place while cadherins are present at the cell membrane.

To determine if there is a possible role for N-cadherin during tooth development or continuous tooth replacement, we studied tooth development in the zebrafish pac^{tm101b} mutant. Tooth $4V^1$ was observed in morphogenesis stage at a much later larval age (104 hpf) than in wild type fish (48-56 hpf), possibly because of the general developmental delay of the animals. We did not observe tooth $4V^1$ in later developmental stages nor the two subsequent developing teeth, $3V^1$ and $5V^1$ at the oldest time point that could be collected. This can be explained in various ways. First, assuming that N-cadherin plays a role during ameloblast and odontoblast differentiation, the absence of N-cadherin in the cells of the enamel organ and condensed mesenchyme would make them unable to differentiate into their functional descendants. This would lead to the arrest of tooth development at morphogenesis stage. A second possibility involves the idea of tooth $4V^1$ as the dental determinant, acting as an initiator of the other first-generation teeth (Huysseune and Witten, 2006). Thus, development of tooth $4V^1$ could be regulated differently from tooth $3V^1$ and $5V^1$ (Laurenti et al., 2004).

We detected tooth 4V[1] in morphogenesis stage at different time points (from 72 hpf until 104 hpf) during development, this suggests that the development of this tooth is indeed hindered by the depletion of N-cadherin. It is necessary to point out that among the serious retardation of the mutants, individual variation is possible. Because of the limited amount of larvae available it is impossible to do statistical analysis. Moreover, it is not achievable to monitor tooth development in each larvae to get a clear insight on when this first tooth is forming and how it will develop. Therefore, it is noteworthy that the presence of tooth 4V[1] in morphogenesis stage at 104 hpf is most likely the result of the developmental arrest but can also be a sign of extreme developmental delay.

The fact that the loss of N-cadherin does not alter the development of teeth is not surprising knowing that N-cadherin knock-down in mice doesn’t disturb the normal development of another epithelial appendage, the mammary gland. Only when N-cadherin was over-expressed in mouse, the mammary gland displayed tumor development. As both murine glands and zebrafish teeth can develop without N-cadherin we can state that there is no role for N-cadherin during the initiation of an epithelial bud in general, neither as an adhesion molecule, nor as a signalling molecule.

In conclusion, our data on zebrafish teeth suggest that N-cadherin might play an important role during ameloblast and odontoblast differentiation, as evidenced by the dramatic increase of N-cadherin expression during cytodifferentiation stage of tooth development. Given that the tooth placode, the successional lamina and the different layers during morphogenesis stage do not express N-cadherin, N-cadherin is likely not necessary for the initiation of a new tooth or for the morphogenesis stage.

2.5. Material and Methods

2.5.1. Zebrafish collection

Wild-type zebrafish were mated and eggs were raised at 28.5°C in a 10h dark / 14h light cycle. The embryos were sacrificed according to the Belgian law on the protection of laboratory animals (KB d.d. 13 September 2004) by an overdose of MS222 (3-aminobenzoic acid ethyl ester) every 4 hours starting at 40 hours post-fertilization (hpf). From adult zebrafish, fifth branchial arches were dissected using a Leica MZ Apo dissecting microscope and microscissors.
Parachute, allele tm101b, mutant zebrafish were ordered from the Nüsslein-Volhard Lab (Tübingen, Germany). The batch was raised at 28.5°C in a 10h dark / 14h light cycle. At specific time points, the embryos with severely disturbed phenotype were sacrificed by an overdose of MS222. This mutation is lethal between 4 and 5 days post-fertilization (dpf); thus the oldest specimens recovered were 104 hpf.

2.5.2. Toluidine blue staining of *Parachute* mutants

Pac mutants were fixed overnight at 4°C in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) and embedded in epon according to standard procedures. 1µm thin sections were made using a diamond knife. The epon sections were stained with toluidine blue (0.5% toluidine blue, 1% borax in bidistilled water) to contrast the different cell types and visualize morphological structures.

2.5.3. Tissue processing

Wild-type embryos and larvae were fixed overnight at 4°C in 4% PFA in PBS, depigmented, dehydrated in an increasing MeOH series and stored in 100% MeOH at -20°C until use.

The dissected fifth branchial arches were also fixed overnight at 4°C in 4% PFA in PBS, decalcified in Morse's solution (10% sodium citrate, 22.5% formic acid) for several days at 4°C, embedded in paraffin according to standard procedures and sectioned at 5 µm.

2.5.4. Immunohistochemistry and *in situ* hybridization

For immunohistochemistry on paraffin sections, the sections were deparaffinized in two parasolve baths, hydrated through a decreasing ethanol series and rinsed with 1xPBS. Endogenous peroxidase was blocked by 1% H_2O_2 in MeOH for 45 minutes in the dark. Subsequently, an antigen retrieval step was performed by putting the sections at 95°C in citrate buffer for 20 minutes. After cooling down to room temperature (RT), the sections were blocked with 1% bovine serum albumin (BSA) and 1% sheep serum in 1xPBS for 2h at RT and afterwards incubated with the primary antibody (mouse anti-N-cadherin, BD Transduction Laboratories, 1/500 or rabbit anti-MMP-13a, AnaSpec, Inc., 1/200) overnight at 4°C. After removal of the primary antibody, the secondary antibody (anti-mouse-biotin labeled antibody, Dako, 1/300; anti-mouse Alexa Fluor 594, Invitrogen, 1/500 or anti-rabbit Alexa Fluor 488, Invitrogen, 1/500) was applied for 1h at RT. Finally the sections were incubated with streptABComplex labeled with peroxidase (Dako) for 45 minutes at RT to enhance the signal. 3,3'-diaminobenzidine (DAB) was used for visualization.

Whole mount immunohistochemistry and whole mount *in situ* hybridization of embryonic and larval stages was performed as described previously in

(Verstraeten et al., In press). The plasmid containing *Cdh-2* probe was kindly provided by Q. Liu (Department of Biology, University of Akron, Akron,OH, USA). Mouse anti-N-cadherin (BD Transduction Laboratories, 1/300) was used to detected zebrafish N-cadherin in whole mount immunohistochemistry.

2.6. Aknowledgements

We gratefully thank the Nüsslein-Volhard Lab (Tübingen, Germany) for providing the *Parachute* mutant zebrafish and Tommy D'heuvaert and Mieke Soenens for technical support. This work was supported by a GOA research grant (BOF08/GOA/019) to ES, JVH and AH. BV acknowledges a grant of the Agency for Innovation by Science and Technology (IWT).

3. Beta-catenin and plakoglobin expression during tooth development and tooth replacement

Manuscript to be submitted

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3.1. Abstract

We analyzed the distribution of two cadherin-associated molecules, β-catenin and plakoglobin, during the different stages of tooth development and tooth replacement in zebrafish. β-catenin expression was found at the cell borders of all cells of the successional lamina but also in the nuclei of surrounding mesenchymal cells. No nuclear β-catenin was found during morphogenesis stage, only membranous β-catenin. However, during cytodifferentiation stage, both nuclear and membrane-bound β-catenin was detected in the layers of the enamel organ as well as in the differentiating odontoblasts. Nuclear β-catenin is an indication of an activated Wnt pathway, therefore suggesting a possible role for Wnt signalling during zebrafish tooth development and replacement. Plakoglobin expression was detected at the plasma membrane already at the onset of tooth development in the epithelial cells of the tooth. This expression pattern remained unaltered during further tooth development. The mesenchymal cells did not show any plakoglobin expression. Plakoglobin morpholino-injected embryos showed normal tooth development with proper initiation and differentiation. Although plakoglobin is strongly expressed during normal tooth development, the loss of plakoglobin does not influence tooth development.

3.2. Introduction

During the various phases of development of the vertebrate body, it is of major importance that cells are capable of communicating with each other and of rearranging themselves. Adhesion molecules play a very important role during the morphogenetic processes that underlie development (Halbleib and Nelson, 2006; Niessen et al., 2011).

There are three major types of cell junctions: tight junctions, adherens junctions and desmosomes. Tight junctions (TJ) form a belt-like structure at the apical side of epithelial polarized cells. Their main function is to prevent intramembrane diffusion of proteins and lipids and therefore favour active transport through the cells (Ebnet, 2008). Moreover, TJ also prevent the mixing of membrane proteins of the apical and basolateral membranes (fence function). Another type of intercellular junction which consists of the cadherin-catenin complex is known as adherens junctions (AJ). Cadherins represent an important family of cell adhesion molecules, which includes classical cadherins and desmosomal cadherins. Classical cadherins are connected to the actin cytoskeleton through β-catenin. At the cell membrane the cadherins cluster laterally to achieve strong adhesive forces. By connecting to cadherins at the neighbouring cell membrane, cells are held closely together (Ivanov et al., 2001; Meng and Takeichi, 2009; van Roy and Berx, 2008). The third type of intercellular junction are desmosomes which provide strong intercellular adhesion by linking to the intermediate filament cytoskeleton, especially in tissues subjected to mechanical stress. Transmembrane desmosomal cadherins, desmocollin or desmoglein, are coupled at the C-terminal end to the linker proteins plakophilin or plakoglobin which together form the desmosomal plaque. This plaque is linked to the intermediate filaments via desmoplakin.

The N-terminal domain of the desmosomal cadherins in one cell binds to the N-terminal domain of the desmosomal cadherin of the neighbouring cell in the intercellular space forming a characteristic highly organized, electron-dense structure (Garrod and Chidgey, 2008).

The cadherin-associated molecules plakoglobin and β-catenin are close homologues. Their sequence is partially similar and they compete for the same binding site at the C-terminal region of classical cadherins (Zhurinsky et al., 2000). While plakoglobin has the capacity to substitute for β-catenin in AJ, β-catenin can also interact with desmosomal cadherins and therefore replace plakoglobin at the desmosomes but only when excessive desmosomal cadherins are present or when there is no plakoglobin available. These interactions suggest a cross-talk between these two kinds of junctions (Choi et al., 2009).

Apart from functioning in cell adhesion, β-catenin is also known to play a role in the Wnt pathway by binding to the LEF/TCF binding site in the nucleus (Behrens, 1999; Bienz, 2005; Nelson and Nusse, 2004). Given the similarity between β-catenin and plakoglobin, it is not surprising that plakoglobin can also bind LEF/TCF. However, the role for these two close homologues in the Wnt signalling appears to be distinct. The precise role of plakoglobin in the Wnt signalling pathway is still controversial as plakoglobin cannot compensate for β-catenin in KO mice (Charpentier et al., 2000; Klymkowsky et al., 1999). The complete KO of either β-catenin or plakoglobin in mice, results in a lethal phenotype. Intriguingly, both phenotypes are distinctive of one another. The loss of β-catenin results in animals missing dorsal structures while the plakoglobin null mouse fails to form correct desmosomal structures causing a failure in heart development (Haegel et al., 1995; Ruiz et al., 1996).

Tooth development has been used for many years as a paradigm for investigating organ development.

Not only do teeth result from multiple, reciprocal interactions between two tissue layers, the epithelium and underlying mesenchyme, but these two tissue layers undergo extensive morphogenetic movements. Tooth formation starts with the establishment of an epithelial thickening, called placode. As the epithelium and underlying mesenchyme pass through different stages of morphogenesis, both their cells need to rearrange before they can differentiate and deposit tooth-specific matrices. How such cell rearrangements are achieved, and what the role of cell adhesion molecules is in this process, has only partially been addressed.

We have set out for a study aiming at elucidating the role of cell adhesion molecules during development and replacement of teeth. To this end, we focus on the zebrafish since this species, contrary to mammalian species, replaces its teeth throughout life. Zebrafish lack jaw teeth in the oral cavity, but have pharyngeal teeth located on the fifth branchial arch. The full zebrafish dentition consists of 11 teeth on each side, divided into three rows. The first tooth starts to develop at two days post-fertilization (dpf), soon followed by the adjacent teeth in the same (i.e., ventral) row. At around 80 hours post-fertilization (hpf) the first developing tooth attaches to the branchial arch, thereby becoming functional. At the same time, a replacement tooth starts to develop from the base of the crypt surrounding the functional tooth (Van der heyden and Huysseune, 2000).

An earlier study on E-cadherin expression and distribution in zebrafish teeth has shown that the epithelial-derived part of the tooth remains E-cadherin positive throughout development. In contrast, the mesenchymal cells never display any E-cadherin expression (Verstraeten et al., 2010b). Because of the absence of down-regulation of E-cadherin during the formation of a new tooth, it was necessary to expand our research to the cadherin-related molecules β-catenin and plakoglobin.

From the literature we found that in another epithelial appendage, the mammary gland, the formation of an epithelial bud was accompanied with the down-regulation of the desmosomal compartment. On the other hand, studies on cancer and tumor development suggested that it might be the loss of β-catenin that can make the cadherin-catenin complex incompetent and thereby loose adhesive strength. Both β-catenin and plakoglobin expression is observed in murine tooth development, but this was limited to a certain developmental stage, based on gene expression data or not solely focussed on these molecules (Kieffer-Combeau et al., 2001; Obara and Lesot, 2004). In zebrafish, β-catenin and plakoglobin have two paralogues, a typical result of the genome duplication that has occurred in zebrafish. Plakoglobin-1b is a shorter transcript than plakoglobin-1a and up till now it has not been linked to a certain function in zebrafish development. Plakoglobin-1a is described to play a critical role during cardiac development and have a signalling role during zebrafish development (Martin et al., 2009). The two β-catenin genes that have been discovered in zebrafish, β-catenin-1 and β-catenin-2, have distinct roles during zebrafish development (Bellipanni et al., 2006).

Here we describe the distribution of both β-catenin and plakoglobin during tooth development and tooth replacement, in first- and later-generation teeth. Using data from knockdown experiments, we analyse the possible function of plakoglobin during tooth development. Finally, we assess the result of complete loss of plakoglobin on β-catenin expression.

3.3. Results

3.3.1. Distribution of plakoglobin during zebrafish tooth development and tooth replacement

At 48 hpf, plakoglobin is present in the pharyngeal epithelium as dots on the plasma membrane, restricted to the apical region of the basolateral membranes of the epithelial cells, i.e., the side towards the pharyngeal lumen. This dotted distribution of plakoglobin reflects the typical appearance of desmosomes. The mesenchymal cells surrounding the pharyngeal epithelium do not show any expression of plakoglobin (Fig. 27A,B). Ventrally on both sides of the midline an epithelial thickening, a placode, is formed. The cells of this placode will start to protrude into the underlying mesenchyme creating the bell-shaped enamel organ of the developing tooth ($4V^1$). During this morphogenesis stage, plakoglobin is strongly expressed at the membrane in the cells of the enamel organ. This is in contrast to the condensed mesenchyme which does not show any plakoglobin expression. In addition, the keratinized pad, arising in the roof of the pharyngeal cavity opposite the site where teeth develop, clearly expresses plakoglobin at the cell membrane. The cells of the pharyngeal epithelium maintain their dotted plakoglobin expression in their apical membrane facing the lumen (Fig. 27C,D).

In 72 hpf specimens, tooth $4V^1$ is flanked by tooth $3V^1$ on its medial and tooth $5V^1$ on its lateral side. Tooth $4V^1$ is now in cytodifferentiation stage, featuring the differentiation of the inner dental epithelium of the enamel organ into ameloblasts and the differentiation of cells of the dental papilla into odontoblasts. Tooth $3V^1$ and $5V^1$ are at the end of morphogenesis. In all teeth present, the expression pattern of plakoglobin is alike: plakoglobin is strongly expressed in both the inner and the outer dental epithelium while the cells of the mesenchymal dental papilla start to express plakoglobin (data not shown).

The keratinized pad maintains its plakoglobin expression during further development and growth of the embryo (Fig. 27E,F).

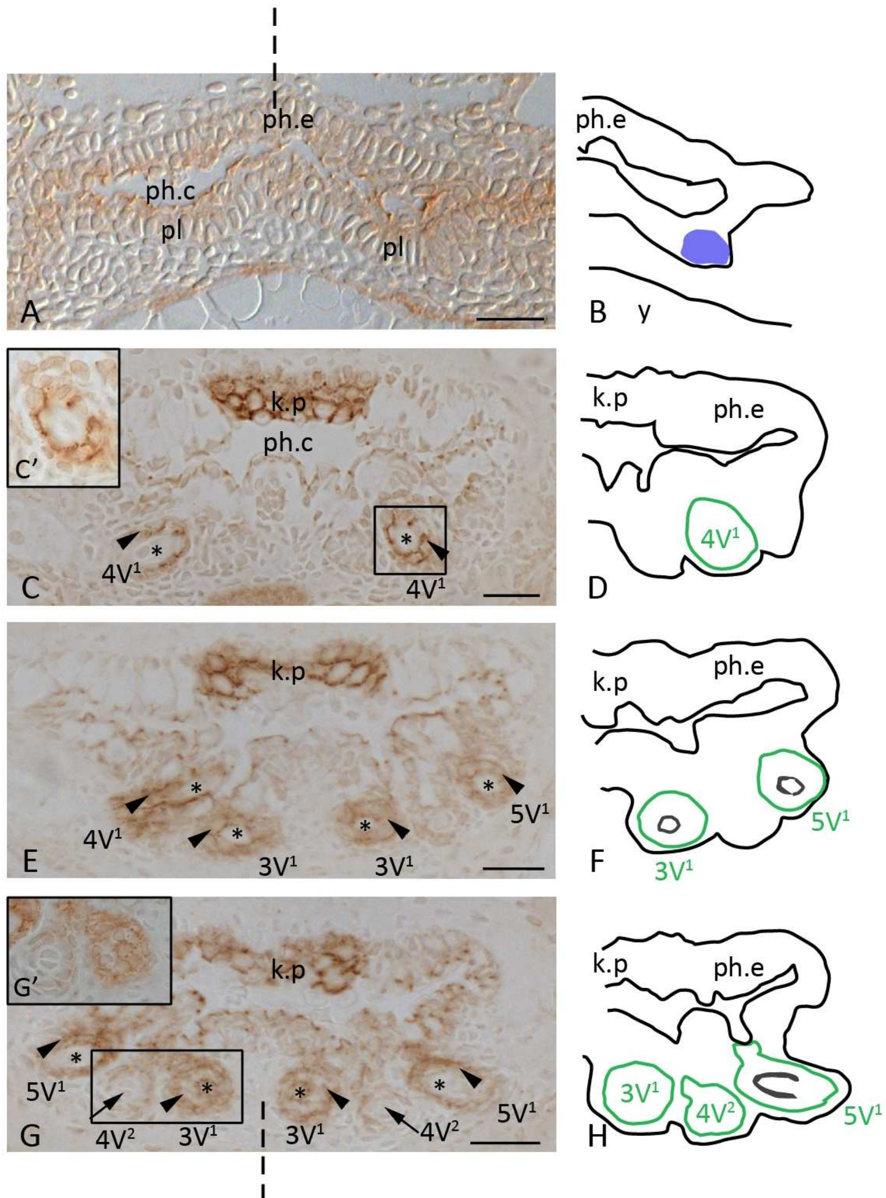

Figure 27. Plakoglobin distribution during the development of first-generation teeth (left page)

Cross-sections through the pharyngeal region of a 48 hpf (A), 72 hpf (B), 96 hpf (C) and 100 hpf (D) zebrafish embryo. A,B: Initiation stage of tooth $4V^1$; the pharyngeal epithelium expresses plakoglobin in contrast to the mesenchymal cells. C,D: Morphogenesis stage of tooth $4V^1$; plakoglobin is clearly expressed at the cell borders of the inner and outer dental epithelium. The keratinized pad strongly expresses plakoglobin. E,F: Tooth $4V^1$ in late cytodifferentiation stage; teeth $3V^1$ and $5V^1$ in early cytodifferentiation stage. All teeth present, display plakoglobin expression limited to the inner and outer dental epithelium. The dental papillae remain negative. G,H: Initiation of the first replacement tooth, $4V^2$. The epithelial outgrowth shows plakoglobin expression while the condensed mesenchyme is negative. Diagrams: blue patch: placode; green line: border of the tooth; dark grey: tooth matrix. Orientation: dorsal to the top, ventral to the bottom of each figure; dashed line indicates mediosagittal plane. ph.c: pharyngeal cavity; ph.e: pharyneal epithelium; pl: placode; k.p: keratinized pad; *: dental papilla; arrowhead: enamel organ; block arrow: initiation replacement tooth. Scale bars=20 µm.

The first replacement tooth, tooth $4V^2$, starts to develop at around 80 hpf. Its initiation coincides with the attachment and eruption of its predecessor. The enamel organ of the replacement tooth expresses plakoglobin (Fig. 27G,H). It is more subtle compared to expression during cytodifferentiation stage, but the signal is undeniable. The reduced enamel organ of the functional tooth is still expressing plakoglobin but less intense. Tooth $3V^1$ and $5V^1$, now in late cytodifferentiation stage, express plakoglobin in the inner and outer dental epithelium and the differentiating odontoblasts (Fig. 27G,G').

Unlike the formation of a first-generation tooth, the initiation of replacement teeth starts with the formation of a so-called successional lamina. The latter yet cannot be distinguished in the first replacement tooth $4V^2$ but becomes prominent in larger juveniles and adults. The successional lamina is an outgrowth of the crypt epithelium surrounding the functional tooth. The cells of the successional lamina express plakoglobin very strongly at the cell membrane while the mesenchymal tissue does not (Fig. 28A).

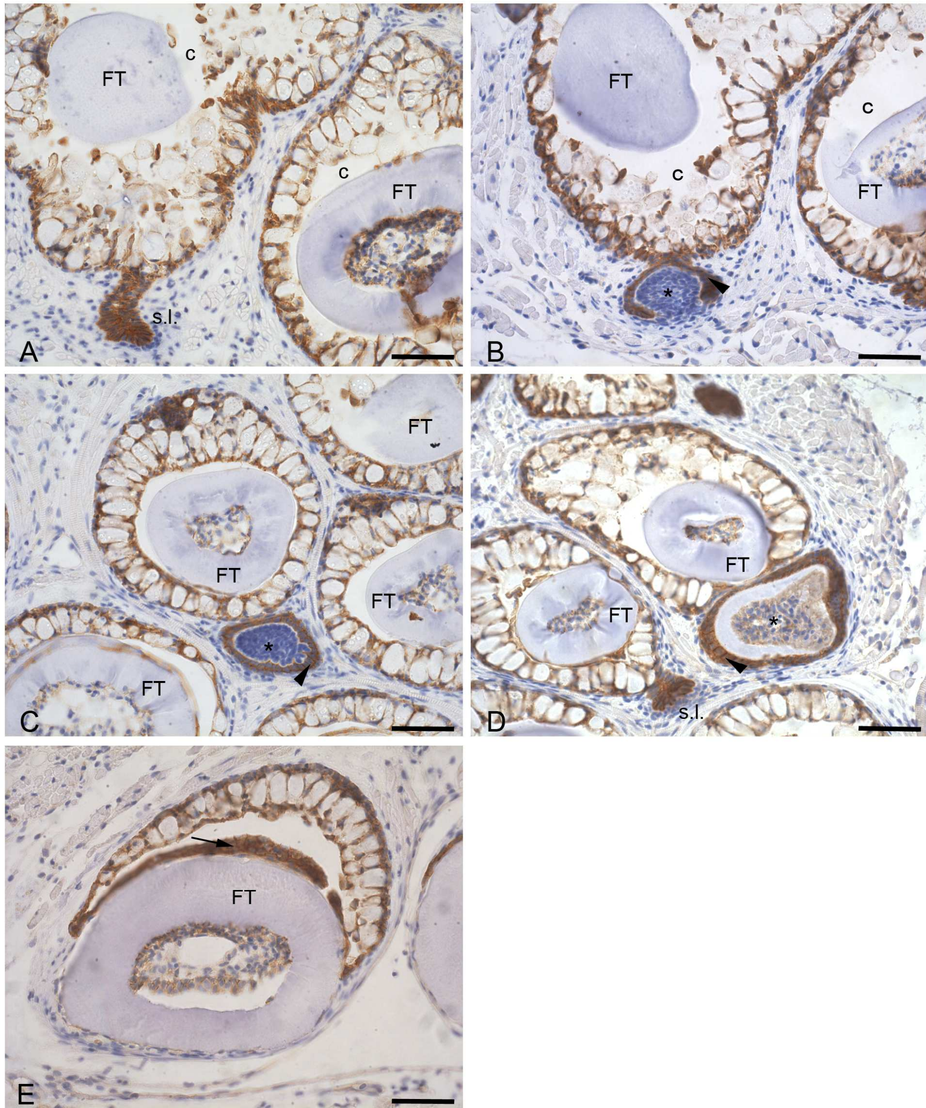
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Figure 28. The distribution of plakoglobin during later-generation teeth (left page)

Cross-sections through one of the paired fifth branchial arches of an adult zebrafish. A: In contrast to the surrounding mesenchyme, all the cells constituting the successional lamina express plakoglobin at their cell membrane. Also the cells of the crypt epithelium express plakoglobin. B: Morphogenesis stage; the condensed mesenchyme constituting the dental papilla is plakoglobin-negative. The enamel organ is strongly expressing plakoglobin. C: During early cytodifferentiation, the expression of plakoglobin remains limited to the inner and outer dental epithelium. D: Tooth in late cytodifferentiation stage, showing plakoglobin expression in the enamel organ. The differentiating odontoblasts do not show any signal. E: Functional tooth with reduced enamel organ, showing that even in a fully developed tooth plakoglobin remains expressed in the reduced enamel organ. Orientation: dorsal to the top, ventral to the bottom, medial to the right and lateral to the left of the figure. c: crypt slightly posterior to the tip of the functional predecessor; FT: functional tooth; *: dental papilla; s.l: successional lamina; arrowhead: enamel organ; arrow: reduced enamel organ. Scale bars=50 µm.

As tooth development progresses, plakoglobin expression remains restricted to the epithelial-derived part of the tooth. During morphogenesis stage, all cells of the enamel organ express plakoglobin. There is no difference of intensity between the cervical loops and the rest of the enamel organ. The clearly condensed mesenchymal cells do not express plakoglobin (Fig. 28B). When cytodifferentiation starts, there is no visible change in expression of plakoglobin in the enamel organ. Both inner and outer dental epithelium express plakoglobin equally strong at the plasma membrane. The differentiating odontoblasts do also express plakoglobin, so do the other cells of the dental papilla (Fig. 28C,D). An adult, functional tooth shows that the reduced enamel organ, after attachment and eruption, still expresses plakoglobin (Fig. 28E). Throughout the different stages of tooth development, the cells of the epithelial crypt surrounding the functional tooth consistently express plakoglobin, with no visible variation in intensity or position of the signal. The mesenchymal tissue surrounding the crypts and the developing teeth never expresses plakoglobin during any stage of tooth development.

Summarizing, both in first-generation and later-generation teeth plakoglobin is strongly present in the different layers of the developing enamel organ. The mesenchymal-derived cells only show any signal for plakoglobin from cytodifferentiation onwards. The formation of a new tooth is not accompanied by the loss this desmosomal component as shown by the strong detection of plakoglobin in all cells of the successional lamina.

3.3.2. Tooth development in plakoglobin morphant zebrafish

Given the strong and specific expression of plakoglobin during the different stages of both first-generation and later-generation teeth, we wanted to determine if there is a role for plakoglobin during zebrafish tooth development and/or replacement.

While plakoglobin-morpholino injected zebrafish display delayed midbrain-hindbrain border formation, reduced heart size and kinked tail (Martin et al., 2009), no attention was paid to the tooth phenotype.

We looked for developing teeth at 72, 80 and 96 hpf both in control-injected as well as in morphant embryos which are both delayed in development compared to wild-type embryos, all raised under the same conditions. At 72 hpf, control-injected embryos display the first tooth, tooth $4V^1$, on both sides of the midline in early cytodifferentiation stage. The morphant embryos of the same age also display normal development of tooth $4V^1$. As in controls, tooth $4V^1$ develops at the right position, and shows differentiation of the enamel organ into inner and outer dental epithelium. Also the start of formation of matrix is observed in the morphant embryos (Fig. 29A,B).

Morphant embryos at 80 hpf display tooth $4V^1$ and $5V^1$, as in control embryos, with tooth $4V^1$ in late and tooth $5V^1$ in early cytodifferentiation stage (Fig. 29C,D). At 96 hpf, all first three tooth positions are occupied and the teeth do not display any abnormalities (Fig. 29E,F).

Thus, our data suggest that in the absence of a functional plakoglobin protein, the teeth of the first generation still develop.

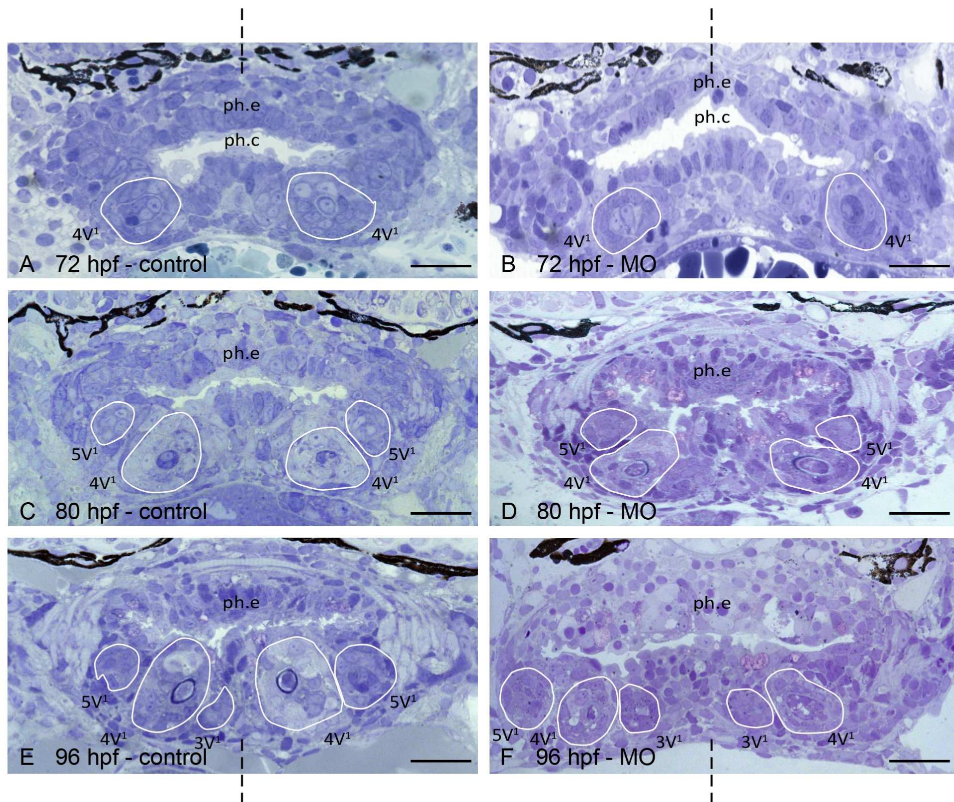

Figure 29. Comparison of control and plakoglobin morpholino-injected zebrafish at different times during tooth development

A,C,E: cross-sections through the pharyngeal cavity of control zebrafish at 72, 80 and 96 hpf, respectively; B,D,F: cross-sections at approximately the same level of plakoglobin morphant (MO) zebrafish at 72, 80 and 96 hpf, respectively.

A,B In both control and morphant embryos the first tooth to develop, $4V^1$ is in early cytodifferentiation stage. C,D: Tooth $4V^1$ has continued to develop in control and morphant embryos. Moreover, tooth $5V^1$ is also present in both dentitions. E,F: The first three tooth positions develop normally in both control and in morphant zebrafish. Orientation: dorsal to the top, ventral to the bottom of each figure; dashed line indicates mediosagittal plane. ph.c: pharyngeal cavity; ph.e: pharyneal epithelium, line: contour of the developing tooth. Scale bars=20 µm.

The different layers of the enamel organ can differentiate and the ameloblasts can produce, together with the odontoblasts, normal amounts of tooth matrix (enameloid). As general development of both control and morphant embryos is slightly delayed compared to wild-type, we were unable to observe the development of the first replacement tooth. As older morpholino-injected zebrafish embryos lose the effect of the injection, we were unable to assess the effect of plakoglobin knockdown in older specimens. Thus, whether or not plakoglobin plays a role during tooth replacement is still unknown.

3.3.3. Distribution of β-catenin during zebrafish tooth development and tooth replacement

The data concerning the distribution of β-catenin during tooth development are based on immunostaining of paraffin sections of adult jaws and are therefore limited to replacement teeth. Whole mount immunostaining on embryos or larvae to collect data on first-generation teeth was never successful as we were unable to obtain a specific color reaction.

β-catenin is expressed at the cell membrane of all cells constituting the successional lamina, whereas β-catenin is not expressed at the plasma membrane of the mesenchymal cells. However, both in the epithelial as well as in the mesenchymal cells of the developing replacement tooth, β-catenin is present in some nuclei (Fig. 30A,A').

During morphogenesis stage, when the epithelium further invaginates and the mesenchyme condenses, β-catenin is expressed in the enamel organ but not in the condensed mesenchyme (data not shown). Both the inner and outer dental epithelium express β-catenin at the plasma membrane. β-catenin is however no longer observed in the nuclei (Fig. 30B).

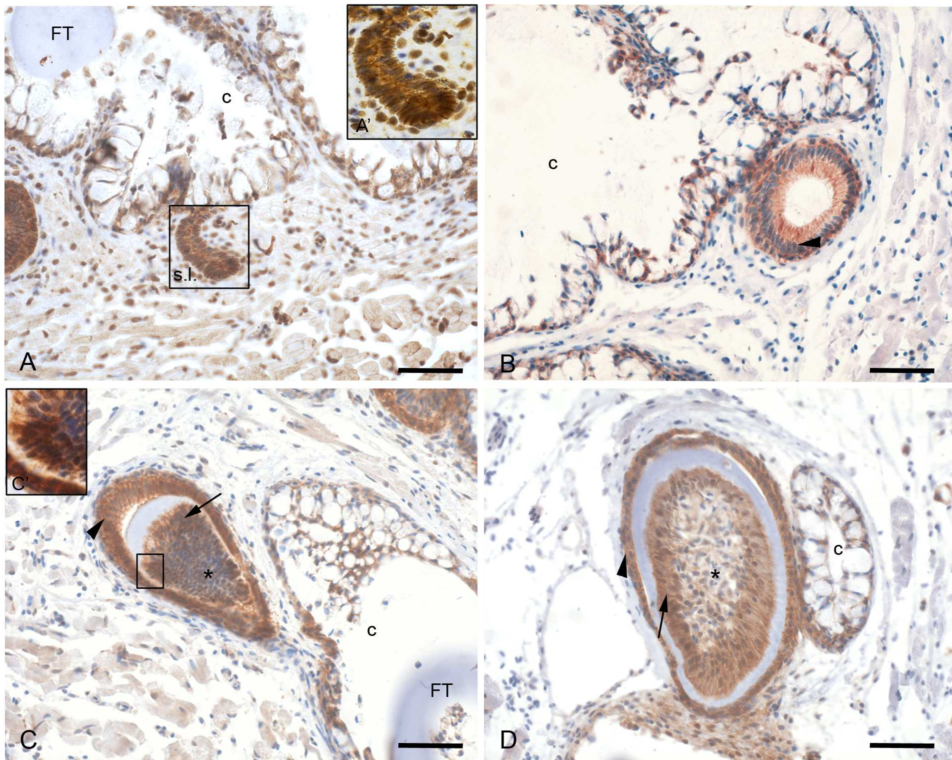

Figure 30. β-catenin distribution during the development of later-generation teeth

A,A': The successional lamina shows expression of β-catenin at the cell membrane and in some nuclei. The mesenchyme also displays nuclear β-catenin. B: β-catenin is detected only at the plasma membrane of the epithelial cells during morphogenesis stage. C: In the enamel organ β-catenin is expressed at the plasma membrane of cells of both inner and outer dental epithelium. C': The dental papilla shows odontoblasts with membrane-bound and nuclear β-catenin expression. D: During late cytodifferentiation stage, β-catenin remains expressed in both the inner and outer dental epithelium as well as in the polarized odontoblasts adjoining the tooth matrix. Orientation: dorsal to the top, ventral to the bottom, medial to the right and lateral to the left of the figure. c: crypt slightly posterior to the tip of the functional predecessor; FT: functional tooth; *: dental papilla; s.l: successional lamina; arrowhead: enamel organ; arrow: differentiated odontoblasts. Scale bars=50 µm.

Once cytodifferentiation starts, the expression of β-catenin expands. While β-catenin remains expressed at the plasma membrane in the inner and outer dental epithelium, it is now also expressed in the differentiated, polarized odontoblasts that line the dentine matrix. In these cells β-catenin is expressed both at the cell membrane and in the nucleus. The β-catenin signal in the centre of the dental papilla is either very low or completely lacking (Fig. 30C,C'). In the functional tooth, β-catenin is expressed in the cells of the reduced enamel organ as well as in the odontoblasts, both in the nucleus and at the plasma membrane. The expression is much weaker in the centre of the dental papilla (now called dental pulp). β-catenin is also expressed at the plasma membrane of the cells constituting the epithelial crypts surrounding the functional tooth (Fig. 30D).

3.4. Discussion

In this study we show the distribution of two closely related cell adhesion molecules during the development and the continuous replacement of zebrafish teeth. β-catenin and plakoglobin are partially expressed by the same cell layers, but β-catenin has a broader distribution. Plakoglobin expression remains restricted to the epithelial-derived part of the tooth during initiation and morphogenesis stage but expands to the dental papilla from cytodifferentiation onwards. β-catenin is also expressed both in the epithelial enamel organ and in the mesenchymal-derived dental papilla but one major difference in expression is that plakoglobin is never found in the nucleus. In addition, β-catenin shows a nuclear expression at certain stages during zebrafish tooth development. The analysis of plakoglobin morphant embryos showed no abnormalities during tooth development as the first three teeth developed normally compared to the control embryos.

Plakoglobin, and desmosomes in general, have been studied less in the context of tooth development compared to β-catenin. The data available on teeth are limited to the mouse model, where the expression of plakoglobin during odontogenesis parallels our data. In both organisms, plakoglobin is expressed in the epithelial part of the developing tooth. In mice, an asymmetrical distribution has been observed, the plakoglobin protein signal being weaker on the medial side of the tooth (Falk, 2010; Kieffer-Combeau et al., 2001). Such an uneven distribution is not observed in zebrafish tooth development.

The distribution of β-catenin during tooth development has been studied previously in mouse and in human and corresponds to the distribution we find during zebrafish tooth development (Lo Muzio et al., 2009; Obara and Lesot, 2004; Obara et al., 2006).

Both in human and in mouse, the enamel organ is expressing β-catenin throughout tooth development with a stronger expression of β-catenin in the enamel knot. The enamel knot is known to be a signalling centre which is especially important for cusp formation. In zebrafish teeth, which display only one single cusp, an enamel knot has not yet been identified. In addition, different from the mouse, expression of β-catenin was found in the cells of the dental papilla. In the mouse, the dental papilla is weakly expressing β-catenin and the differentiating odontoblasts are described to be negative. Our data resemble those on human teeth, which also show β-catenin expression in the dental papilla and the odontoblasts (Lo Muzio et al., 2009; Obara and Lesot, 2004; Obara et al., 2006).

Jamora and co-workers proposed the hypothesis that a down-regulation of E-cadherin is necessary to enable the formation of an epithelial bud. This hypothesis was based on their data on hair follicle development in mice (Jamora et al., 2003). Hair follicles, gland primordia and teeth all share the formation of an epithelial thickening, called placode, at the onset of development, followed by a series of different developmental stages in order to finally become a functional unit (Mikkola, 2007; Sharpe, 2001). Our data on the expression pattern of both cadherin-related cell adhesion molecules nicely matches the expression data in other epithelial appendages, demonstrating that tooth development can be a model for epithelial morphogenesis (Kurzen et al., 1998; Menko et al., 2002; Nanba et al., 2001; Ridanpaa et al., 2001).

In contrast to the hypothesis of Jamora et al (2003), previous studies have shown that there is no down-regulation of E-cadherin at the onset of the formation of both first-generation or later-generation zebrafish teeth (Verstraeten et al., 2010a; Verstraeten et al., 2010b). Tooth development is a process of intense morphogenetic movements during which cell rearrangements are necessary.

Each replacement cycle is accompanied with an epithelial invagination. If there is no detectable down-regulation of E-cadherin, how does this process occur? Two possible explanations have been suggested with regard to other epithelial appendages. In early morphogenesis of the mammary gland and of hair placodes in mice, E-cadherin and β-catenin are present at all stages. Yet, instead of a reduction of adherens junctions (AJ), the desmosomal components were down-regulated during early development (Nanba et al., 2000; Nanba et al., 2001). According to our data, plakoglobin remains expressed in all stages of tooth development. Second, studies on cancer and tumor development suggest that the loss of β-catenin could make the cadherin-catenin complex incompetent, causing the loss of adhesive strength (Da Silva et al., 2008; Sundfeldt, 2003). In zebrafish E-cadherin is present throughout tooth development at the plasma membrane, as is β-catenin, suggesting the coupling to a classical cadherin. If, how and which cell adhesion molecules are regulated during zebrafish tooth development needs to be further examined.

Since plakoglobin showed such a strong and specific expression during zebrafish tooth development, plakoglobin morpholino-injected zebrafish were studied to determine a possible role for this molecule during tooth development or tooth replacement. These plakoglobin morphants are described to have reduced heart size with reduction of blood circulation, severe oedema and a kinked tail (Martin and Grealy, 2004; Martin et al., 2009). Thus, control and morphant zebrafish were examined for aberrant tooth development. Until 96 hpf, there was no detectable difference between control and morphant larvae. Both morphant and control zebrafish developed teeth at the correct tooth positions. Hence, reduction of plakoglobin did not alter the possibility to initiate and further develop teeth. Furthermore, the morphant teeth were also capable of differentiating, as enameloid was formed.

This indicates that plakoglobin is not necessary for the differentiation of the inner dental epithelium or the odontoblasts (which participate in enameloid formation, cf. Huysseune (2006); Laurenti et al. (2004)). In general, we can state that the loss of plakoglobin has no effect on the development of teeth in zebrafish. The fact that tooth development is not altered despite the loss of an important component of the desmosomes, suggests that the absence of plakoglobin is rescued in some way. Plakoglobin resembles β-catenin as they both contain multiple Armadillo (Arm) repeats. Therefore, β-catenin and plakoglobin functions are possibly overlapping such as contributing to the cadherin-catenin complex and regulating the Wnt pathway. The affinity of β-catenin for binding E-cadherin is much stronger than that of plakoglobin for E-cadherin (Fukunaga et al., 2005). Moreover, it has been shown that plakoglobin has a higher affinity for desmosomal cadherins compared to classical cadherins (Teuliere et al., 2004). However, when β-catenin is depleted, plakoglobin will compensate for β-catenin in AJ (Fukunaga et al., 2005). When lacking plakoglobin completely, β-catenin can replace plakoglobin in the desmosomes (Bierkamp et al., 1999). This is a plausible explanation why the absence of plakoglobin does not alter tooth development. β-catenin is found in all cells that are expressing plakoglobin during zebrafish tooth development. Therefore, it is possible that β-catenin rescues the integrity of the desmosomes in the developing teeth by substituting for plakoglobin.

Plakoglobin KO mice die because of severe defects in the developing heart due to the lack of desmosomes. The intestinal epithelium and hair follicle development of these plakoglobin null embryos was not altered and AJ were unaffected demonstrating that epithelial-derived tissues can develop normally without plakoglobin (Bierkamp et al., 1999; Teuliere et al., 2004).

Cell culture experiments have shown that, when plakoglobin expression was inhibited, the presence of the cadherin typical for the cell type at the plasma membrane remained largely unchanged. Yet, the cells showed a significant reduction of adhesive strength (Schnittler et al., 1997). This can match our observations that without plakoglobin, there can be adhesive reduction while at the same time the cadherin-catenin complex remains functional. Therefore, tooth initiation, which is assumed to require a decrease in adhesion, can progress normally in plakoglobin morphants.

Next to its important function in cell adhesion, β-catenin is also known to be a key mediator of Wnt signalling. When Wnt signalling is active, β-catenin accumulates in the nucleus, binds to LEF/TCF and activates multiple target genes, including E-cadherin. Thus, the presence of nuclear β-catenin is considered a valid proxy for active Wnt signalling. Since this pathway can be induced and suppressed in many ways, Wnt signalling has been studied extensively in numerous developmental processes including the development of epithelial appendages. Wnt signalling is required for the induction and further development of mammary placodes and for the initiation of hair follicle development (Andl et al., 2002; Chu et al., 2004). Studies on Wnt signalling during tooth development have shown that ectopic Dickkopf 1 (*Dkk1*), a Wnt-signal repressor, blocks the formation of teeth (Jernvall and Thesleff, 2000). Tooth buds fail to develop in K14- *Dkk1* transgenic mouse embryos indicating that Wnt signalling is necessary for the conversion from dental lamina to bud stage tooth germs (Sarkar and Sharpe, 1999). Moreover, *Lef1* is essential in the epithelium for the transition from bud to cap stage (Kratochwil et al., 1996). When β-catenin is stabilized in the dental epithelium, thus producing a state at which Wnt signalling is incessantly active, continuous tooth generations are induced by the formation of ectopic enamel knot signalling centres (Järvinen et al., 2006).

Chen et al. (2009) stated that β-catenin is required in the epithelium to induce primary enamel knot formation, and in the mesenchyme for tooth morphogenesis beyond bud stage. They also demonstrated that β-catenin is regulated in order to obtain proper differentiation of ameloblasts and odontoblasts while β-catenin-regulated Wnt-signals also provide an odontogenic potential to the mesenchyme (Chen et al., 2009). Our immunohistochemistry results show that nuclear β-catenin is present in different stages of the developing tooth. At initiation stage, nuclear β-catenin was observed both in the epithelium and in the mesenchyme. No nuclear signal was observed at morphogenesis stage but from cytodifferentiation onwards, nuclear β-catenin was detected in some of the odontoblasts. These observations correspond to a previous study of Chen and colleagues and therefore suggests that also in zebrafish tooth development Wnt signalling provides cues necessary for normal tooth development. We were unable to determine the role of β-catenin during tooth development since β-catenin MO-injected zebrafish do not live long enough to start tooth formation. Using the β-catenin mutant *ichabod* zebrafish embryos is not useful as they are deficient for maternal, but not zygotic, expression of β-*catenin-2* (Bellipanni et al., 2006).

While β-catenin and plakoglobin share at least some functions, plakoglobin was long thought not to be involved in Wnt signalling. Only later it was found that plakoglobin can influence the Wnt signalling pathway on its own also by binding to LEF/TCF in the nucleus (Charpentier et al., 2000; Garrod and Chidgey, 2008; Klymkowsky et al., 1999). As we did not detect plakoglobin in the nucleus, we suggest that tooth development in zebrafish is not influenced by plakoglobin-induced Wnt signalling.

This study shows that both β-catenin and plakoglobin are expressed during the different stages of zebrafish tooth development. The enamel organ, both IDE and ODE, simultaneously express β-catenin and plakoglobin. The dental papilla

only expresses β-catenin and no plakoglobin expression could be determined in the mesenchymal tissue throughout tooth development.

During initiation and cytodifferentiation stage, β-catenin signal was detected in numerous nuclei suggesting that Wnt signalling is active during at least those developmental stages. These observations suggest that Wnt signalling provides cues necessary for normal zebrafish tooth development. Although plakoglobin is strongly expressed in developing teeth, the loss of plakoglobin does not seem to have an effect on tooth development. Plakoglobin morphants show no tooth defect as their teeth develop at the correct time points, normal position, contain all defined layers and are able to differentiate and form enameloid. Neither β-catenin nor plakoglobin is down-regulated during initiation of a new tooth placode. How and which cell adhesion molecules are regulated to obtain the fine balance between reduced adhesion and proper development, needs to be further examined.

3.5. Material and Methods

3.5.1. Zebrafish collection

Wild-type (wt) zebrafish embryos were raised in a 14h light / 10h dark light regime at the standard temperature of 28.5°C (Westerfield, 1995). The embryos were sacrificed by an overdose of MS222 (3-aminobenzoic acid ethyl ester) according to the Belgian law on the protection of laboratory animals (KB d.d. 13 September 2004). Embryos and larvae were collected every 4 hours, starting at 40 hpf. In addition, fifth branchial arches were dissected from adult zebrafish, using a Leica MZ Apo dissecting microscope and microscissors.

3.5.2. Plakoglobin morphant zebrafish

Plakoglobin morpholino-injected, control morpholino-injected and wild-type zebrafish were obtained from the lab of Dr. M. Grealy (Galway, Ireland). One- to 2-cell stage embryos have been injected with 5 ng of a morpholino constructed to prevent translation of plakoglobin-1a mRNA or with a 5 base pair (bp) mismatch morpholino as control (Martin et al., 2009). The batch was raised under standard conditions and sacrificed at specific time points.

3.5.3. Histological analysis of plakoglobin morphants

Plakoglobin MO-injected embryos and larvae were fixed overnight at 4°C in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) and embedded in epon according to standard procedures. Semithin (1 µm) sections were made using a diamond knife. Next, the epon sections were stained with toluidine blue (0.5% toluidine blue, 1% borax in bidistilled water) for detailed histological analysis.

3.5.4. Immunohistochemistry and *in situ* hybridization

Wild-type embryos and larvae were fixed overnight at 4°C in 4% PFA in PBS, depigmented, dehydrated in an increasing MeOH series and stored in 100% MeOH at -20°C for whole-mount analysis.

The dissected fifth branchial arches were fixed overnight at 4°C in 4% PFA in PBS, decalcified in Morse's solution (10% sodium citrate, 22,5% formic acid) for several days at 4°C, embedded in paraffin according to standard procedures and sectioned at 5 µm with a Prosan Microm HM360 microtome.

To generate data on first-generation teeth and the first replacement tooth (tooth $4V^2$), embryos and larvae were processed for both whole mount *in situ* hybridization and whole mount immunohistochemistry as described previously (Verstraeten et al., In press). Primary antibodies used included anti-plakoglobin clone 15 (1/500, BD Transduction Laboratories) and anti-β-catenin (1/300, Sigma). This anti-plakoglobin antibody recognizes only plakoglobin 1a of the two paralogues and was validated by western blot (Martin et al., 2009). Secondary antibodies of goat-anti-mouse-biotin labeled IgG (1/300, Dako) and goat-anti-rabbit-biotin labelled IgG (1/300, Dako) were used. The signal was enhanced by the use of streptABComplex-horseradish peroxidase (Dako) and 3,3'-Diaminobenzidine (DAB) is used for visualization.

Data on tooth replacement were generated from the dissected branchial arches through immunohistochemistry on paraffin sections. After deparaffination and hydration, the sections were rinsed with 1xPBS before blocking endogenous peroxidase in 1% H_2O_2 in MeOH for 45 minutes in the dark. Next, the sections were submerged in citrate buffer at 95°C for 20 minutes to retrieve the epitopes.

After cooling to room temperature (RT), the sections were blocked with 1% Bovine serum albumin (BSA) and 1% sheep serum in 1xPBS for 2h at RT and next incubated overnight at 4°C with the primary antibody: anti-plakoglobin clone 15 (1/500, BD Transduction Laboratories) or anti-β-catenin (1/300, Sigma). After washing, the secondary antibody goat-anti-mouse-biotin labelled IgG (1/300, Dako) or goat-anti-rabbit-biotin labelled IgG (1/300, Dako) was applied for 1h at RT. Afterwards, the sections were incubated with streptABComplex labelled with peroxidase (Dako) for 45 minutes at RT to increase the signal. Finally, 3,3'-Diaminobenzidine (DAB) was used for visualization.

3.6. Acknowledgments

We would like to thank Dr. M. Grealy (Department of Pharmacology & Therapeutics, National University of Ireland, Galway) for providing the plakoglobin morpholino-injected zebrafish, Ellen Sanders, Tommy D'heuvaert and Mieke Soenens for technical support and Sam Vandenplas and Jeroen Crucke for critical reading of the manuscript. This work was supported by a GOA research grant (BOF08/GOA/019) to JVH and AH. BV acknowledges a grant of the Agency for Innovation by Science and Technology (IWT).

4. Molecular cloning and developmental expression of p120catenin (Ctnnd1) in zebrafish

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4.1. Abstract

In this study, we report on the expression of zfp120ctn during the different stages of tooth development in zebrafish. During initiation of a first-generation tooth, the tooth placode expresses zfp120ctn as does the surrounding mesenchyme. Also during morphogenesis stage does the enamel organ and the condensed mesenchyme express zfp120ctn. The differentiating ameloblasts and odontoblasts keep expressing zfp120ctn during tooth cytodifferentiation. The overall expression level in the surrounding mesenchyme appears to have diminished. The first replacement tooth is also expressing zfp120ctn. To determine whether zfp120ctn also binds to membrane-bound cadherins we overexpressed zfp120ctn in the MCF7 cell line. This resulted into a branching phenotype, typical for overexpressing p120ctn in general and we were also able to detect zfp120ctn near the membrane. These observations suggest that zfp120ctn probably has the same functions as in human and mouse.

4.2. Introduction

For proper tissue integrity and functioning, many tissues depend on the presence of specialized cell-cell contacts, called adherens junctions (AJ). AJ are composed of clusters of cadherin-catenin complexes of with the extracellular part on one cell couples to the extracellular part of the complex on the neighbouring cell. In epithelia, e.g. in the skin epidermis, they form adhesion belts located underneath the tight junctions. The type of cadherin present in the AJ is tissue-specific. The membrane-bound cadherin is linked via β-catenin and α-catenin to the actin cytoskeleton (Alberts et al., 2002; Green et al., 2010; Yap et al., 1997).

Previously, we have studied the distribution of two classical cadherins, E- and N-cadherin, during zebrafish tooth development (Verstraeten et al., 2010b, and unpublished results Chapter 2). We expanded our research to the cadherin-associated molecules, β-catenin and plakoglobin in order to study the link with the cytoskeleton and therefore the functionality of these AJ during tooth development (unpublished results Chapter 3). To complete our research on the cadherin-catenin complex during zebrafish tooth development and tooth replacement, we gained interest another catenin that is linked to the membrane-bound cadherin, p120catenin.

The p120 catenin (hereafter p120ctn) together with Armadillo Repeat gene deleted in Velo-Cardio-Facial syndrome (ARVCF), p0071 and δ-catenin is a member of the p120ctn subfamily. Members of this subfamily share several similar features such as the presence of 9 armadillo (Arm) repeats, the possibility to be transferred to the nucleus and the ability to bind to the cytoplasmic tail of different cadherins (McCrea and Park, 2007).

In addition, p120ctn is known to stabilize classical cadherins at the plasma membrane (Ireton et al., 2002).

This is the result of the ability of p120ctn to bind to the juxtamembrane domain (JMD) of the intracellular part. Hence, the loss of p120ctn results in the inability of stable positioning of the cadherin at the membrane and marks it for degradation (Davis et al., 2003; Xiao et al., 2007). Apart from its well known adhesive function, it was found that p120ctn also contains a nuclear localization signal and can shuttle to the nucleus modulate the transcriptional activity of Kaiso (Daniel and Reynolds, 1999; van Hengel et al., 1999). Furthermore, p120ctn also has a role in signal transduction by mediating the activity of Rac1, RhoA and Cdc42 small GTPases (Noren et al., 2000). Finally, p120ctn also plays a significant role during tumor development as it might act as a tumor and/or metastasis suppressor (Reynolds and Roczniak-Ferguson, 2004; van Hengel and van Roy, 2007).

Both in human and in mouse, p120ctn has different isoforms as a result of alternative splicing, each starting with a different start codon. Alternative splicing can occur both in the N-terminal and C-terminal region. N-terminal alternative splicing results in the use of four different start codons giving rise to p120ctn isoform type 1, 2, 3 or 4 according to which one of the start codons is used as translational start. Splicing at the C-terminal end leads to the incorporation of exon A, exon B, both exon A and B or none of them. Sometimes a third exon, exon C, is included. This exon is positioned within Arm repeat 6. Combinations of a specific start codon and exons generate a considerable number of possible p120ctn isoforms (Keirsebilck et al., 1998). In contrast to the N- and C-terminal domain, which are subjected to splicing, the central Arm domain remains intact and thus free to interact with cadherins. Depending on the cell type, different p120ctn isoforms are expressed, which implies functional differences between the isoforms. Motile cells such as fibroblasts preferentially express p120ctn 1A isoform, in contrast to epithelial cells which express smaller isoforms such as p120ctn 3A.

This differential expression of isoforms suggests a specific function for each of them, e.g. recruiting different cadherins to the membrane (Keirsebilck et al., 1998; Mo and Reynolds, 1996; van Hengel and van Roy, 2007).

In zebrafish, research has been performed on different tissues and organs concerning different cadherins and their expression throughout development (Babb-Clendenon et al., 2006; Babb and Marrs, 2004; Bagatto et al., 2006). In addition, β-catenin has been a valuable topic over the years with most emphasis on its role during the Wnt signalling pathway, but also on its role in cell-cell adhesion where it has proven to be important for the formation of dorsal structures and neurectoderm during zebrafish development (Cerda et al., 1999; Kelly et al., 2000; Yin et al., 2011). Nonetheless, despite studies on the presence of the cadherin-catenin complex in zebrafish, at present data regarding p120ctn is lacking. The p120ctn homologue in zebrafish is not yet studied despite its role during development and its involvement in cancer in mouse and human.

Teeth develop as a result of reciprocal interactions between epithelial and mesenchymal cell layers. Zebrafish lack oral teeth but possess pharyngeal teeth located on the fifth branchial arch. There are 11 teeth on each side of the body which develop in three rows: a ventral row (V) with 5 teeth, a mediodorsal row with 4 teeth and a dorsal row with only 2 teeth. Like in other tooth-possessing non-mammalians, the teeth are continuously replaced throughout life. The first tooth (first-generation tooth $4V^1$) starts to develop already at 48 hours post-fertilization (hpf) and at the same tooth position a replacement tooth starts to form already 32 hours later, at 80 hpf. Shortly after the onset of formation of tooth $4V^1$ the two neighbouring teeth on the ventral row, tooth $3V^1$ and $5V^1$, start to develop. Developing teeth pass through several developmental stages (early and late morphogenesis, early and late cytodifferentiation, cf. Laurenti et al., 2004) before becoming an attached and functional tooth.

While first-generation teeth develop from a placode directly from the pharyngeal epithelium, a replacement tooth develops from a successional lamina which is an outgrowth of the epithelial crypt surrounding the erupted tip of the functional predecessor tooth. In early-generation replacement teeth this successional lamina is hardly recognizable because of its small size but in later-generation replacement teeth this transient structure is clearly visible (Huysseune, 2006).

In this study, we focus on the expression pattern of p120ctn during zebrafish tooth development and tooth replacement. In the appendix, data is included concerning the cloning of zebrafish p120ctn, a description of its possible isoforms and their expression throughout ontogeny and in several organs. These data are obtained in collaboration with the lab of Prof. Dr. F. van Roy and Dr. J. van Hengel (DMBR, VIB Ghent, Belgium).

4.3. Results

4.3.1. zfp120ctn mRNA expression throughout the different stages of zebrafish tooth development

Tooth initiation

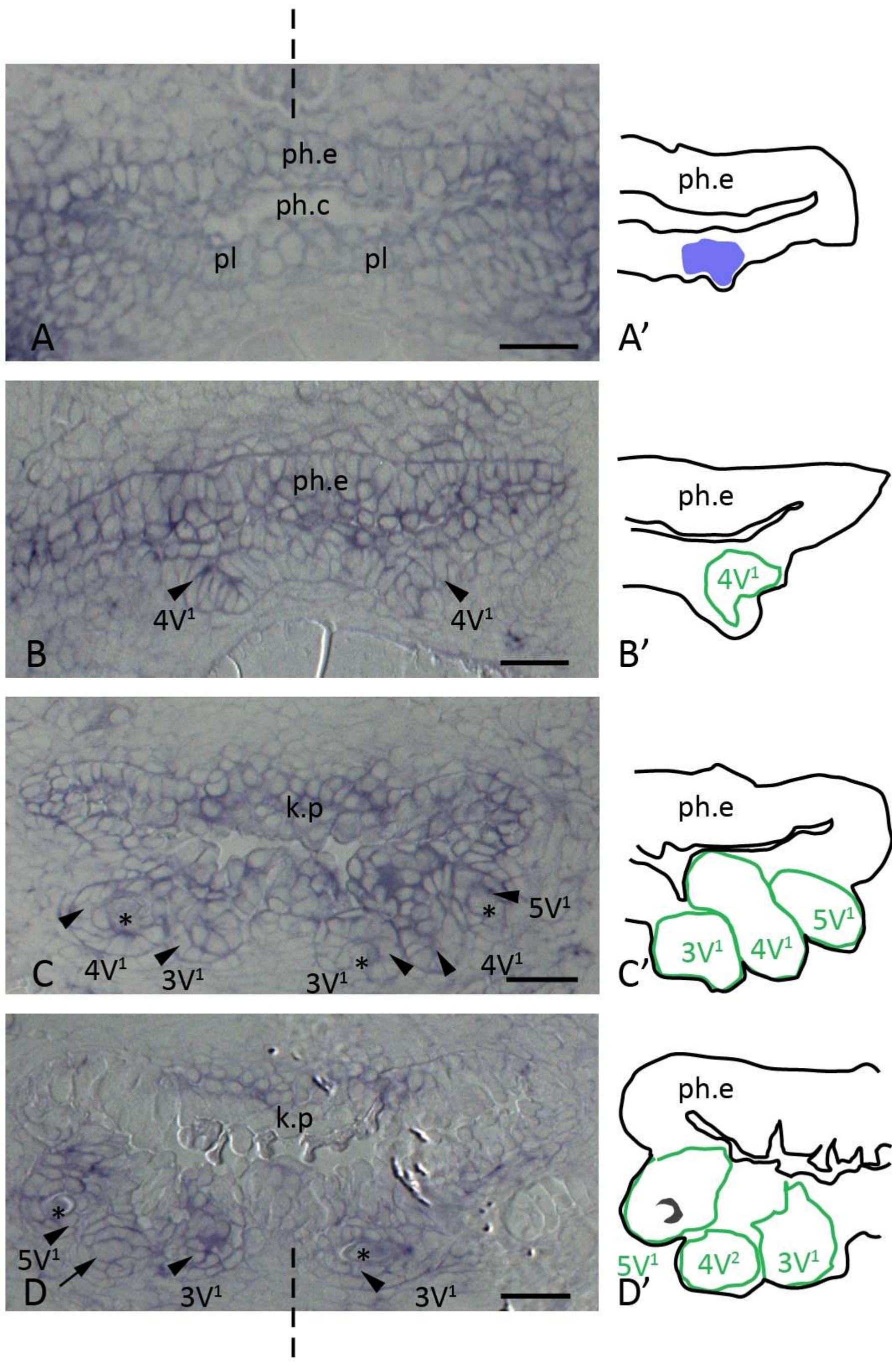
The initiation of a first-generation tooth in zebrafish always starts with the formation of an epithelial thickening, the tooth placode. These polarized epithelial cells express p120ctn in the same way the cells of the complete pharyngeal epithelium show a positive signal for p120ctn. The surrounding mesenchyme is also expressing p120ctn (Fig. 31A,A').

Morphogenesis

During morphogenesis stage, the epithelial cells grow deeper into the underlying mesenchyme and thereby surround mesenchymal cells. The enamel organ consists of two different layers: the inner dental epithelium (IDE) and the outer dental epithelium (ODE). Figures 31B and diagram 31B' clearly show that p120ctn is expressed in all the cells of the pharyngeal epithelium, and in the mesenchymal cells lying dorsal of the pharyngeal cavity. Both layers of the enamel organ of the developing tooth $4V^1$ express p120ctn equally strong. The mesenchymal cells constituting the early dental papilla are also expressing p120ctn, but less pronounced.

Early and late cytodifferentiation

This stage is marked by the differentiation of the IDE into ameloblasts and cells of the dental papilla into odontoblasts. Ameloblasts and odontoblasts together will form enameloid. Therefore, the presence of a small amount of matrix is evidence that the cells have progressed beyond early cytodifferentiation.


ph.e
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A
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3V1
4V1
C'
k.p
5V1
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D
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4V2
3V1
D'

Figure 31. mRNA expression of zebrafish p120 catenin during development of first-generation teeth and the first replacement tooth (left page)

A,A’: At 48 hpf, the placode of $4V^1$ expresses p120ctn. The pharyngeal epithelium and the surrounding mesenchyme also express p120ctn. B,B’: The epithelial cells of tooth $4V^1$ protrude into the underlying mesenchyme while in morphogenesis stage (52 hpf). Both the epithelial and the mesenchymal cells express p120ctn but the expression in the developing enamel organ is the strongest. C,C’: At 72 hpf tooth $4V^1$ is in late cytodifferentiation stage while the neighbouring teeth $3V^1$ and $5V^1$ are in early cytodifferentiation stage. All teeth present express p120ctn in the differentiating ameloblasts and odontoblasts and in all other cells of the enamel organ and dental papilla. D,D’: at 80 hpf, tooth $3V^1$ and $5V^1$ display a distinct amount of enameloid but are not attached yet while at tooth position 4V the first replacement tooth develops. The replacement tooth expresses p120ctn strongly in the epithelial-derived part, the expression in the mesenchyme is less pronounced but present. Diagrams: blue patch: placode; green line: border of the tooth; dark grey: tooth matrix. Orientation: dorsal to the top, ventral to the bottom of each figure; dashed line indicates mediosagittal plane. ph.c: pharyngeal cavity; ph.e: pharyneal epithelium; pl: placode; k.p: keratinized pad; *: dental papilla; arrowhead: enamel organ; block arrow: morphogenesis of replacement tooth. Scale bars=20 µm

Figure 31C and diagram 31C’ illustrate tooth $4V^1$ in late cytodifferentiation stage while at the same time tooth $3V^1$ and $5V^1$ are in early cytodifferentiation stage. During differentiation both ameloblasts and odontoblasts maintain expression of p120ctn. Likewise, the ODE and the pharyngeal epithelium show the presence of p120ctn transcripts.

As enameloid production proceeds, a considerable amount of matrix becomes visible, indicating that the teeth have well progressed into late cytodifferentiation stage. In Figure 31D, tooth $3V^1$ and $5V^1$ are in late cytodifferentiation while tooth $4V^1$ is attached to the fifth branchial arch, has erupted and has become functional. Also during late cytodifferentiation, the expression pattern of p120ctn remains since all layers of the enamel organ and the odontoblasts express p120ctn.

Overall, the expression level in the surrounding mesenchyme appears to have diminished, and expression in the tooth germs appears to have more attenuated. A strong focal expression is also seen in the deeper layers of the keratinous pad opposite the teeth (Fig. 31D,D').

Replacement teeth

At around 80 hpf the first replacement tooth, tooth $4V^2$, develops medial and slightly caudal to tooth $4V^1$. Figure 31D and diagram 31D' show the morphogenesis stage of the first-replacement tooth that is clearly expressing p120ctn. The first replacement cycles do not show a successional lamina. That is why we can only observe morphogenesis as the earliest detectable stage of the replacement tooth.

4.3.2. Overexpression of zf p120catenin in MCF7 cells

Full length zebrafish p120ctn was cloned into pCS2+MT, which includes 6 copies of the Myc epitope, and transfected into a confluent layer of MCF7 cells. Around 50% of all cells were successfully transfected and showed strong expression of the Myc-epitope (Fig 32A). In general, the transfected cells showed the typical branching phenotype as described multiple times for overexpression of p120ctn (Derycke and Bracke, 2004; Grosheva et al., 2001; Kelly et al., 2004; Reynolds et al., 1996). An affinity-purified rabbit polyclonal antibody against the central domain of p120ctn was generated but this antibody proved to be unsuitable for both whole mount immunohistochemistry and immunohistochemistry on paraffin and cryosections. Yet, it was capable of detecting p120ctn on western blot and in cells transfected with zebrafish p120ctn-Myc albeit with a significant amount of background. Double immunostaining showed that cells positive for the Myc-epitope could also be

detected using the custom-made rabbit anti-p120ctn antibody (Fig. 32B). This antibody was not able to detect p120ctn at the plasma membrane. Therefore, we transfected the cells with 10 times and 20 times less plasmid than in the original setup to obtain a functional p120ctn that can traffic to the cell membrane. In both cases, Myc-epitope could be detected at the cell membrane and cell-cell contacts (Fig. 32C).

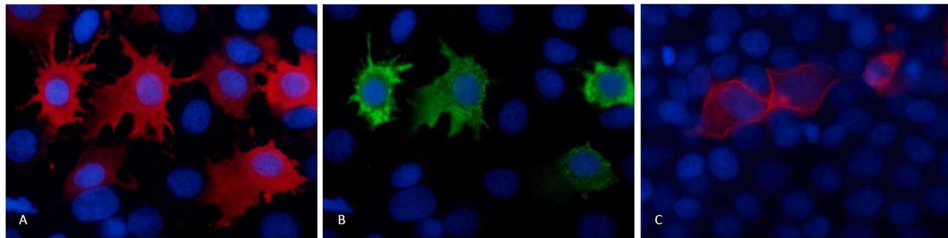


Figure 32. Overexpression of zebrafish p120ctn tagged to a Myc-epitope in MCF-7 cell line

A, B: Detection of Myc-epitope (A) and of zebrafish p120ctn (B) The transfected MCF-7 cells show the branching phenotype typical for overexpression of p120ctn. C: When transfected with 10 times less plasmid, Myc-epitope is detected at the cell membrane of the cells suggesting the functional presence of p120ctn at the cell membrane.

4.4. Discussion

By performing whole mount *in situ* hybridization on zebrafish larvae varying from 48 hpf until 6 dpf, we could study the mRNA expression of zebrafish p120ctn during the development of first-generation teeth from initiation until attachment and eruption. p120ctn is expressed in the pharyngeal epithelium and the keratinized pad opposite the teeth throughout development. p120ctn is expressed in the epithelial part of the developing tooth uninterruptedly from initiation stage at 48 hpf until attachment. Also the mesenchymal cells constituting the dental papilla express p120ctn.

p120ctn is known to stabilize classical cadherin at the plasma membrane and so to provide strong cell-cell adhesion force between neighbouring cells. Our previous studies have shown the distribution of the classical cadherins, E-cadherin and N-cadherin, during zebrafish tooth development (Verstraeten et al., 2010b). E-cadherin is expressed in the enamel organ throughout tooth development and N-cadherin is localized in the differentiating ameloblasts and odontoblasts. Furthermore, the pharyngeal epithelium and typical keratinized pad opposite the teeth express both E-cadherin and p120ctn. Since p120ctn stabilizes the cadherin at the membrane and therefore resulting in the functionality of the cadherin-catenin complex, our current finding that p120ctn is expressed in all the cells of the enamel organ and in the dental papilla is not surprising.

Our overexpression data show that zebrafish p120ctn in MCF-7 cells induces a branching phenotype typical for overexpression of p120ctn in general. This cell phenotype has been described before and is characterized by the formation of dentritic processes and filopodial extensions (Noren et al., 2000; van Hengel et al., 1999).

p120ctn is known to affect the actin cytoskeleton by mediating Rho-family GTPases. This branching phenotype while overexpressing p120ctn, can occur as a result of the disassembly of the stress fibers and focal adhesion which contributes to an increased migration. The loss of these stress fibers is a result of a decrease in RhoA activity which is inhibited by p120ctn. p120ctn is not only inhibiting RhoA activity, it can also activate Cdc42 and Rac1. These two Rho-family members are also associated with increased cell motility (Noren et al., 2000). According to our data p120ctn also traffics to the cell membrane and therefore we propose that zebrafish p120ctn has the same function in adherens junctions, stabilizing the cadherin at the plasma membrane and preventing its degradation.

p120ctn has been intensively studied in human and mouse. In mammals, its function is more complex than solely servicing for proper cell adhesion. p120ctn is known to interact with the Rho family GTPases and therefore regulating the organization of the actin cytoskeleton (Anastasiadis, 2007; Grosheva et al., 2001; Noren et al., 2000). Additionally, it has also been described to translocate to the nucleus and bind the transcription factor Kaiso (Daniel and Reynolds, 1999; Park et al., 2005; van Hengel et al., 1999). Whether these functions of p120ctn contribute to the morphogenetic movements typical for tooth replacement, needs to be addressed in the future.

Recently, a study was performed on the cKO of p120ctn in the epithelial part of the teeth in mice. Without p120ctn the enamel was distorted whereas the development of the teeth itself appeared normal. Only when the ameloblasts became secretory, they lost their cell polarity and became disorganized (Bartlett et al., 2010). p120ctn cKO mice under a Wnt1 promoter, deletes this catenin all neural crest-derived tissues, one of them are the mesenchyme constituting the dental papilla in the teeth.

In contrast to the mice with the loss of p120ctn in the epithelial part of the tooth, these mice did not show any tooth phenotype (A. Huysseune, personal observation). Whether any tooth phenotype can occur by loss of p120ctn in zebrafish needs to be examined in the future. Moreover, it would be interesting to investigate if the depletion of p120ctn affects tooth replacement.

4.5. Material and Methods

4.5.1. Zebrafish

Adult wild-type zebrafish (*Danio rerio*) were maintained and mated at 28.5°C standard temperature in a 10h dark / 14h light cycle. Eggs and embryos were raised at the same standard temperature and light regime. Starting from 40 hours post-fertilization, embryos were sacrificed every four hours by an overdose of MS222 (3-aminobenzoic acid ethyl ester), in accordance with the Belgian law on the protection of laboratory animals (KB d.d. 13 September 2004).

4.5.2. Probe design

Total RNA was extracted from an adult zebrafish using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 1µg of total RNA using iScript cDNA Synthesis Kit (Bio-Rad) as described in the manufacturer's guidelines.

A 1021bp fragment from the central part of the zebrafish p120ctn sequence was amplified via PCR using Taq polymerase (Invitrogen) and the primers 5' GATTACCCCACCTCCACTGTC 3' and 5' TCTCCTCTCCGCCTTCATT 3'. This fragment was cloned into pGEMTeasy vector (Promega) according to the manufacturer's instructions. The anti-sense probe was generated by linearising the plasmid with NCO I and transcription with Sp6-polymerase. Sal I and T7-polymerase were used to prepare the sense probe. Both sense and anti-sense probes were labeled with Digoxigenin using a Digoxigenin RNA labeling kit (Roche Diagnostics). The probe used did not distinguish between different p120ctn isoforms.

4.5.3. Whole mount *in situ* hybridization

The whole mount *in situ* hybridization was performed according to (Verstraeten et al., In press). The probes were detected with NBT/BCIP. After staining, the specimens were fixed overnight at 4°C in 4% PFA. Next, they were dehydrated, embedded in epon, and serially sectioned at 4 µm. The sections were mounted with Depex. All sections were examined using a Zeiss Axio Imager Microscope equipped for DIC and photographed using an Axiocam MRC videocamera.

4.5.4. Generation of polyclonal ctnnd1 antibody

Antibodies were produced against both a central (KNDKVKSEVRRLKGIPAL) and C-terminal domain (GFKELRRTLEKDGWKKTD) of zebrafish ctnnd1. Rabbits were immunized against a KLH-conjugated protein and affinity purification was performed (ThermoFisher Scientific).

4.5.5. MCF7 cell culture, transfection and immunostainings

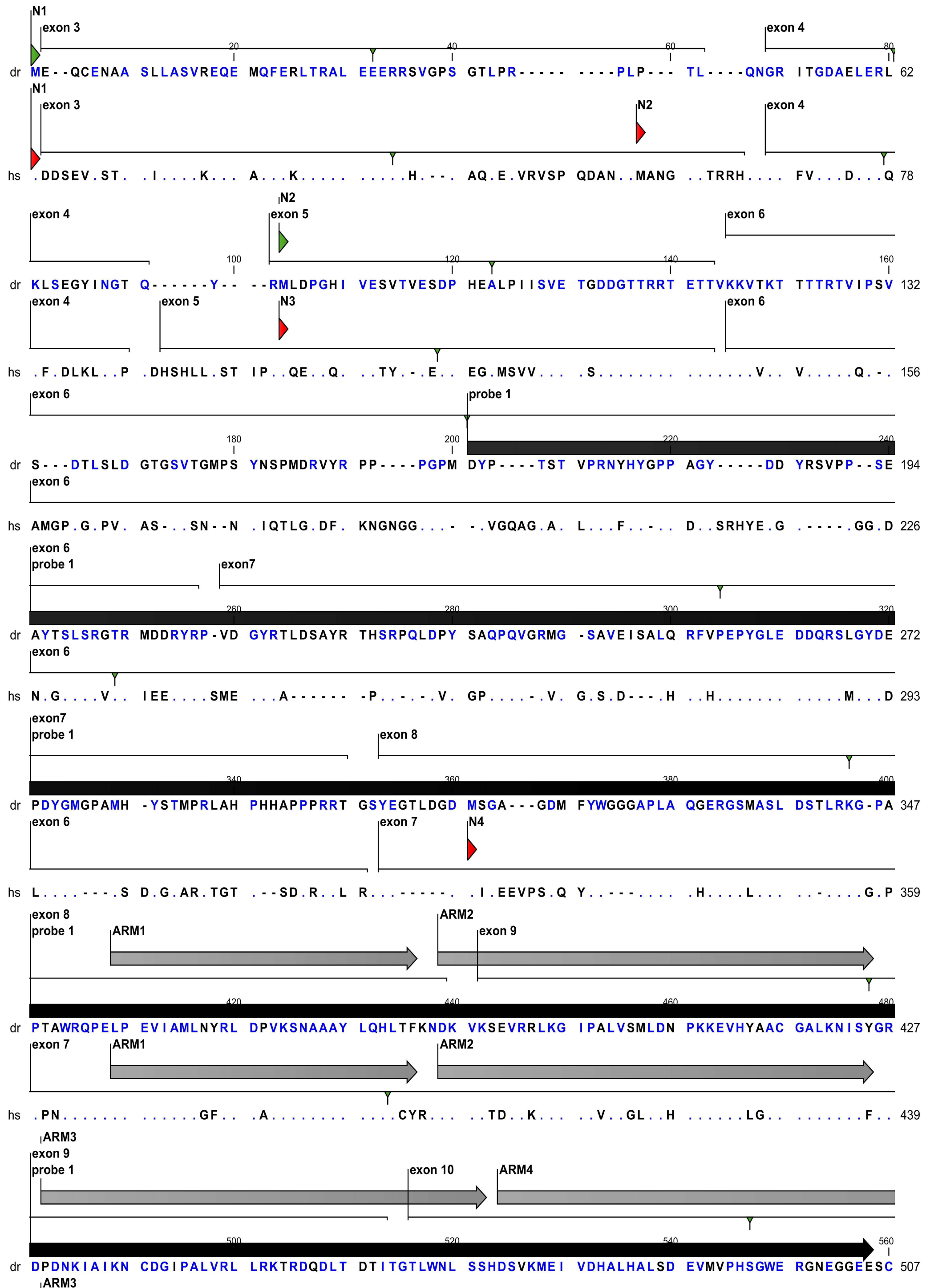
MCF-7 cells were cultured in basic DMEM medium supplemented 5% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.4 mM sodium pyruvate. For MCF-7 cells, non-essential amino acids and 6 ng/ml bovine insulin was added. All cells were grown in a humified atmosphere with 5% CO2 in air. MCF-7 cells were transfected with Fugene® HD transfection Reagent (Roche) in a 24 well plate setting according to a 8:2 ratio of transfecting reagent (µl) to DNA (µg). The transfection complex was made in Opti-MEMI Reduced Serum Medium. All cells were fixed with methanol when they reached a confluent layer. Expression was assessed by immunfluorescence. The cells were fixed with ice-cold 100% methanol for 10 minutes at -20°C and the primary antibody was applied for 1h at room temperature (RT).

The zebrafish ctnnd1 antibody was 1:100 diluted in 0.4% gelatin in 1xphosphate buffered saline (PBS). Anti-rabbit-DyeLight (Abcam) was used at 1:1000 dilution for 1h at RT, protected from light. Coverslips were mounted with Vectorshield with DAPI (Vector labs) and observed on a Zeiss Axio Imager Microscope equipped for epifluorescence, photographed using an Axiocam MRC videocamera.

4.6. Acknowledgements

We would like to thank Tommy D'heuvaert, Mieke Soenens and Dennis Vlaeminck for technical support and Jeroen Crucke for critical reading. This work was supported by a GOA research grant (BOF08/GOA/019) to ES, JVH and AH. BV acknowledges a grant of the Agency for Innovation by Science and Technology (IWT).

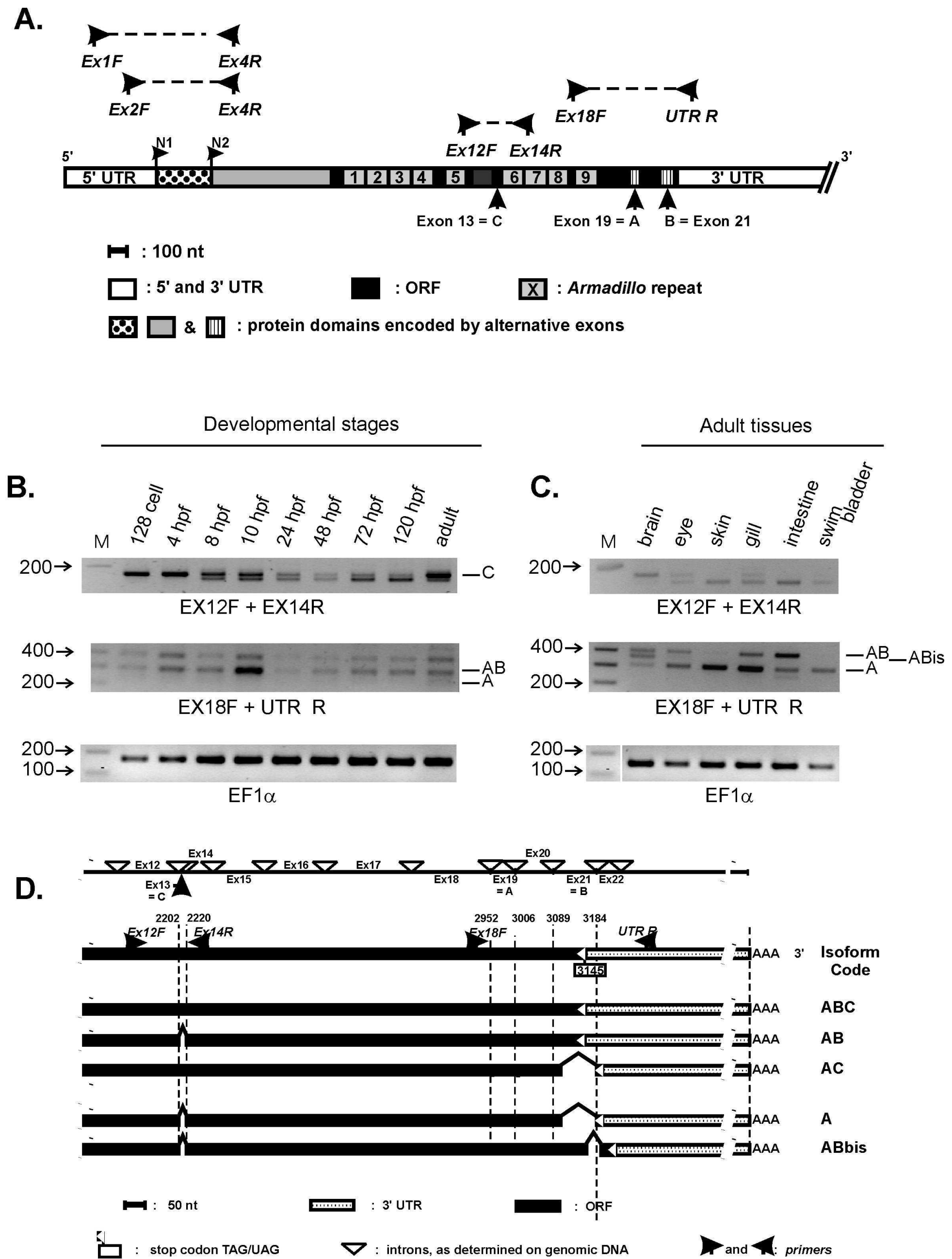
APPENDIX



N1
exon 3
exon 4
dr ME--QCENAA SLLASVREQE MQFERLTRAL EEERRSVGPS GTLPR----- ----PLP--- TL----QNGR ITGDAELERL 62
N1
exon 3
N2
exon 4
hs .DDSEV.ST. .I....K... A...K..... .....H.-.. AQ.E.VRVSP QDAN..MANG ..TRRH.... FV...D...Q 78
exon 4
N2
exon 5
exon 6
dr KLSEGYINGT Q------Y-- --RMLDPGHI VESVTVESDP HEALPIISVE TGDDGTTRRT ETTVKKVTKT TTTRTVIPSV 132
exon 4
exon 5
N3
exon 6
hs .F.DLKL..P .DHSHLL.ST IP..QE..Q. ..TY.-.E.. EG.MSVV... .S........ ....V.. V.....Q.-. 156
exon 6
probe 1
dr S---DTLSLD GTGSVTGMPS YNSPMDRVYR PP----PGPM DYP----TST VPRNYHYGPP AGY-----DD YRSVPP--SE 194
exon 6
hs AMGP.G.PV. AS-..SN--N .IQTLG.DF. KNGNGG...- -.VGGQAG.A. L...F..-.. D..SRHYE.G .----.GG.D 226
exon 6
probe 1
exon7
dr AYTSLSRGTR MDDRYRP-VD GYRTLDSAYR THSRPQLDPY SAQPQVGRMG -SAVEISALQ RFVPEPYGLE DDQRSLGYDE 272
exon 6
hs N.G....V.. IEE....SME ...A------ -P.-.-.V. GP....-.V. G.S.D---.H ..H....... .....M...D 293
exon7
probe 1
exon 8
dr PDYGMGPAMH -YSTMPRLAH PHHAPPPRRT GSYEGTLDGD MSGA---GDM FYWGGGAPLA QGERGSMASL DSTLRKG-PA 347
exon 6
exon 7
N4
hs L....---.S D.G.AR.TGT .--SD.R..L R....-----. .I.EEVPS.Q Y...--.... .H....L... ..-.....G.P 359
exon 8
probe 1
ARM1
ARM2
exon 9
dr PTAWRQPELP EVIAMLNYRL DPVKSNAAAY LQHLTFKNDK VKSEVRRLKG IPALVSMLDN PKKEVHYAAC GALKNISYGR 427
exon 7
ARM1
ARM2
hs .PN....... ......GF.. .A........ ....CYR... ..TD..K... ..V..GL..H ......LG.. ....F.. 439
ARM3
exon 9
probe 1
exon 10
ARM4
dr DPDNKIAIKN CDGIPALVRL LRKTRDQDLT DTITGTLWNL SSHDSVKMEI VDHALHALSD EVMVPHSGWE RGNEGGEESC 507
ARM3

Figure 33. Predicted amino acid sequence of zebrafish p120ctn

compared to the human p120ctn isoform 1ABC (Genbank Accesion no: NP_00107927). Parameters according to muscle alignment. The zebrafish sequence is based on the longest ORF in the corresponding cDNA sequence. For the human sequence (indicated by hs on the left), identical residues are shown as dots, dashes indicate lacking residues.Triangles represents alternatively used startcodons, numbered N1 till N4. For zebrafish, there are only 2 startcodons N1 and N2. Armadillo repeats ARM1 to ARM9 (Marchler-Bauer A. et al. Nucleic Acids Res. 2011 Jan;39) are visualised by grey arrows. Exons are represented by lines, alternatively used exons 13 (C), exon 19 (A) and exon 21 (B) are boxed. Probe 1, used for in situ hybridisation is represented by a black arrow.



Sanders et al., Fig.2

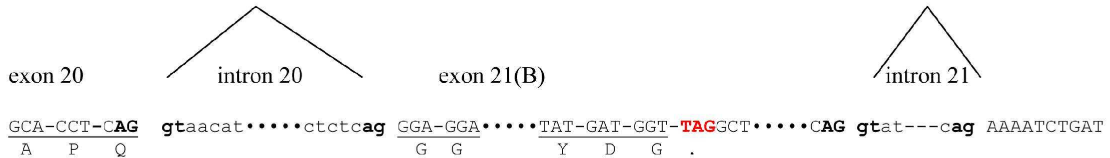
Figure 34. RT-PCR experiments used to examine alternative splicing events in zebrafish p120ctn mRNA (previous page)

(A) Schematic overview of primer names and their positions used for RT-PCR. Exon 13 (C), exon 19 (A) and exon 21 (B) are expected to be alternatively used. Agarose gel electrophoresis of RT-PCR products obtained from **(B)** various stadia throughout development and **(C)** several adult tissues.
For RT-PCR products obtained by the use of primers EX12F plus EX14R; a product of 173 bp will be observed if exon 13 (exon C) is present. When exon 13 is spliced out a product of 155 bp is expected. For RT-PCR products obtained by the use of primers EX18F plus UTR R, a product of 394 bp is expected when both exon B and exon A sequences are present, wheras a 349 bp product is expected upon removal of the exon A sequences. A product of 299 bp is expected upon removal of exon B sequences and a 245 bp product upon removal of both exon A and B sequences. Isoform ABbis is formed by an interexonic splicing event.Zebrafish *EF1α* was used as a loading control. **(D)** Schematic representation of the alternatively used exons in the 3' region of the p120ctn mRNA. Some examples of codes for the encoded protein isoforms are shown on the right. The scheme on top shows the exon structure of the corresponding region as determined on genomic DNA.

Figure 35. Schematic overview of the alternative splicing of exon 21 and the use of a splice donor sequence within exon 21 (next page)

Removal of exon 21 (exon B) results in a shortened ORF while interexonic splicing results in a frameshift and prolonged ORF.

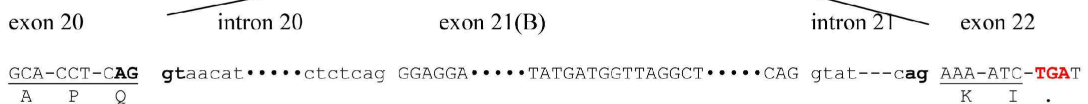
Standard splicing



exon 20 intron 20 exon 21(B) intron 21

```
GCA-CCT-CAG  gtaacat•••••ctctcag GGA-GGA•••••TAT-GAT-GGT-TAGGCT•••••CAG gtat---cag AAAATCTGAT
 A   P   Q                         G   G       Y   D   G  .
```

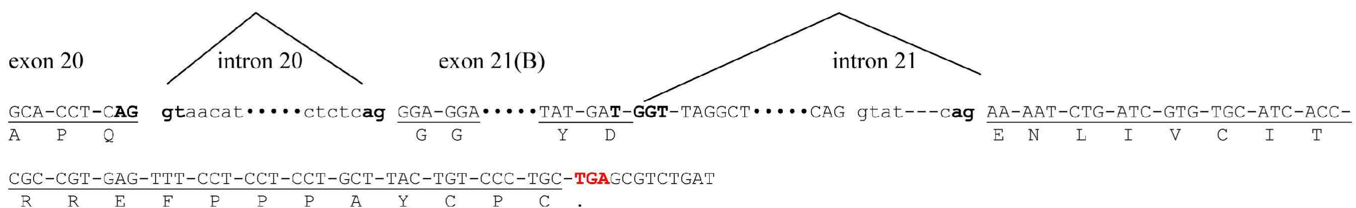
Alternative splicing exon B



exon 20 intron 20 exon 21(B) intron 21 exon 22

```
GCA-CCT-CAG  gtaacat•••••ctctcag GGAGGA•••••TATGATGGTTAGGCT•••••CAG gtat---cag AAA-ATC-TGAT
 A   P   Q                                                               K   I  .
```

Interexonic splicing exon B



exon 20 intron 20 exon 21(B) intron 21

```
GCA-CCT-CAG  gtaacat•••••ctctcag GGA-GGA•••••TAT-GAT-GGT-TAGGCT•••••CAG gtat---cag AA-AAT-CTG-ATC-GTG-TGC-ATC-ACC-
A   P   Q                          G   G       Y   D                                 E   N   L   I   V   C   I   T
CGC-CGT-GAG-TTT-CCT-CCT-CCT-GCT-TAC-TGT-CCC-TGC-TGAGCGTCTGAT
 R   R   E   F   P   P   P   A   Y   C   P   C  .
```

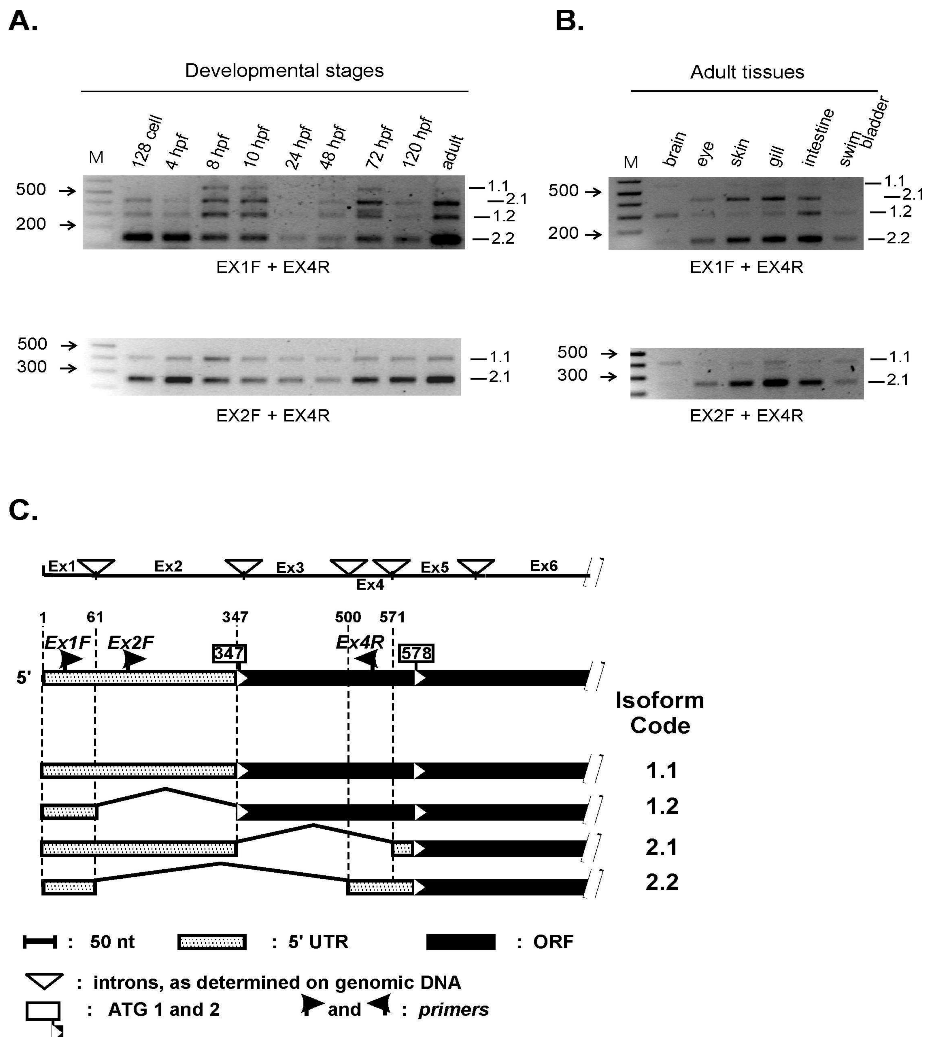


Figure 36. Analysis by RT-PCR of alternative splicing events occcuring in the 3’ region of the zebrafish p120ctn mRNA

Agarose gel electrophoresis of RT-PCR products obtained from **(A)** various stadia throughout development and **(B)** several adult tissues. With the use of primers EX1F plus EX4R, a product of 580 bp is expected when exons one until four are present, a product of 427 bp is expected upon removal of exon 3, 294 bp upon removal of exon 2 and 142 bp if both exons 2 and 3 are spliced. Using primers EX2F plus EX4R, a 410 bp fragment is generated when exon 2, 3 and 4 are present, whereas a 350 bp product is detected upon removal of exon 3. **(C)** Schematic representation of the alternatively used exons in the 5’ region of the p120ctn mRNA. Some examples of codes for the encoded protein isoforms are shown on the right. Startcodon N2 is used if exon 3 is spliced out, resulting in isoforms 2.1 and 2.2.

Expression of zebrafish p120ctn mRNA isoforms in various developmental stages and tissues

RNA source	1.1 580 bp	1.2 294 bp	2.1 427 bp	2.2 142 bp	A	AB 394 bp	B spliced 299 bp	C 173 bp	C spliced 155 bp
128 cell	+	++	++	+++	+++	++	++	++	-
4hpf	+	++	++	+++	+++	++	++	++	-
8hpf	+	++	++	+++	+++	++	++	++	++
10 hpf	+	++	++	+++	+++	++	+++	++	++
24 hpf	+	++	++	+++	+++	++	++	++	++
48hpf	+	++	++	++	+++	++	++	++	++
72 hpf	+	++	++	++	+++	++	++	++	++
120 hpf	+	++	++	+++	+++	++	++	++	++
adult	+	++	++	+++	+++	++	++	+++	++
brain	+	++	+	+	+++	+	+	++	-
eye	+	++	++	++	+++	+	+	+	+
skin	+	++	+	+++	+++	-	++	-	++
gill	+	++	+	+++	+++	+	++	+	+
intestine	+	++	++	+++	+++	++	+	-	++
swim bladder	+	++	++	+++	+++	-	+	+	+

Table 4. Relative expression of the different zebrafish p120ctn mRNA isoforms

For all isoforms, the relative amounts of the different RT-PCR products are estimated per gel lane and refelected by the number of plus signs. Below the isoform codes, the sizes of the diagnostic PCR fragments are indicated.

SECTION V

GENERAL DISCUSSION AND PERSPECTIVES

The **development and replacement of teeth** is a process of pronounced morphogenetic movements that issue from intense interactions between epithelial and mesenchymal cells. In zebrafish, first-generation teeth develop from the pharyngeal epithelium, which interacts with the underlying mesenchyme to form a functional tooth. The initiation starts with the formation of an epithelial placode (Huysseune et al., 1998). Replacement teeth do not develop from the pharyngeal epithelium but from the successional lamina, an outgrowth of the epithelial crypt surrounding the tip of the functional predecessor (Huysseune, 2006). The formation of a tooth placode and the formation of a successional lamina in each replacement cycle, starts with the formation a thickening of the basal epithelial layer from which the successor will develop, i.e., the pharyngeal epithelium, and the crypt epithelium, respectively. The cells that constitute this thickening next undergo invagination, followed by important morphogenetic movements (Huysseune et al., 1998; Van der heyden et al., 2000). The cell rearrangements and associated cell-cell interactions that characterize early tooth formation occur repeatedly throughout the zebrafish lifespan, as these animals display continuous tooth replacement (Huysseune and Thesleff, 2004; Van der heyden and Huysseune, 2000; Van der heyden et al., 2001). Thus, we wondered how these intense cellular movements occur in the light of cell-cell adhesion.

Jamora and colleagues (2003), carrying out research on early hair follicle development, proposed that the formation of an epithelial bud can only take place when accompanied with the down-regulation of the main epithelial cadherin, **E-cadherin**. While their results were based on the development of hair follicles, they suggested that a similar mechanism could operate for the formation of other integumental appendages, including tooth buds (Barrandon, 2003; Jamora et al., 2003).

To test the hypothesis raised by Jamora and colleagues (2003) on the relationship between epithelial bud formation and E-cadherin down-regulation, we embarked on a study investigating the expression and distribution pattern of E-cadherin during the development of first-generation teeth and replacement teeth in zebrafish (Section IV.1). We found that E-cadherin is present from the start onwards (in the tooth placode) and throughout the development of first-generation teeth in the epithelial part of the tooth, the enamel organ. The mesenchymal-derived dental papilla did not show any E-cadherin signal in any developmental stage (Fig. 37).

Developing replacement teeth showed exactly the same distribution for E-cadherin as first-generation teeth (Fig. 37). Strikingly, the formation of the epithelial placode (in first-generation teeth) or the successional lamina (in replacement teeth) was not accompanied by a down-regulation of E-cadherin. Unlike the hair follicle, both epithelial tooth anlagen showed strong E-cadherin staining. Thus, we concluded that no down-regulation of E-cadherin is observed during the formation of a tooth placode or successional lamina in zebrafish.

Not only teeth and hair follicles, but also mammary and salivary glands start their development with the formation of an epithelial thickening as the result of interactions between the epithelium and mesenchyme. Both mammary and salivary glands express E-cadherin and β-catenin at the plasma membrane at the epithelial bud stage and during further development (Hieda et al., 1996; Menko et al., 2002; Nanba et al., 2001). These studies on the formation of these other epithelial buds correspond to our data.

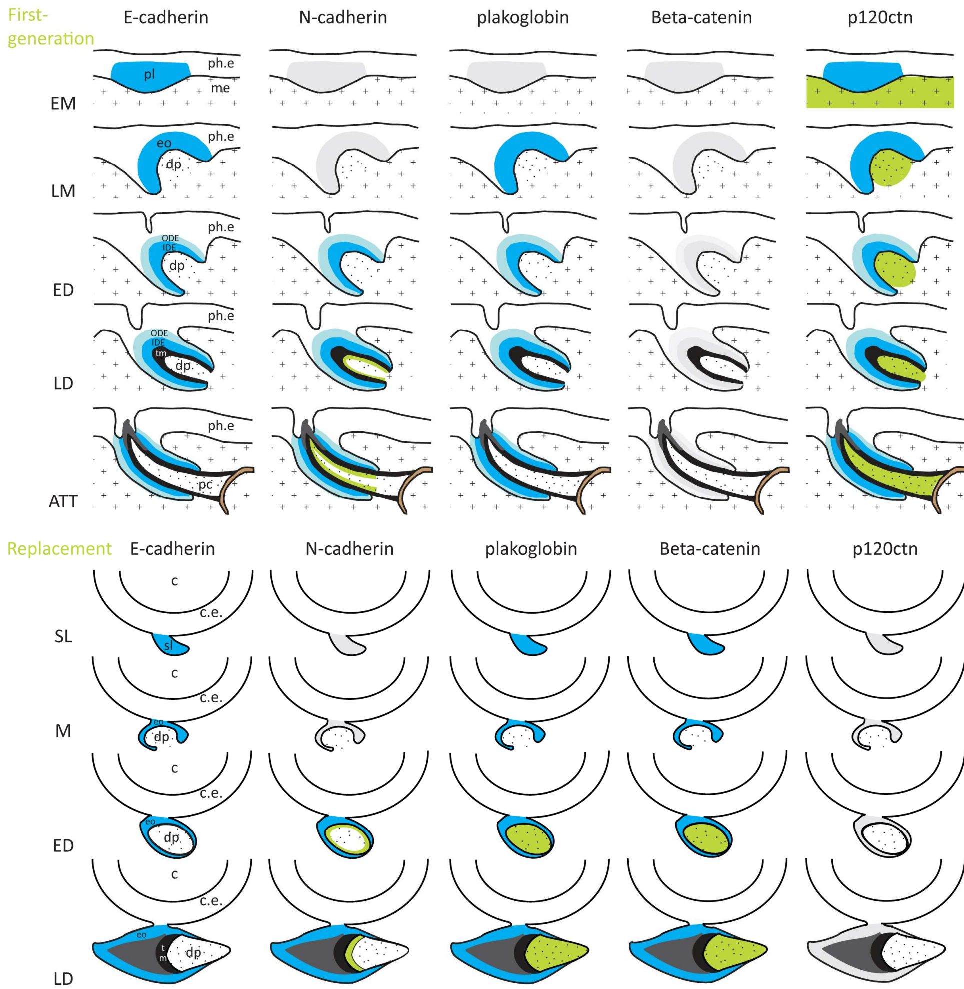


Figure 37. Overview of expression patterns of the cell adhesion molecules analyzed in this PhD thesis during the different stages of both first-generation and later-generation teeth

Color codes first-generation teeth: no epithelial expression (grey), epithelial expression in ODE (light blue) and IDE (darker blue); no expression dental papilla (dotted, white), expression odontoblasts/dental papilla (dotted, green).
Color codes later-generation teeth: no epithelial expression (grey), expression in the enamel organ (blue); no expression dental papilla (dotted, white), expression odontoblasts/dental papilla (dotted, green).

When comparing our data with data available on teeth of other species, we found that in mouse molars (and also in zebrafish teeth, see below) the epithelial tooth placode and the following bud stage show that β-catenin is present at the membrane (Obara and Lesot, 2004). The presence of β-catenin at the cell membrane is a strong indication for its binding to a classical cadherin at the membrane and hence for a functional adherens junction (Heuberger and Birchmeier, 2010). Based on our data and those of the studies mentioned above concerning mammary and salivary gland and development of mouse molars, it is acceptable to suggest that the morphogenetic movements resulting in the formation of an epithelial bud can occur without the loss of E-cadherin from the plasma membrane. An example of the fact that intense movements can occur while E-cadherin is present, is zebrafish epiboly (Babb et al., 2001; Babb and Marrs, 2004). E-cadherin depletion during early zebrafish development impairs all typical gastrulation movements (Babb and Marrs, 2004; Shimizu et al., 2005).

While E-cadherin is an interesting protein because of its multiple functions, other classical cadherins are also involved in various processes. During mouse tooth development, the epithelial-derived enamel organ interacts with the neural crest-derived mesenchyme (Chai et al., 2000; Rothova et al., 2011). **N-cadherin** is known to be expressed in neural crest-derived, mesenchymal tissues (such as the dental papilla) (Derycke and Bracke, 2004; Hatta et al., 1987; Takeichi, 1988) and is considered to be a marker for differentiation (Gravdal et al., 2007; Koh et al., 2008; Larue et al., 1996; Nakazora et al., 2010). Not only does N-cadherin have an important role as a cell adhesion molecule (Bagatto et al., 2006; Derycke and Bracke, 2004; Lefort et al., 2011; Matsunaga et al., 1988; Sakamoto et al., 2008), it is also involved in multiple processes such as migration and signal transduction (Derycke and Bracke, 2004; Niessen et al.,

2011). As could be expected, the expression and distribution of N-cadherin during zebrafish tooth development and replacement is clearly different from that of E-cadherin (Section IV.2). N-cadherin is not expressed in the early stages of tooth development (initiation and morphogenesis stage) (Fig. 37). Only when cytodifferentiation starts, i.e., when the cells of the inner dental epithelium start to differentiate into ameloblasts and the cells of the dental papilla start to become odontoblasts, does N-cadherin appear (Fig. 37). This suggests that N-cadherin is important during differentiation into these specialized cell types. Once a tooth has become functional, N-cadherin expression is maintained in the odontoblasts while the reduced enamel organ (i.e. the ameloblasts and the other remaining cells of the enamel organ) has lost its expression. This could be related to the fact that even in attached and erupted teeth, odontoblasts continue to deposit dentine matrix (Huysseune, 2006). In older functional teeth, the odontoblasts lose their polarity and also lose their N-cadherin expression. The presence of N-cadherin has repeatedly been linked to the process of differentiation (Derycke and Bracke, 2004; Ferreira-Cornwell et al., 2000; George-Weinstein et al., 1997; Leonard et al., 2011; Nakazora et al., 2010; Shin et al., 2005). During salivary gland development the expression of N-cadherin starts simultaneously with the differentiation into salivary tissue-specific structures (Menko et al., 2002). During human tooth development, N-cadherin is upregulated in the differentiating ameloblasts and odontoblasts (Heymann et al., 2002) which nicely corresponds to our observation on zebrafish tooth development.

To test our hypothesis that N-cadherin has a role in the differentiation process of ameloblasts and odontoblasts, we examined the development of teeth in the *parachute (pac)*^{tm101b} mutant, a zebrafish mutant defective for N-cadherin (Section IV.2) (Lele et al., 2002).

The first tooth that normally develops, tooth $4V^1$, could only be detected up to morphogenesis stage, but not beyond. Moreover, we were also unable to detect tooth $3V^1$ and $5V^1$ flanking tooth $4V^1$. Thus, without N-cadherin it seems that tooth development is arrested at the morphogenesis stage and further development into cytodifferentiation stage cannot be accomplished, suggesting that N-cadherin is necessary for ameloblast and odontoblast differentiation and thus for proper tooth development. The observation that the two adjacent teeth are lacking suggests that N-cadherin might also have a role in the onset of formation of these neighbouring teeth. One possible explanation is that the cleavage of N-cadherin is necessary for differentiation of the ameloblasts and odontoblasts. Bartlett and colleagues (2011) described recently that cleavage of E-cadherin by MMP20 is necessary for ameloblasts differentiation during mouse molar development. The authors suggest that the hydrolysis of the E-cadherin ectodomain by MMP20 and the subsequent release of β-catenin into the cytoplasm and localization of this protein to the nucleus are necessary for proper ameloblasts development (Bartlett et al., 2011). We can only speculate on how the cleavage of N-cadherin can affect differentiation in zebrafish. One possible mechanism is that the soluble, extracellular part functions as a signalling molecule. It has been shown that cleaved ectodomains can influence various biological functions. For example, the cleaved ectodomain of JAM-C promotes angiogenesis (Rabquer et al., 2010; van Kilsdonk et al., 2010). More specific, shedding of the N-cadherin ectodomain has been linked to several processes such as angiogenesis, synapse formation and chondrocyte differentiation (Latefi et al., 2009; Nakazora et al., 2010; Niessen et al., 2011). A second possibility is that, instead of the extracellular part, the intracellular part of the cleaved protein can stimulate signalization. It has been shown that the C-terminal domain of desmoglein-2 regulates apoptosis in the intestinal epithelium (Nava et al., 2007).

It has also been suggested that chondrocyte differentiation is possibly regulated by the shed N-cadherin intracellular part (Nakazora et al., 2010). When a functional cadherin is cleaved, the proteins associated with that cadherin (β-catenin and p120ctn) are set free into the cytoplasm and can influence signalling events. Thus, the cadherin-associated proteins β-catenin and p120catenin are able to transfer to the nucleus and influence gene transcription (Daniel, 2007; Huelsken and Held, 2009; Klaus and Birchmeier, 2008). Such a role for N-cadherin has already been proposed during neural crest delamination and ameloblasts development (Bartlett et al., 2011; Shoval et al., 2007). This study shows that during tooth development cleavage of cadherins can be essential for normal development.

Not only parts, but also the entire N-cadherin molecule can influence signalling events as a functional adhesion molecule at the membrane and thus regulate developmental processes. It has been suggested that *cis*-dimerization of N-cadherin facilitates the dimerization of the fibroblast growth factor receptor (FGFR), initiating the signalling pathway, at least during the outgrowth of neurites (Wheelock and Johnson, 2003a). Upon stimulation of this complex with Fgf, the MAPK signalling pathway is activated. The MAPK pathway regulates various cellular activities such as gene expression, proliferation, apoptosis and also differentiation (Chang and Karin, 2001). Moreover, the MAPK signalling also affects the expression of MMPs (O'Brien et al., 2004; Suyama et al., 2002). The detection of MMP-13a in the differentiating ameloblasts and odontoblasts during zebrafish tooth development (B. Verstraeten, unpublished results), indicates that one of the possibilities above or a combination can be the underlying mechanism for inducing cytodifferentiation.

The observation that the two neighbouring teeth are lacking, suggests that N-cadherin might also have a role in the onset of formation of these neighbouring teeth. It is striking that initiation of tooth $3V^1$ and $5V^1$ starts when tooth $4V^1$ is in early cytodifferentiation stage. If the cleavage of N-cadherin is necessary for proper differentiation, it is also possible that this cleavage event can induce the start of tooth formation of the two neighbouring teeth. There is no evidence that soluble N-cadherin plays a role in placode formation but it is known to function in different pathways (Derycke and Bracke, 2004; Latefi et al., 2009).

Since we did not observe a down-regulation of E-cadherin during tooth placode formation and start of morphogenesis, and since N-cadherin is not present during early stages of tooth development, we expanded our research in order to obtain insights on how cell adhesion is regulated during tooth development and replacement. Several possible mechanisms could be envisaged of how these morphogenetic movements can occur without the apparent loss of the cadherin-catenin complex. It is important to indicate the term morphogenetic movements, used several times in this thesis, does not necessarily implies the strict movement of individual cells with hallmarks such as the construction of a leading edge and filopodia. There is no indication that cells are actually migrating during the first stages of tooth development. During the morphogenetic movements described in this thesis the cells most likely move because of proliferation events. Proliferation studies on zebrafish tooth development are ongoing in our lab but the first suggestion for the influence of this process in zebrafish tooth development has been made by Huysseune (1989). A first possible mechanism underlying these movements is based on studies on cancer and tumor development which suggest that the loss of β-catenin could make the cadherin-catenin complex incompetent (Da Silva et al., 2008; Sundfeldt, 2003).

Thus, it is possible that E-cadherin is present at the membrane but because of the possible absence of the normal link with the actin cytoskeleton, there is no adhesive strength. A second possible mechanism has been discovered in studies on mammary gland development. Like teeth, these glands start their development with the formation of an epithelial bud. No down-regulation of the cadherin-catenin cell adhesion system was observed in this phase. However, in contrast to the cadherin-catenin, desmosomal cell adhesion showed a dramatic decrease during the formation of the mammary epithelial bud (Nanba et al., 2000; Nanba et al., 2001).

In order to test whether one of these possible mechanisms is acting during tooth formation in zebrafish, we expanded our studies to the cadherin-associated molecules **β-catenin and plakoglobin**. β-catenin is a protein that binds at the C-terminal region of all types of membrane-bound classical cadherins, and provides a link with actin, via the binding of α-catenin (Meng and Takeichi, 2009). Plakoglobin, on the other hand, is first and foremost a component of the desmosomes. It binds to the desmosomal cadherins desmocollin or desmoglein and provides a link with the intermediate filaments via the linker protein desmoplakin (Delva et al., 2009). In addition, because of the homology between plakoglobin and β-catenin, both proteins compete for the binding of the classical cadherins (Troyanovsky et al., 1996; Zhurinsky et al., 2000). β-catenin can also substitute for plakoglobin in the desmosomes, but only when plakoglobin is deficient (Bierkamp et al., 1999; Choi et al., 2009). We examined the distribution of β-catenin during zebrafish replacement tooth development to test the possibility that the cadherin-catenin complex is not functional during these morphogenetic movements (Section IV.3). Examination of β-catenin distribution revealed that this catenin is strongly expressed at the plasma membrane of all cells of the successional lamina (Fig. 37).

During further development of the tooth, β-catenin remains present at the membrane of the cells of the epithelial-derived enamel organ, also when the tooth becomes functional and the enamel organ becomes significantly reduced (Fig.37). In the dental papilla, β-catenin becomes expressed only from cytodifferentiation onwards in the polarized odontoblasts that line the dentine matrix (Fig. 37). β-catenin was not only localized at the membrane, it was also nuclearly expressed in the cells of the successional lamina and the differentiating odontoblasts. Based on these results we can draw several conclusions. First, because β-catenin is clearly localized at the membrane of the successional lamina, there is no evidence that the cadherin-catenin complex might not be functional, falsifying our first hypothesis regarding a non-functional cadherin-catenin adhesion complex. The observation that β-catenin is present in the nucleus of the cells of the successional lamina and the polarized odontoblasts is a valid proxy for active Wnt signalling (Logan and Nusse, 2004). Wnt signalling is known to play an important role during tooth development (Chen et al., 2009; Järvinen et al., 2006; Sarkar and Sharpe, 1999; Wang et al., 2009). Wnt signalling appears to be crucial for the conversion of the tooth placode to bud stage in murine tooth germs (Jernvall and Thesleff, 2000; Sarkar and Sharpe, 1999). By blocking Wnt signalling the formation of teeth is arrested (Chen et al., 2009; Liu et al., 2008), while overexpression of Wnt in the epithelium results in the formation of multiple tooth buds and teeth (Järvinen et al., 2006; Wang et al., 2009). However, unlike the situation in the mouse, overexpression of Wnt, mimicked by a mutation in axin 1, in the masterblind zebrafish mutant (van de Water et al., 2001), results in normal tooth development (Huysseune et al., unpublished observations). How Wnt signalling is regulated during zebrafish tooth development needs further research.

To test the second hypothesis, stating that the formation of a tooth is accompanied by the loss of the desmosomal cell adhesion system rather than by a down-regulation of the cadherin-catenin system, we examined the distribution of plakoglobin throughout zebrafish tooth development and replacement (Section IV.3). During initiation of first-generation teeth, plakoglobin is present in a dotted pattern, typical for desmosomal distribution. During further development, plakoglobin continues to be expressed in the different layers of the epithelial enamel organ. The condensed mesenchyme, and later the cells of the dental papilla showed plakoglobin expression from cytodifferentiation onwards (Fig. 37). In later-generation teeth, all the cells of the successional lamina are positive for plakoglobin (Fig. 37). The distribution of plakoglobin furthermore displayed the same pattern as in first-generation teeth, i.e. the epithelial part of the tooth is plakoglobin-positive throughout development. The mesenchymal part only becomes positive when cytodifferentiation starts (Fig.37). Thus, we could not find any sign of a loss of desmosomes during the formation of a tooth placode, successional lamina or any further step during tooth development, rejecting the hypothesis of desmosomal down-regulation as suggested for mammary glands (Nanba et al., 2000; Nanba et al., 2001). Because of the dual role of plakoglobin in cell adhesion (it can be incorporated in both the desmosomes and the cadherin-catenin complex), the distribution of this protein by immunohistochemistry cannot distinguish between both cell adhesion systems. Thus, while most of the plakoglobin present is part of the desmosomes (Zhurinsky et al., 2000), we cannot exclude that a smaller portion of the plakoglobin that is detected, is included in the cadherin-catenin complex. In order to obtain a more clear insight on desmosome distribution (and possible down-regulation) during zebrafish tooth development, detection of one of the desmosomal cadherins might give more information.

Additionally, we could also perform immuno-electron microscopy in order to visualize the subcellular distribution of the desmosomal proteins. However, this technique is technically challenging, expensive, and requires rigorous optimization of tissue fixation and processing methods. Although immune-electron microscopy is already used to study mouse and rat teeth, this technique has never been used for zebrafish dentition.
Given the strong and specific expression of plakoglobin during the different stages of both first-generation and later-generation teeth, we wanted to determine if there is a role for plakoglobin during zebrafish tooth development and/or replacement. Therefore, we examined plakoglobin-morpholino injected larvae at different developmental stages and compared their tooth development with that in control-injected (five base pair mismatch morpholino) zebrafish at the same time points (Section IV.3). Both control- and morpholino-injected zebrafish displayed a general delay in development compared to wild-type zebrafish, suggesting also a delayed onset of tooth formation in both groups. Plakoglobin-morpholino injected and control-injected zebrafish started the development of the first tooth, tooth $4V^1$, simultaneously. At 96 hpf, all first three tooth positions were occupied and the teeth did not display any abnormalities. Thus, the different layers of the enamel organ had differentiated and normal amounts of tooth matrix (enameloid) had been produced by the ameloblasts in collaboration with the odontoblasts. Thus, the loss of plakoglobin did not seem to have influenced tooth development. In conclusion, although plakoglobin is strongly expressed during tooth development, its loss does not alter this morphogenetic process. A possible explanation is that β-catenin substitutes for plakoglobin in the nascent desmosome systems, a mechanism also found in plakoglobin deficient tissues, such as the skin of plakoglobin null mice (Bierkamp et al., 1996; Bierkamp et al.,

1999). Such a substitution does not occur under normal circumstances (Choi et al., 2009).

The pattern of desmosomal distribution is also observed in other integumental appendages. Contrary to what we observed for plakoglobin in developing zebrafish teeth, early stages of developing glands and hair follicles in the mouse barely express components of the desmosomal adhesion systems. The occurrence of desmosomes significantly increases during later developmental stages. By investigating the distribution of plakoglobin, we mainly detect desmosomes but we cannot exclude that a portion of the plakoglobin detected is part of cadherin-catenin complex. Thus, it is possible that desmosomes are absent in the placode and successional lamina of zebrafish teeth, but we were unable to differentiate between plakoglobin in desmosomes or in the cadherin-catenin complex. A much more complete view on the distribution of desmosomes during the early stages of zebrafish tooth development can be obtained by examining the distribution of desmoglein or desmocollin. In addition, transmission electron microscopy can reveal the presence of desmosomes. Preliminary observations showed few desmosomes between the cells of the placode and successional lamina (B. Verstraeten and A. Huysseune, personal observations). A possible lack of desmosomes in early stages should not be surprising. When establishing epithelial cell-cell contacts and therefore cell adhesion, the first adhesion system to appear is always the adherens junctions (Baum and Georgiou, 2011; Ebnet, 2008; Yap et al., 1997). It provides a close aggregation of the cell membranes of neighbouring cells enabling other adhesion complexes, such as desmosomes and tight junctions, to form (Yin and Green, 2004).

Each of the components of the cadherin-catenin complex is important for a specific characteristic of this junction: cadherins at the membrane provide contact between adjacent cells while β-catenin and plakoglobin secure the linkage of the cadherin with the cytoskeleton (Meng and Takeichi, 2009). However, yet another catenin is indispensible for proper adherens junction functionality, **p120ctn**. In human and mouse, p120ctn has multiple functions but in the cell adhesion complex it binds the juxtamembrane domain of any classical cadherin, thus stabilizing the entire cadherin-catenin complex at the membrane (Ireton et al., 2002). Without p120ctn, the cadherin is not maintained at the membrane and becomes rapidly internalized and degraded (Davis et al., 2003). Zebrafish p120ctn (zfp120ctn) has not been studied before in any developmental stage or tissue. Analysis of zfp120ctn showed that the gene contains 22 exons (21 exons in human and mouse) and that alternative splicing of different exons (exon 13, 19 and 21) combined with the presence of two different translation initiation sites can result in different zfp120ctn isoforms during ontogeny and in specific tissues. Its gene organisation is comparable with mouse and human p120ctn, which combines four possible translation sites with the alternative splicing of four possible exons (Aho et al., 1999; Keirsebilck et al., 1998; Pieters et al., 2012b). Because of the low sequence homology between human and zebrafish nuclear localization signal (NLS) and nuclear export signal (NES), it can be assumed that the corresponding NLS and NES signals are not present in zebrafish. No other NLS and NES signals could be detected (Appendix). To investigate if zfp120ctn also shuttles to the cell membrane to bind the classical cadherins, we overexpressed zfp120ctn coupled to a Myc-tag in human MCF7-cells. The localization of the Myc-tag showed that zfp120ctn is indeed present near the membrane, suggesting that zfp120ctn is also part of the cadherin-catenin complex.

Moreover, it is very likely that it fulfils the same function as in human and mouse (Pieters et al., 2012b). For example, the overexpression of zfp120ctn induced the cells to exhibit a branching phenotype, found to be typical for overexpressing p120ctn in general (Noren et al., 2000; van Hengel et al., 1999). This branching phenotype has been related to the disassembly of stress fibers and focal adhesion, which contributes to increased migration. p120ctn is known to inhibit RhoA activity and to activate Cdc42 and Rac1, all of which are Rho-family GTPases, known for their role in cell migration and reorganization of the actin cytoskeleton (Anastasiadis, 2007; Braga and Yap, 2005; Noren et al., 2000). The presence of this typical cell phenotype obtained by overexpressing zfp120ctn suggests that zfp120ctn also interacts with Rho GTPases and has the ability to regulate the actin cytoskeleton.

To complete our research on the distribution of cadherin-catenin complex during zebrafish tooth development and replacement, we looked at the expression pattern of p120ctn (Section IV.4). Zfp120ctn transcripts appear to be present already at the early stage of tooth development (Fig. 37). As tooth development progresses this catenin is continuously expressed in both the epithelial and mesenchymal part of the tooth. During morphogenesis stage of the first tooth in the dentition to be formed, expression of zfp120ctn is observed in both the enamel organ and in the condensed mesenchyme (Fig. 37). Because of our previous findings on the distribution of E- and N-cadherin in the different cell types of the developing zebrafish teeth, and the fact that zfp120ctn is linked to these cadherins, this overall expression pattern is not surprising. How crucial this protein is for zebrafish tooth development, and whether it can be substituted for, is not addressed here. Nevertheless, this would be an interesting topic to study in the future, especially in view of the recent paper showing that the cKO of p120ctn in the epithelial part of the developing mouse molar results in a severe enamel phenotype (Bartlett et al.,

2010). Teeth in these cKO mice developed normally until the ameloblasts become secretory. The secretory ameloblasts become flattened and separate from the surrounding tissue as they have lost most, if not all, of their intracellular adhesion resulting in impaired enamel formation (Bartlett et al., 2010). When p120ctn is knocked-out in the dental papilla (using a p120:Wnt1-Cre cKO, abolishing p120ctn protein in all neural crest-derived tissues, including the mesenchymal dental papilla), no obvious tooth phenotype could be detected (A. Huysseune and H. Tian, personal communication). No p120ctn zebrafish mutant has yet been identified. For functional studies in the future, the most widely used method of gene knockdown in zebrafish is morpholino-injection, antisense oligonucleotides which either block translation of mRNAs or interfere with correct splicing of mRNA. Although they have proven to be a powerful tool, their short-lived nature has limited their use. To circumvent this disadvantage, RNA interference (RNAi) has also been used for targeted gene knockdown in zebrafish but the results are disappointing. Recently, promising results for targeted gene knockout in zebrafish have been obtained by injecting Zinc Finger Nucleases and TALENs.

It is clear that we have embarked on a new field yet to be discovered as not much data are available on zfp120ctn. For future research it would be very interesting to look closer into the tissue-specific distribution of the zfp120ctn isoforms and compare it with the distribution in human and mouse. It would be of great interest to check if this protein has the same signalling functions such as the interaction with the transcription factor Kaiso in zebrafish as described for mouse.

Based on the data found concerning the distribution of the components of the cadherin-catenin complex, we can conclude that the cellular movements related to the formation of an epithelial placode and subsequently bud, or of

the formation of a successional lamina, can occur without the loss or even reduction of adhesion complex expression. Apparently, the polarized epithelial cells of the pharyngeal epithelium or crypt epithelium that will give rise to the tooth placode or successional lamina, respectively are able to make the typical morphogenetic movements without losing contact between the cells. In addition, we have to consider that the function of these adhesion molecules is not restricted to mere cell adhesion. Apart from their role in the cadherin-catenin complex, cadherins are in control of the assembly of the actin cytoskeleton which is essential for specific morphogenetic movements (Bryant and Mostov, 2008; Halbleib and Nelson, 2006; Niessen et al., 2011).

A question that is left unaddressed is how cells of the basal layer of the pharyngeal epithelium become polarized to form the epithelial placode, and how placodal cells next transform from columnar cell shape to a wedge or cone shape (Huysseune et al., 1998). The latter change in cell shape is not only observed during tooth placode formation but is a feature typical for placode formation and invagination in general (Lecuit and Lenne, 2007; Martin et al., 2010). This columnar-to-wedge-shaped cell shape change that results in epithelial morphogenesis is called apical constriction and results in a reduction of the apical domain of the cell (Sawyer et al., 2010). Coordinated apical constriction of mechanically coupled cells promotes epithelial folding and tissue invagination (Odell et al., 1981). Therefore, apical constriction can be used to generate a variety of epithelial morphogenetic changes including folding, pits and tube formation (Fristrom, 1988; Hogan and Kolodziej, 2002). The reduction in the apical domain of the cell is accomplished by actinomyosin-driven contraction (Chung and Andrew, 2008; Dawes-Hoang et al., 2005) of which the resulting tension transmits its effect on the cell surface via the linkage of actin with the adherens junctions (Dawes-Hoang et al., 2005; Lecuit and Lenne, 2007).

An apical contractile actinomyosin network that spans the junctional area needs to be established, followed by the activation of myosin II by Rho GTPases (Lecuit and Lenne, 2007). A study of the tissue movements of the neural and non-neural ectoderm in *Xenopus* showed that the classical cadherins as a group have the ability to promote actin assembly at the cell surface (Nandadasa et al., 2009). More specific, the presence of N-cadherin is required for the morphogenetic movements concurrent with neurulation. Without N-cadherin the *Xenopus* embryos failed to undergo normal invagination movements. It was demonstrated that in these embryos the activated myosin light chain did not occur at the apical surface. Therefore, N-cadherin-mediated actin assembly is required for the apical localization of activated myosin light chain which will result in the necessary myosin-mediated apical constriction (Nandadasa et al., 2009).

Apical constriction is an important driving force for tissue bending and invagination in several developmental contexts. Examples are *Drosophila* gastrulation where it is important for the internalization of the presumptive mesoderm (Oda and Tsukita, 2001) and *Drosophila* salivary gland formation (Myat and Andrew, 2000). In vertebrates, a good example is neural tube formation and closure in which several populations of cells undergo apical constriction to fold the neural plate into a tube. In these cells, actin and myosin II are organized as into a circumferential cable that directly associates with the AJs of the cells. Constriction of the apex of the cells is the result of a shortening and thickening of the cable (Colas and Schoenwolf, 2001; Martin, 2010).

It was shown that the presence of AJs at the cell membrane is necessary to resist the tension provoked by the apical constriction. When for some reason the AJs are weakened, then apical constriction leads to epithelial tears and tissue retraction. It was suggested that all components of the AJs are required for epithelial tension (Martin et al., 2010). This can explain why we did not

observe a change in expression of components of the AJs during tooth placode and successional lamina formation. We speculate that the cadherin-catenin complex is needed to resist forces originated by the apical constriction of the cells. Further research is necessary particularly on how these shape changes are accomplished with specific attention for the regulation of the actin cytoskeleton during epithelial morphogenesis. Therefore, the distribution and alterations of the actin cytoskeleton can provide a better insight in this matter. It would be also interesting to look closer to the apparent movement of the epithelial cells and to be able to distinguish if these cells are actually moving or if they are passively displaced by proliferation and/or cell shape changes of the neighbouring cells. To determine which mechanism is at the basis of placode and bud formation, proliferation studies can provide more knowledge on this matter. This combined with the detection of structures and/or molecules related to cell movement such as the formation of lamellipodia or the detection of Rho GTPases.

Wautier et al. (2001) showed by detailed morphologic research that functional zebrafish teeth are cone-shaped with additional cusps. Cusp formation in mouse and human is related to the signalling activity of the enamel knot (Jernvall et al., 1994; Jernvall and Thesleff, 2000) but so far, no enamel knot equivalent is found in zebrafish tooth development. How are these additional cusps and the associated morphogenetic changes accomplished? Most of the attention has been paid to the epithelial part of the tooth, but it would be of great interest to find out if and how the mesenchymal cells contribute to the morphological changes during zebrafish tooth development. A more general question can also be raised: is tooth formation a morphogenetic process that is really a unique process or can tooth epithelial morphogenesis somehow show a deep homology to the formation of other appendages?

SECTION VI

SUMMARY

ENGLISH SUMMARY

Interactions between epithelium and mesenchyme characterize the development of various integumental appendages including hairs, mammary glands and teeth. During formation of these organs, both the epithelium and mesenchyme undergo a series of morphogenetic changes, mediated in part by changes in cell adhesion. Like hairs in mammals, teeth in zebrafish undergo cyclical renewal, as they replace their teeth throughout life. Thus, zebrafish provide a useful model to study some cellular aspects of epithelial and mesenchymal morphogenesis and differentiation in different tooth generations at various life stages of the animal.

In the zebrafish, the first-generation teeth arise from a thickening of the pharyngeal epithelium, called a tooth placode. A replacement tooth, in contrast, develops from an outgrowth of the epithelial crypt that surrounds the tip of the erupted predecessor. This outgrowth is called the successional lamina. After initiation, the developing tooth passes through different, partially overlapping, stages, termed early and late morphogenesis, early and late cytodifferentiation, and finally attachment and eruption.

In this PhD-thesis, I have focused on the distribution of the cadherin-catenin complex during the development of teeth in the zebrafish. This complex comprises a membrane-bound classical cadherin that is stabilized at the membrane by linkage of p120ctn. The C-terminal domain of the cadherin provides a binding site for β-catenin that links the cadherin to the actin cytoskeleton via binding to α-catenin. Via interaction with cadherins at the membrane of neighboring cells, a strong adhesive force is established between the cells.

Each tooth replacement cycle in the zebrafish is accompanied by the formation of an epithelial outgrowth and the development of each tooth includes considerable cellular rearrangements. Therefore I have examined how these processes can occur in the light of cell adhesion, more particularly the cadherin-catenin complex.

I wished to test the hypothesis proposed by Jamora et al. (2003, Nature. 422, 317-22.) that the formation of an epithelial bud is accompanied by the down-regulation of E-cadherin. Therefore, I started my research with the examination of the distribution of **E-cadherin**, a major component of adhesion junctions, during the different stages of development of first-generation and replacement teeth. I paid special attention to its distribution during the formation of a tooth placode (start of a first-generation tooth) and the successional lamina (onset of a replacement tooth). I examined the distribution of E-cadherin both at the transcript and protein level. During initiation stage of first-generation teeth, the tooth placode showed strong E-cadherin expression in all of its constituent cells. Subsequent developmental stages all showed a similar E-cadherin expression; the epithelial-derived part of the tooth showed a strong E-cadherin signal at the cell membrane while the mesenchymal-derived part remained negative for E-cadherin. Opposite to the proposed hypothesis, I did not find any evidence for down-regulation of E-cadherin during the morphogenetic movements displayed by first-generation teeth. I also looked at E-cadherin expression during the development of later-generation teeth. These teeth and their associated successional lamina are larger and include more cells enabling to dissect the expression signals at a finer scale. As for the first-generation teeth, I was unable to detect any loss of E-cadherin during the initiation stage of a replacement tooth.

Likewise, the following stages of tooth development did not show any deviation in E-cadherin distribution compared to the pattern found in first-generation teeth. The pattern of E-cadherin expression and distribution observed in first-generation and replacement teeth in zebrafish coincides with that observed in teeth of human and mouse. Minor differences can likely be explained by the differences in tooth morphology. The lack of down-regulation of E-cadherin during the formation of a tooth placode or successional lamina stands in sharp contrast to what is observed in the development of another epithelial appendage, the hair follicle.

Given that part of the tooth is composed of mesenchymal tissue, the dental papilla, I next focused on another classical cadherin, **N-cadherin**. N-cadherin is known to be expressed in neural crest-derived, mesenchymal tissues, such as the dental papilla. In addition to its role in cell adhesion, N-cadherin is also involved in multiple other processes such as embryogenesis, migration and signal transduction. Like for E-cadherin, I examined this molecule during both first-generation and replacement tooth development. At the onset of tooth development, no N-cadherin signal could be detected, neither in the first-generation tooth placode, nor in the successional lamina of later-generation teeth. During morphogenesis stage, the enamel organ and the dental papilla likewise did not show any signal for N-cadherin. Only from cytodifferentiation onwards, N-cadherin became detectable in the differentiating ameloblasts and odontoblasts. Once the tooth became attached, the reduced enamel organ lost its expression but N-cadherin expression persisted in the odontoblasts. Older, mature odontoblasts, which lose their polarized aspect, eventually also lost their N-cadherin expression. Thus, the start of N-cadherin expression clearly is concurrent with the start of differentiation of the ameloblasts and odontoblasts.

To obtain insights into the possible role of N-cadherin during tooth development, I studied N-cadherin deficient zebrafish mutants. In these *parachute* (*pac*) mutants, I could only detect the presence of tooth $4V^1$ in morphogenesis stage. The two neighbouring teeth, $3V^1$ and $5V^1$, were not present in any sample, suggesting a requirement for N-cadherin in the differentiation of ameloblasts and odontoblasts. This idea is supported by the fact that N-cadherin has previously been correlated to other differentiation processes such as in the development of the salivary glands, and during chondrocyte differentiation.

To collect information on the functionality of the cadherin-catenin complex, and on the presence and possible regulation of the desmosomes during the development of teeth, I next examined the distribution of two cadherin-associated molecules, **β-catenin and plakoglobin**. β-catenin provides the link between the membrane-bound classical cadherin and the actin cytoskeleton but can also be present independently of the adherens junctions and act as a component of the Wnt-signalling pathway. Plakoglobin is likewise known for its possible involvement in the Wnt-signalling pathway but, most importantly, constitutes an essential structural component of the desmosomes. β-catenin signal was detected in the successional lamina both at the membrane and in the nucleus whereas the surrounding mesenchyme did only show nuclear β-catenin. During morphogenesis stage, β-catenin was detected at the membrane of the cells of the enamel organ. Once cytodifferentiation started, β-catenin remained expressed at the membrane of the ameloblasts, ODE and odontoblast but it became also expressed as a nuclear signal in differentiating odontoblasts lining the dentine matrix. In the functional teeth, I observed β-catenin in the cells of the reduced enamel organ as well as in the odontoblasts.

These observations on β-catenin distribution in zebrafish teeth correspond to its distribution as described in the mouse dentition. The nuclear β-catenin signal that I detected during initiation and cytodifferentiation suggests that Wnt-signalling is active during at least those developmental stages. In other integumental appendages, such as mammary gland and hair, this signalling pathway is required for proper development. Wnt signals are essential for the initiation of both hair follicle and mammary placode, and for the initiation of the mammary gland morphogenesis.

The distribution of plakoglobin during zebrafish tooth development partially resembles that of β-catenin. The first plakoglobin signal was already detected during initiation stage in a dot-like pattern on the apical sides of the cells of the pharyngeal epithelium, including the polarized cells of the tooth placode. The plakoglobin signal became stronger at the cell membrane around the cell in both layers of the enamel organ during morphogenesis stage. When the tooth reached cytodifferentiation, plakoglobin was expressed in the ameloblasts and ODE. The dental papilla did not show any plakoglobin signal. A functional first-generation tooth showed a weaker distribution of this protein in the reduced enamel organ compared to the plakoglobin signal in the enamel organ before tooth eruption. All cells constituting the successional lamina of later-generation teeth expressed plakoglobin. This suggests that the formation of a new epithelial protrusion in the underlying mesenchyme during tooth replacement is not accompanied by the loss of desmosomes as seen in mammary gland development. The data available on plakoglobin in teeth of other species are limited to the mouse, where the expression of plakoglobin during odontogenesis parallels our data. Given the strong and specific expression of plakoglobin during the different stages of both first-generation and later-generation teeth, I wished to determine its possible role. To this end, I studied tooth development in plakoglobin morpholino-injected zebrafish.

Morpholino-injected zebrafish did not show any abnormality in tooth development compared to control-injected fish, and the teeth developed in their correct positions. The lack of plakoglobin did not prevent the initiation and progression of development of the teeth. The different layers of the enamel organ differentiated properly and normal amounts of enameloid and dentine were observed. The fact that, despite the loss of an important component of the desmosomes, normal tooth development occurs, suggests that the absence of plakoglobin is rescued. A good candidate for this is β-catenin as it is closely related to plakoglobin and they resemble each other structurally. Both are known for their ability to bind to classical cadherins and regulate the Wnt-pathway. That β-catenin may rescue the integrity of the desmosomes by substituting for plakoglobin during tooth development is supported by studies on other epithelial-derived organs such as hair and the intestinal epithelium, which also develop normally in the absence of plakoglobin.

Finally, I wished to explore one further component of the cadherin-catenin complex, not previously investigated in zebrafish, **p120catenin** (p120ctn). In human and mouse, p120ctn has multiple functions but in the cell adhesion complex it binds the juxtamembrane domain of any classical cadherin, thus stabilizing the entire cadherin-catenin complex at the membrane. Cytoplasmic p120ctn can interact with small Rho GTPases and can also bind the transcription factor Kaiso and modulate its transcriptional activity. p120ctn has different isoforms which result from four possible start codons in combination with the splicing of four possible exons. Zebrafish p120ctn (zfp120ctn) was cloned and the gene analyzed. Zfp120ctn contains 22 exons and alternative splicing of different exons (exon 13, 19 and 21) combined with the presence of two different translation initiation sites can result in different zfp120ctn isoforms.

We designed a probe to detect zfp120ctn mRNA during the different stages of tooth development. *In situ* hybridization showed that zfp120ctn was present both in the tooth placode and surrounding mesenchyme during tooth initiation. During morphogenesis stage, these two tissues retained their zfp120ctn expression. Cytodifferentiation of the different cell types did not alter zfp120ctn expression pattern and zfp120ctn was observed in the ODE, ameloblasts and odontoblasts. The first replacement tooth also showed zfp120ctn expression. This overall expression during tooth development is consistent with p120ctn particularly being known to bind and stabilize classical cadherins, which we showed to be present during tooth development. To check if zfp120ctn protein indeed binds classical cadherins near the membrane, we overexpressed zfp120ctn in the human MCF7 cell line. We observed zfp120ctn localized at the plasma membrane suggesting that it indeed links the membrane-bound cadherin. Furthermore, the transfected cells displayed a branching phenotype, formerly described to be typical for p120ctn overexpression as the result of the effect of p120ctn on Rho GTPases. This suggests that zfp120ctn can also regulate Rho GTPases. Further research is needed to shed a light on the different functions of zfp120ctn during tooth development.

NEDERLANDSE SAMENVATTING

De ontwikkeling van verschillende integumentale aanhangsels, zoals haren, borstklieren en tanden, wordt gekarakteriseerd door de wederzijdse interactie tussen epitheel en mesenchym. Tijdens de vorming van deze organen ondergaan zowel het epitheel als het mesenchym een reeks morfologische veranderingen, deels onder invloed van veranderingen in celadhesie. Net zoals haren bij zoogdieren, worden zebravis tanden cyclisch vernieuwd aangezien zij continu hun tanden vervangen. Zebravissen zijn dus een zeer bruikbaar model organisme om meerdere cellulaire aspecten van epitheliale en mesenchymale morfogenese en differentiatie te onderzoeken, dit in de verschillende tandgeneraties gedurende verschillende stadia tijdens de levenscyclus.

In de zebravis ontstaan de eerste generatie tanden uit een verdikking van het faryngeaal epitheel, de tand placode. Een vervangingstand echter ontwikkelt vanuit een uitwas van de epitheliale crypte die de tandtip van de doorgekomen voorganger. Deze aanwas wordt de successionele lamina genoemd. Na de initiatie doorloopt de ontwikkelende tand meerdere, deels overlappende stadia zoals vroege en late morfogenese, vroege en late cytodifferentiatie en finaal de aanhechting en het doorkomen.

In deze doctoraatsthesis heb ik mij gericht op de verspreiding van het cadherine-catenine complex tijdens de ontwikkeling van de tanden bij de zebravis. Dit complex omvat een membraan-gebonden klassiek cadherine dat aan de membraan gestabiliseerd wordt door middel van de binding met p120ctn. Het C-terminale gedeelte van de cadherine bevat een bindingsplaats voor β-catenine dat zorgt voor de link van het cadherine met het actine cytoskelet via de binding met α-catenine. Door middel van de interactie van membraan-gebonden cadherines van aanpalende cellen wordt een sterke adhesie kracht gevestigd.

Elke tandvervangingscyclys in zebravissen gaat samen met de vorming van een epitheliale uitwas en de ontwikkeling van elke tand omvat aanzienlijke herschikking van de cellen. Dit is de reden waarom ik deze specifieke processen in relatie tot celadhesie heb bestudeerd.

Ik begon mijn onderzoek met het bestuderen van de verspreiding van **E-cadherine**, een belangrijke component van de adherens juncties, tijdens de verschillende stadia van tandontwikkeling van eerste generatie en vervangingstanden. Ik lette speciaal op zijn distributie tijdens de vorming van de placode (start van een eerst generatie tand) en van de successionele lamina (aanzet van een vervangingstand). Meer in het bijzonder wenste ik de hypothese die voorgesteld was door Jamora et al. (2003, Nature. 422, 317-22), aangaande dat de vorming van een epithelial knop samengaat met de downregulatie van E-cadherine, te testen. Ik onderzocht de distributie van E-cadherine zowel op transcriptie als op eiwit niveau. Tijdens de initiatie fase van eerste generatie tanden vertoonde de tand placode in alle cellen een sterke expressie van E-cadherine. De daaropvolgende stadia vertoonden allemaal een gelijkaardige expressie patroon; het epitheliale deel van de tand had een sterk E-cadherine signaal aan de plasma membraan terwijl het mesenchymale gedeelte negatief bleef voor E-cadherine. In tegenstelling tot de voorgestelde hypothese, vond ik geen aanwijzing voor een eventuele downregulatie van E-cadherine tijdens de morfogenetische bewegingen in eerste generatie tanden. Ik bekeek ook de E-cadherine expressie tijdens de ontwikkeling van latere generatie tanden. Deze tanden en hun bijhorende successionele laminas zijn groter en bevatten meer cellen wat ons in staat stelt om de expressie signalen beter te kunnen bekijken. Net zoals in de eerste generatie tanden was er geen verlies aan E-cadherine waarneembaar tijdens de initiatie van een vervangingstand.

De volgende tandontwikkelingsstadia vertoonden eveneens geen enkele afwijking van de E-cadherine verspreiding vergeleken met het distributie patroon in eerste generatie tanden. Het E-cadherine expressie patroon en de distributie van het eiwit gevonden tijdens de eerste generatie en vervangingstanden bij zebravissen, komt overeen met wat er werd waargenomen in tanden van mensen en muizen. Minieme verschillen kunnen naar alle waarschijnlijkheid worden verklaard door de verschillen in tandmorfologie. Het gebrek aan downregulatie van E-cadherine tijdens de vorming van een tand placode of successionele lamina staat in groot contrast met de observaties tijdens de ontwikkeling van een ander epitheliaal appendage, de haarfollicle.

Aangezien een gedeelte van de tand bestaat uit mesenchymaal weefsel, de dentale papil, concentreerde ik mij vervolgens op een ander klassiek cadherine, **N-cadherine**. N-cadherine komt tot expressie in mesenchymale weefsels afgeleid van de neurale kammen, zoals de dentale papil. Naast zijn rol in celadhesie is N-cadherine ook betrokken bij meerdere andere processen zoals embryogenese, migratie en signaal transductie. Net zoals voor E-cadherine, heb ik deze molecule bestudeerd tijdens de tandontwikkeling zowel eerst generatie tanden als vervangingstanden. Bij de start van tandontwikkeling kon er geen N-cadherine signaal worden waargenomen, niet in de eerste generatie tand placode en niet in de successionele lamina van latere generatie tanden. Tijdens morfogenese vertoonden het emailorgaan en de dentale papil ook geen signaal voor N-cadherine. Enkel vanaf cytodifferentiatie werd N-cadherine waarneembaar in de differentiërende ameloblasten en odontoblasten. Wanneer de tand vasthechtte verloor het overblijvende emailorgaan zijn N-cadherine expressie maar de odontoblasten behielden hun N-cadherine expressie.

Oudere, meer mature odontoblasten, die hun gepolariseerde aspect verloren hebben, verloren uiteindelijk ook hun N-cadherine expressie. Dus het begin van de N-cadherine expressie is gelijktijdig met de start van differentiatie van de ameloblasten en de odontoblasten. Om een beter inzicht te krijgen in een mogelijke rol voor N-cadherine tijdens tandontwikkeling bestudeerde ik N-cadherine deficiënte zebravis mutanten. In deze *parachute* (*pac*) mutanten kon ik enkel tand $4V^1$ in morfogenese vinden. De twee naburige tanden, $3V^1$ en $5V^1$, konden niet gevonden worden wat de vereiste van N-cadherine suggereert voor de differentiatie van ameloblasten en odontoblasten. Dit idee wordt ondersteund door het feit dat N-cadherine eerder al is gecorreleerd met andere differentiatie processen zoals in de ontwikkeling van speekselklieren en chondrocyt differentiatie.

Om informatie te winnen over de functionaliteit van het cadherine-catenine complex en over de aanwezigheid en mogelijke regulatie van desmosomen gedurende de ontwikkeling van tanden onderzocht ik vervolgens de verspreiding van twee cadherine-geassocieerde moleculen, **β-catenine en plakoglobine**. β-catenine is de verbinding tussen het membraan-gebonden klassieke cadherine en het actine cytoskelet maar kan ook onafhankelijk van de adherens juncties voorkomen en functioneren als een onderdeel van de Wnt signalisatie pathway. Plakoglobine is eveneens gekend voor zijn mogelijke betrokkenheid in de Wnt signalisatie pathway maar belangrijker, is een essentieel structurele component van de desmosomen. β-catenine signaal werd gedetecteerd in de successionele lamina zowel aan de membraan als in de kern in tegenstelling tot het omgevende mesenchyme dat geen nucleair β-catenine vertoonde. Tijdens morfogenese stadium, werd β-catenine gedetecteerd aan de membraan van alle cellen van het emailorgaan.

Wanneer cytodifferentiatie startte, bleef β-catenine geëxpresseerd aan de membraan van de ameloblasten, het buitenste emailorgaan epitheel en de odontoblasten maar het kon ook worden waargenomen in de kernen van differentiërende odontoblasten die de dentine matrix aflijnen. In de functionele tanden observeerde ik β-catenine in de cellen van het overblijvende emailorgaan en in de odontoblasten. Deze waarnemingen betreffende β-catenine distributie in zebravis tanden komt overeen met de verspreiding beschreven in muizentanden. Het nucleaire β-catenine signaal dat ik vond tijdens initiatie en cytodifferentiatie suggereert dat Wnt signalisatie actief is althans tijdens deze ontwikkelingsstadia. In ander integumentale aanhangsels, zoals de borstklier en haar, is deze signalisatie pathway vereist voor de goede ontwikkeling. Wnt signalen zijn essentieel voor de initiatie van zowel haarfollicle als borstklier placode, en voor de aanzet tot morfogenese van de borstklieren.

De distributie van plakoglobine tijdens zebravis tandontwikkeling lijkt gedeeltelijk op die van β-catenine. Het eerste plakoglobine signaal werd reeds gedetecteerd tijdens het initiatie stadium als een punt patroon aan de apicale zijde van de cellen van het faryngeale epitheel, inclusief de gepolariseerde cellen van de tand placode. Het plakoglobine signaal werd sterker aan de celmembraan rond de cellen, in beide lagen van het emailorgaan gedurende morfogenese. Wanneer de tand cytodifferentiatie stadium bereikte, was plakoglobine geëxpresseerd in de ameloblasten en het buitenste emailorgaan epitheel. De dental papil vertoonde geen plakoglobine signaal. Een functionele eeste generatie tand vertoonde een verminderde distributie van dit eiwit in het overblijvende emailorgaan in vergelijking met het plakoglobine signaal in het emailorgaan voor de eruptie van de tand. Alle cellen van de successionele lamina van latere generatie tanden expresseerden plakoglobine.

Dit suggereert dat de vorming van een nieuwe epitheliale uitstulping in het onderliggende mesenchym tijdens tandontwikkeling niet samengaat met het verlies van de desmosomen zoals geobserveerd werd tijdens borstklier ontiwikkeling. De beschikbare data in verband met plakoglobine in tanden van andere species zijn beperkt to muis gegevens, waarin de expressie van plakoglobine tijdens de odontogenese gelijkaardig verloopt met onze data. Aangezien de sterke en specifieke expressie van plakoglobine tijdens de verschillende stadia van zowel eerste generatie als latere generatie tanden, wou ik ook de mogelijke functie onderzoeken. Daarvoor bestudeerde ik de tandontwikkeling in plakoglobine morfolino-geinjecteerde zebravissen. De morfolino-geinjecteerde zebravissen vertoonden geen abnormale tandontwikkeling in vergelijking met de controle-geinjecteerde vissen, en de tanden ontwikkelden op de correcte positie. Het gebrek aan plakoglobine heeft de aanzet en de vooruitgang van de tandontwikkeling niet gehinderd. De verschillende lagen van het emailorgaan differentieerden goed en er werden normale hoeveelheden enameloid en dentine waargenomen. Het feit dat, desondanks het verlies van een belangrijke component van de desmosomen, normale tandontwikkeling voorkomt suggeert dat de afwezigheid van plakoglobine wordt opgevangen. Een prima kandidaat hiervoor is β-catenine aangezien die erg gerelateerd is aan plakoglobine en ze structureel ook op elkaar lijken. Ze zijn beiden gekend voor hun mogelijkheid om te binden met klassieke cadherines en om de Wnt pathway te reguleren. De suggestie dat β-catenine de integriteit van de desmosomen kan garanderen door plakoglobine te vervangen tijdens tandontwikkeling wordt ondersteund door studies van andere organen die van het epitheel afkomstig zijn, zoals haren en het intestinaal epitheel, die zich ook normaal ontwikkelen in de afwezigheid van plakoglobine.

Als laatste wou ik nog een component van het cadherine-catenine complex ontdekken, dat nog nooit bij zebravissen onderzocht werd, **p120ctn**. Bij mens en muis heeft p120ctn meerdere functies maar in het celadhesie complex bindt het aan het juxtamembranaire domain van een klassiek cadherine waardoor het gehele cadherine-catenine complex aan de membraan gestabiliseerd wordt. Cytoplasmatisch p120ctn kan interageren met kleine Rho GTPasen en kan ook binden met de transcriptie factor Kaiso en zo zijn transcriptionele activiteit beinvloeden. p120ctn bestaat in verschillende isovormen die het resultaat zijn van vier mogelijke startcodons in combinatie met de splicing van vier mogelijke exonen. Zebravis p120ctn (zfp120ctn) is gecloneerd en het gen geanalyseerd. Zfp120ctn omvat 22 exonen en de alternatieve splicing van meerdere exonen (exon 13, 19 en 21) gecombineerd met de aanwezigheid van twee verschillende translatie initiatie plaatsen wat kan leiden tot verschillende zfp120ctn isovormen. We ontwierpen een probe om zfp120ctn mRNA te detecteren tijdens de verschillende stadia van tandontwikkeling. *In situ* hybridisatie toonde aan dat zfp120ctn aanwezig is zowel in de tand placode als in het omgevende mesenchym tijdens tand initiatie. Tijdens morfogenese stadium behielden deze twee weefsels hun zfp120ctn expressie. Cytodifferentiatie van de verschillende celtypes veranderde niets aan het expressie patroon van zfp120ctn en werd dus waargenomen in het buitenste emailorgaan epitheel, ameloblasten en odontoblasten. De eerste vervangingstand vertoonde ook zfp120ctn expressie. Deze algemene expressie tijdens tandontwikkeling is consistent met de kennis dat p120ctn hoofdzakelijk gebonden is aan en een stabiliserende function heeft op klassieke cadherines die wij reeds hebben aangetoond tijdens tandontwikkeling. Om na te gaan of zfp120ctn eiwit weldegelijk de klassieke cadherines bindt aan de plasma membraan, hebben we zfp120ctn overgeëxpreseerd in de humane MCF7 cellijn. We konden zfp120ctn aan de membraan localiseren wat suggereert dat

het inderdaad bindt aan de klassieke cadherines ter hoogte van de membraan. De getransfecteerde cellen vertoonden ook nog een vertakkend fenotype dat in het verleden beschreven is als typisch voor p120ctn overexpressie ten gevolge van het effect van p120ctn op Rho GTPasen. Deze observatie suggereert dat zfp120ctn ook de mogelijkheid heeft om Rho GTPasen te reguleren. Verder onderzoek is noodzakelijk om een licht te werpen op de verscheidenheid aan functies van zfp120ctn tijdens tandontwikkeling.

SECTION VII

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SECTION VIII

CURRICULUM VITAE

PUBLICATION LIST

° A1 publications:

Verstraeten, B., Sanders, E., van Hengel, J., Huysseune, A., 2010a. Expression pattern of E-cadherin during development of the first tooth in zebrafish (Danio rerio). Journal of Applied Ichthyology. 26**,** 202-204.

Verstraeten, B., Sanders, E., van Hengel, J., Huysseune, A., 2010b. Zebrafish teeth as a model for repetitive epithelial morphogenesis: dynamics of E-cadherin expression. BMC Dev Biol. 10**,** 58.

° B2 publications:

Verstraeten, B., Sanders, E., Huysseune, A., Whole mount immunohistochemistry and *in situ* hybridization of larval and adult zebrafish dental tissues
In: C. Kioussi, (Ed.), Odontogenesis: Methods and Protocols, Methods in Molecular Biology, Vol. 887. Springer Science+Business Media, New York, 2012.

CONTRIBUTION TO INTERNATIONAL CONFERENCES:

° Academic year 2008 – 2009

- 15th Benelux Congress of Zoology (Liege, Belgium);
  Oral presentation
  Verstraeten, B., Sanders, E., van Hengel, J., Huysseune, A.
  Expression of E-cadherin during the development and continuous replacement of teeth in the zebrafish (*Danio rerio*).
- Workshop Interdisciplinary Approaches in Fish Skeletal Biology (Tavira, Portugal); Poster
  Verstraeten, B., Sanders, E., van Hengel, J., Huysseune, A.
  Expression of E-cadherin during the development and continuous replacement of teeth in the zebrafish (*Danio rerio*).
- Gordon Research Conference: Cell contact and adhesion (Watervill Valley, NH, USA); Poster
  Verstraeten, B., Sanders, E., van Hengel, J., van Roy, F., Huysseune, A.
  Expression of cell adhesion molecules during tooth development in zebrafish: a model for repetitive renewing structures.

° Academic year 2009 – 2010

- 16th Benelux Congress of Zoology (Wageningen, Netherlands);
  Oral presentation
  Verstraeten, B., Sanders, E., van Hengel, J., Huysseune, A.
  Expression of E-cadherin during the development and continuous replacement of teeth in the zebrafish (*Danio rerio*).
- Gordon Research Conference: Craniofacial Morphogenesis & Tissue Regeneration (Lucca, Italy);
  Poster
  Verstraeten, B., Sanders, E., van Hengel, J., Huysseune, A.
  Zebrafish teeth as a model for repetitive epithelial morphogenesis: the involvement of E-cadherin.
- 10th TMD Tooth Morphogenesis and Differentiation (Berlin, Germany);
  Oral presentation
  Verstraeten, B., Sanders, E., D'heuvaert, T., van Hengel, J., Huysseune, A.
  The cadherin-catenin complex during zebrafish tooth development.

° Academic year 2010 – 2011

- 17th Benelux Congress of Zoology (Ghent, Belgium);
  Oral presentation
  Verstraeten, B., Sanders, E., D'heuvaert, T., van Hengel, J., Huysseune, A.
  The cadherin-catenin complex during zebrafish tooth development.
- Workshop Interdisciplinary Approaches in Fish Skeletal Biology (Tavira, Portugal); Oral presentation
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