Studies of $\alpha 11 \beta 1$ integrin in the mouse and human periodontal ligament

Malgorzata M. Barczyk

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Acknowledgements

List of papers

The present thesis is based on the following papers and will be referred to in the text by their Roman numerals.

Paper I: Popova SN, <u>Barczyk M</u>, Tiger CF, Beertsen W, Zigrino P, Aszodi A, Miosge N, Forsberg E, Gullberg D**. α11β1 integrin-dependent regulation of periodontal ligament function in the erupting mouse incisor.** Mol Cell Biol. 2007, 27: 4306-4316.

Paper II: <u>Barczyk MM</u>, Popova SN, Bolstad AI, and Gullberg, D. **α11β1 integrin-mediated collagen lattice contraction by mouse incisor periodontal ligament fibroblasts requires MMP-13.** Manuscript.

Paper III: <u>Barczyk MM</u>, Borge Olsen L-H, da Franca P, Loos BG, Mustafa K, Gullberg D and Bolstad AI. **A role for α11β1 integrin in the human periodontal ligament.** J Dent Res. *In press.*

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Abbreviations

ALP	Alkaline phosphatase
BM	Basement membrane
BMP	Bone morphogenetic protein
BSP	Bone sialoprotein
CAF	Carcinoma-associated fibroblast
CD	Cytoplasmic domain
CSF	Colony-stimulating factor
E(x)	Embryonic day (x) e.g. E14
EBS	ETS-binding site
ECM	Extracellular matrix
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated protein kinase
ES cell	Embryonic stem cell
ETS	E26 transforming-specific protein
FAK	Focal adhesion kinase
FGF-10	Fibroblast growth factor 10
GFFAH	Gly-Phe-Phe-Ala-His
GFFKR	Gly-Phe-Phe-Lys-Arg
GFFRS	Gly-Phe-Phe-Arg-Ser
GFOGER	Gly-Phe-Hyp-Gly-Glu-Arg
GLPGER	Gly-Leu-Pro-Gly-Glu-Arg
HERS	Hertwig's epithelial root sheath
hPDLF	Human periodontal ligament fibroblast
hGF	Human gingival fibroblast
I-domain	Inserted domain
IFNγ	Interferon gamma
IGF-II	Insulin-like growth factor 2
IL	Interleukin
ITGA	Human integrin alpha gene
Itga	Mouse integrin alpha gene
MAPK	Mitogen activated protein kinase
MEF	Mouse embryonic fibroblast
MIDAS	Metal ion-dependent adhesion site
MMP	Matrix metalloproteinase
OC	Osteocalcin
OP	Osteopontin
p38	p38 kinase
PDGF-BB	Platelet-derived growth factor-BB
PDL	Periodontal ligament
PSI-domain	Plexin, semaphorin and integrin domain
Runx-2	Runt-related transcription factor 2
SBS	Sp1/3-binding site
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
Sp	Specificity protein
SPARC	Secreted protein, acidic, cysteine-rich
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitor of metalloproteinase
TM	Transmembrane domain
TNF-α	Tumor necrosis factor alpha
TSS	Transcription start site

Introduction

1. Cell-extracellular matrix interactions

1.1. Extracellular matrix

In multicellular organisms the cellular and tissue organization is mediated by the extracellular matrix (ECM), a structure composed of a number of complex macromolecules. Cells in tissues interact with other cells, or with ECM proteins, via specific receptors, like integrins. Different types of ECMs are usually divided into two major groups: the interstitial matrix and the basement membrane.

The interstitial matrix is a hydrated gel containing polysaccharides and fibrous proteins, which fill the interstitial space. The type of collagens and proteoglycans define the structure and properties of interstitial matrices [1]. Collagen I is the most abundant of all collagens found in interstitial matrices except for cartilage which contains mainly collagen II. The interstitial matrices also contain non-collagenous proteins contributing to the structural organization of the ECMs, such as fibronectin [2], elastin [3] and fibrillin [4].

Basement membranes (BM) are the ECMs formed in sheet-like depositions oriented basolaterally to monolayers of epithelium and endothelium, providing separation from underlying connective tissue and providing support to the cells. The BMs also surround cells such as fat cells, individual muscle cells and Schwann cells. Collagen IV, laminins, nidogens and proteoglycans are major components of BMs [5]. The BMs in different tissues can contain specific isoforms of collagen IV or laminins, which reflects the specific functions of particular tissues. Other minor components of BMs include agrin, fibulins, collagen XV, collagen XVIII and SPARC, which all contribute to the tissue-dependent heterogeneity of BMs [6].

The cell-ECM interactions regulate gene expression which further influences processes like cell differentiation and cell proliferation. These processes are important for tissue development, tissue homeostasis, tissue regeneration and in pathological conditions [7, 8]. Apart from the structure and composition of the ECM it is important to understand the complex dynamics of ECM. A number of pathological conditions such as tumor invasion and metastasis, rheumatoid arthritis and periodontal disease are characterized by destruction of the ECM by proteolytic enzymes such as matrix metalloproteinases (MMPs) [9].

1.2. Cell-ECM interactions

1.2.1. Integrins

Integrins are composed of α- and β-subunits forming non-covalent heterodimers [10]. Each subunit is a type I transmembrane glycoprotein with a large N-terminal extracellular multi-domain structure and short intracellular tail joined by a transmembrane domain. In vertebrates, there are 18 α-subunits and 8 β-subunits, forming 24 different integrin heterodimers **(Figure 1)**. The name integrin comes from the "integrating" nature of these receptors [11]. They physically link the cytoskeleton of the cell to the ECM or to other cells, allowing activation of a number of signaling pathways [12]. Integrin receptors bind a number of ligands present in the ECM and on cell surfaces [13].
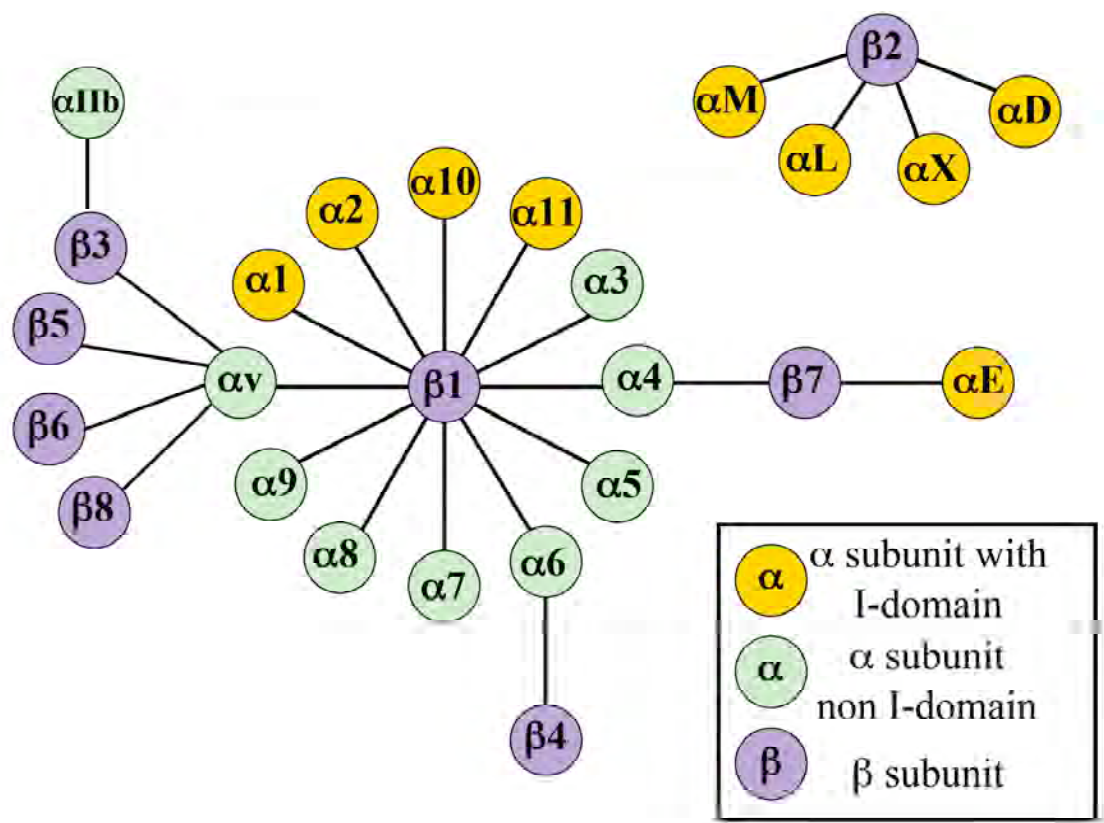
Figure 1. The integrin family. The figure is adopted and modified from Hynes, 2002 [14]. $\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$ and $\alpha11\beta1$ are collagen-binding receptors.

1.2.2. Integrin structure

The integrin α-chains can structurally be divided into two groups depending on whether they contain or do not contain the inserted αI-domain. Nine of 18 α-subunits, $\alpha1$, $\alpha2$, $\alpha10$, $\alpha11$,

αM, αL, αD, αX and αE, contain the αI-domain, composed of approximately 200 amino acids. The αI-domain is inserted between blades 2-3 of the N-terminal β-propeller [15]. In the α-subunit the ligand-binding sites differ between the two groups of α-chains. In αI-domain containing α-chains the ligand-binding site largely resides in the αI-domain and it includes a crucially positioned Mg^{2+} ion. This latter site has been named the metal ion-dependent adhesion site (MIDAS) [16]. The interactions of the αI-domain with the β-chain are required for proper folding of some αI-domains [17]. In the α-chains lacking αI-domain, the ligand-binding site is confined to an interface between the β-propeller in the α-subunit and the βI-domain of the β-subunit [18].

The integrin β-subunit has a very different structure compared to the α-chains **(Figure 2)**. Instead of a β-propeller, the β-chain head consists of an N-terminal cysteine-rich region called PSI (Plexin, semaphorin and integrin)-domain [19] with an inserted βI-domain [20]. The C-terminal end of the extracellular region contains four epidermal growth factor (EGF)-like cysteine-rich domains, which are thought to play an important role in the activation of integrins [21, 22]. The cytoplasmic tails of the β-chains are longer compared to the α-chains and are involved in anchorage to the cytoskeleton and interactions with signaling molecules [23]. The longest cytoplasmic tail, which is over 1000 amino acid long, has been described in the β4 subunit [24].
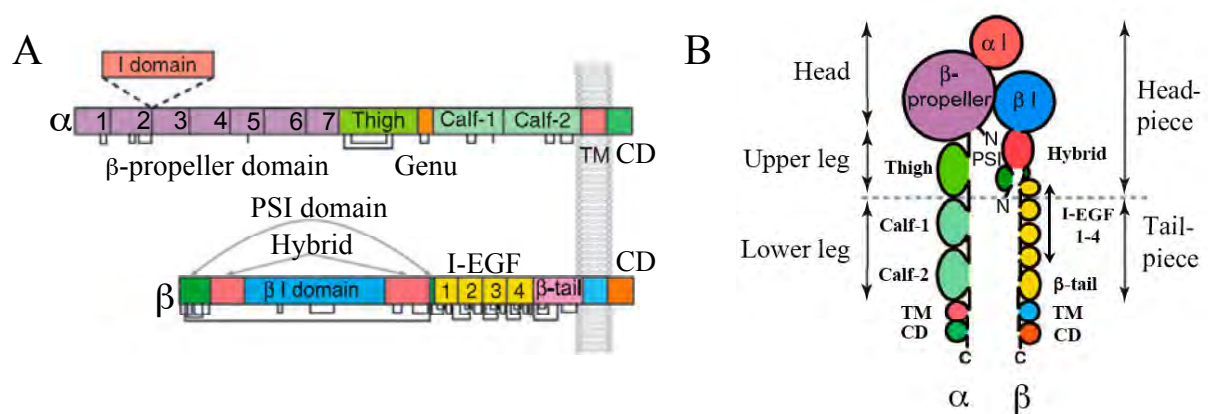
Figure 2. Schematic structure of an αI-domain integrin. (A) Schematic representation of integrin α- and β-subunits. (B) Schematic illustration of the α- and β-subunit domain arrangement from N- to C-terminal ends. Each domain color is the same as in figure A. TM refers to transmembrane domain and CD refers to cytoplasmic domain. Figure is adopted and modified from Luo et al., 2007 [25].

1.2.3. The collagen-binding integrins

Integrins can be grouped in subfamilies on the basis of their β-subunit content, characteristics of their α-subunit or based on the ligands that they bind. All four collagen-binding integrins α1β1, α2β1, α10β1 and α11β1 belong to the subfamily of β1-subunit containing integrins [26, 27]. Collagen-binding integrins bind native collagen via their αI-domains which recognize a GFOGER motif [28-30] or similar sequences, depending on the collagen type [31]. Studies of collagen-binding integrins have shown that they are involved in cell adhesion, cell migration, remodeling of collagen lattices and regulation of collagen synthesis. These receptors can also affect cell proliferation, cell differentiation, angiogenesis, platelets adhesion and aggregation and endothelial tubulogenesis [26]. The α3β1 integrin initially was classified as a collagen receptor but further studies showed it was mainly a laminin-332 receptor [32, 33]. Later, it was demonstrated that α3β1 could regulate α2β1 activity through receptor cross-talk [34].

Integrin α1β1 was initially identified on a subpopulation of cultured activated T-cells [35, 36]. A characteristic feature of the α1 chain is a 20 amino acid insertion of unknown function, which is positioned at the beginning of 6th β-propeller blade [37]. The α1 chain contains a short cytoplasmic tail of 9 amino acids, the shortest of all integrins [26]. Experiments with transfected cells or isolated αI-domains showed that integrin α1β1 is a receptor for collagen I, collagen IV and laminins [38, 39]. α1β1 binds the network-forming collagen IV with higher affinity compared to the affinity to fibrillar collagen I [40]. Other ligands are matrilin-1 [41] and collagen XIII [42]. Integrin α1β1 is predominantly found in mesenchymally-derived tissues and is highly expressed *in vivo* on certain cell types such as capillary endothelial cells and smooth muscle cells [43]. Mice lacking α1 integrin subunit are viable. *In vitro* analysis of α1-deficient mouse embryonic fibroblasts (MEFs) revealed reduced proliferation rate of these cells compared to control MEFs when cells are plated on collagen I or mixture of collagens I and IV [44]. A characteristic feature of the dermis of α1-*null* animals is increased levels of collagen synthesis, but the dermal thickness is not increased, due to increased collagenase expression [45].

Another collagen-binding integrin is α2β1. Similarly to α1β1 integrin, α2β1 was identified on activated T-cells *in vitro* [36]. α2β1 has been identified on a number of cells such as platelets, fibroblasts, Schwann cells of glia and on neuroglia, endothelial and epithelial cells [46]. The α2β1 integrin is a receptor for most of mammalian collagens and it was shown in certain

studies that it binds monomeric forms of collagen I, collagen II, collagen III, collagen V [47] and collagen XI [48]. Other collagens recognized by the α2β1 integrin include collagen IV [49], collagen IX [50], collagen XVI [51], collagen X [52] and collagen VII [47]. This receptor displays the highest binding affinity to fibrillar collagens [49] over lattice-forming collagens and it has been reported to bind to fibrils of collagens I-III [31, 53]. The α2β1 integrin can also recognize some forms of laminins [39]. It is involved in generation of mechanical forces and mediates collagen gel contraction [54-56]. α2β1 was also shown to participate in collagen fibrillogenesis [57, 58] and cell cycle progression [59, 60]. α2-deficient mice display no obvious defects during development and are fertile, however, they display diminished mammary gland branching [61] and a reduced response of platelets to collagen I [61, 62]. Upon vascular injury in α2-deficient mice, bleeding time is prolonged but no spontaneous bleeding has been observed [63]. A lack of the α2-subunit has also been reported to reduce the growth of thrombi after endothelial injury [64]. Finally, during excisional wound healing, the absence of the α2-subunit leads to enhanced neoangiogenesis while re-epithelialization remains normal whereas wound tensile strain is reduced [65, 66].

Integrin α10β1 was originally identified as a collagen II-binding integrin on chondrocytes [67]. Immunohistochemical analysis of embryonic murine tissues has shown that the α10-subunit is detected in collagen II-expressing tissues. It was found mainly on chondrocytes in the cartilage of joints, vertebral column, trachea and the cartilage supporting the bronchi. Furthermore, α10β1 was detected on fibroblasts in specialized fibrous tissues such as tendons, fascia of skeletal muscle or heart valves [68]. Studies using recombinant α10 I-domain showed that it recognizes collagens I-VI, laminin-111 [53] and collagen IX [50]. Similarly to the α1 I-domain, the α10 I-domain prefers network-forming collagens to the fibrillar collagens [53]. The expression of α10-subunit is turned on during the initiation of chondrogenesis and is highly expressed during the different development changes suggesting that α10β1 is important during cartilage development. Surprisingly, α10-deficient mice display only a mild cartilage phenotype [69].

1.2.4. Integrin α11β1

Integrin α11β1 is the last addition to the integrin family and is most structurally related to integrin α10β1, but functionally it displays different collagen-binding specificity. The integrin α11-subunit associates only with the β1-subunit [70].

1.2.4.1. Identification of the α11β1 integrin

α11 integrin chain was initially detected as an extra protein band in immunoprecipitation experiments from differentiated cultures of human fetal myoblasts [71]. Cloning of α11 using human fetal muscle cDNA and uterus cDNA libraries revealed a typical I-domain integrin with the mature chain consisting of 1167 amino acids migrating as a 145 kDa band in SDS-PAGE under non-reducing conditions. α11 is the longest integrin α-subunit identified [70]. The extracellular domain contains seven FG-GAP repeats in the N-terminal end with an inserted I-domain between repeats 2 and 3. The α11 I-domain consists of 195 amino acids and includes a conserved MIDAS motif. A characteristic feature distinguishing α11 integrin chain from other integrin α-subunits is the presence of a 22 amino acid insert in the calf-1 domain in the extracellular stalk region, at amino acids 804-826. The 23 amino acid long transmembrane region (amino acids 1142-1164) is followed by a cytoplasmic tail of 24 amino acids. Similarly to the α10 subunit, the α11 subunit lacks conserved GFFKR sequence and instead contains the sequence GFFRS [70]. In the human α10 subunit the corresponding sequence is GFFAH [72]. A comparison of the α11 chain with other collagen-binding integrin subunits showed that the α11 subunit displayed 42% sequence identity to the α10 subunit, followed by 37% identity to the α1 subunit and 35% identity to the α2 subunit [70]. Regarding comparison to non I-domain containing integrins, α4 and α9 subunits show the highest sequence identity to α11. Human α11 has 86% identity to mouse α11 at the nucleotide level and 89% identity on the protein level [30].

1.2.4.2. α11 integrin gene and α11 promoter region

The human α11 gene (*ITGA11*) and the mouse α11 gene (*Itga11*) have been mapped to chromosomes 15q23 and 9, respectively [70, 73]. No polymorphisms or mutations related to diseases have been mapped to the integrin α11 gene so far. The *ITGA11* gene covers 130 kb of genomic sequence and the complete *ITGA11* is assembled of 30 exons and 29 introns **(Figure 3).** The *ITGA11* transcription start site (TSS) was mapped 30 nucleotides upstream of the translation start site. Gene analysis *in silico* suggested several potential splice variants, which have not been validated at the RNA level yet [74]. The *ITGA11* transcript is composed of a 30-nucleotide (nt) 5' untranslated region, a 3564-nucleotide open reading frame, and a 329-nucleotide 3' noncoding sequence including the polyA tail.
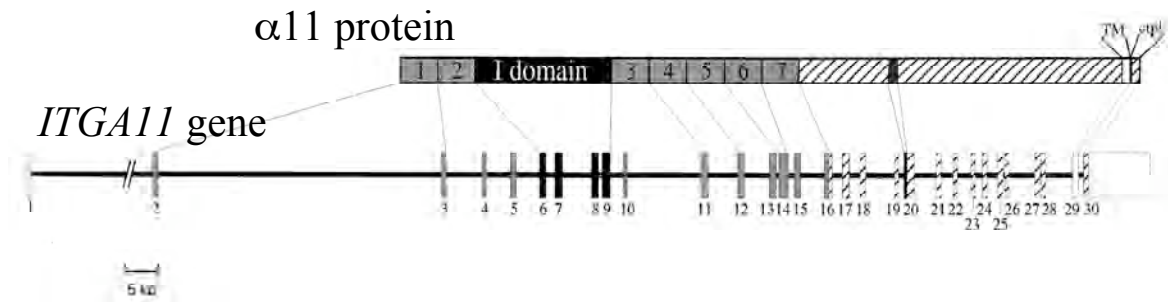
Figure 3. Schematic structure of integrin α11 protein and *ITGA11* gene. In the protein representation, the 7 FG-GAP repeats (1-7), the transmembrane part (TM) and the cytoplasmic tail (ctpl) are marked. In the gene, exonic sequences representing untranslated regions are open boxes and the unknown size in intron 1 is marked with //. The figure is adopted and modified from Zhang et al., 2002 [74].

In the *ITGA11* promoter studies, a 3kb-long sequence of 5'-flanking region (nt -2962/+25, +1 refers to the TSS) of the *ITGA11* has been cloned by genomic polymerase chain reaction (PCR). Putative binding sites for a number of transcription factors including Sp1 sites were identified within the construct. Many of these binding sites were predicted within 1.5 kb upstream of the transcription start site [74]. For further analysis of promoter activity three additional promoter regions (nt -1519/+25, nt -400/+25 and nt -127/+25) were cloned into Luciferase reporter vectors. A region covering nt -127/+25 has been shown to have core promoter activity [74]. The *ITGA11* promoter lacks TATA- and CCAAG-boxes in proximal promoter region, which is typical for the majority of integrin promoters [75]. Instead, it contains tandem Sp1-binding sites (SBS) and an ETS-binding site (EBS). Two SBS were located within nt regions -140/-134 and -122/-116, whereas the EBS was identified within nt-113/-110 [76]. From the collagen-binding integrins subfamily *ITGA2* and *ITGA10* are also regulated by Sp1 or Ets-family members [77-80]. The work with *ITGA11* has shown that the basal promoter is regulated by Sp1/Sp3/Ets-1 binding sites [76].

1.2.4.3. *ITGA11* regulation

Recent studies have demonstrated that the *ITGA11* proximal promoter is differently active within α11-expressing and non-expressing cells, which suggests that this specific part of the promoter decides whether α11 integrin will, or will not, be expressed by certain cell types. SBS and EBS within the proximal promoter are involved in the regulation of transcription of

α11 integrin subunit in α11-expressing cells, such as HT1080 (fibrosarcoma cell line) and MEFs [76].

1.2.4.4. Expression and ligands of the α11β1 integrin

α11β1 was initially detected as a major integrin in cultured skeletal muscle cells [71]. α11 is up-regulated on mRNA and protein level during myogenic differentiation of human myoblast cultures *in vitro*. Analysis of adult human tissues revealed wide expression pattern of α11 mRNA. The highest expression levels were detected in uterus, heart and skeletal muscle [70]. Based on these data, it was initially suggested that α11 would be expressed on muscle cells *in vivo*. However, analysis of α11 mRNA and protein expression and distribution in human [81] and mouse embryos [82] revealed a restricted expression on mesenchymal non-muscle cells in areas of highly organized interstitial collagen networks. Strong expression was detected in areas adjacent to forming cartilage. α11 protein was found in ectomesenchyme in the head, in periosteum around ribs, around vertebrae and tendons. High expression of α11 was also detected in intervertebral discs and in keratocytes of embryonic cornea where collagens are well organized in precise bundles and in multilayer arrangement [83]. No expression of α11 was detected in muscle cells. In general, α11β1 integrin is expressed in mesenchymally-derived cells *in vitro,* and *in vivo* on fibroblasts at sites of highly organized collagen structures.

1.2.4.5. α11β1 integrin function

In order to study the specific properties of α11β1 in a cellular context, *in vitro* studies in α11-[81, 84], and α11-EGFP (unpublished) transfected C2C12 cells (mouse myoblast cell line) lacking endogenous collagen-binding integrins were performed. Cell attachment assays showed that α11β1 integrin preferred collagen I to collagen IV [81] and studies of α11 I-domain binding to collagens have confirmed these finding [30]. The α11 also binds recombinant bacterial Scl proteins harboring a GLPGER motif [84]. C2C12 cells transfected with α2β1 or α11β1 integrins are able to contract collagen lattices [81]. This ability is likely to be important *in vivo* and contribute to collagen reorganization in pathological processes. PDGF-BB and serum stimulate collagen-dependent chemotaxis of α11-over-expressing C2C12 cells [81].

α11β1 integrin function *in vivo* and an α11-deficient mouse model is described in details in **Paper I**.

2.   Matrix metalloproteinases

There are 24 MMPs identified in mice. None of the MMP-deficient mice are embryonic lethal, which indicates enzymatic redundancy, enzymatic compensation or adaptive development [9]. *In vitro* studies have demonstrated that MMPs have multiple overlapping substrates [85].

2.1. The matrix metalloproteinase family

The MMPs belong to the metzincin group of proteases named after the zinc ion and the conserved methionine residue at the active site [86]. Mammalian MMPs share a conserved domain structure **(Figure 4)**.
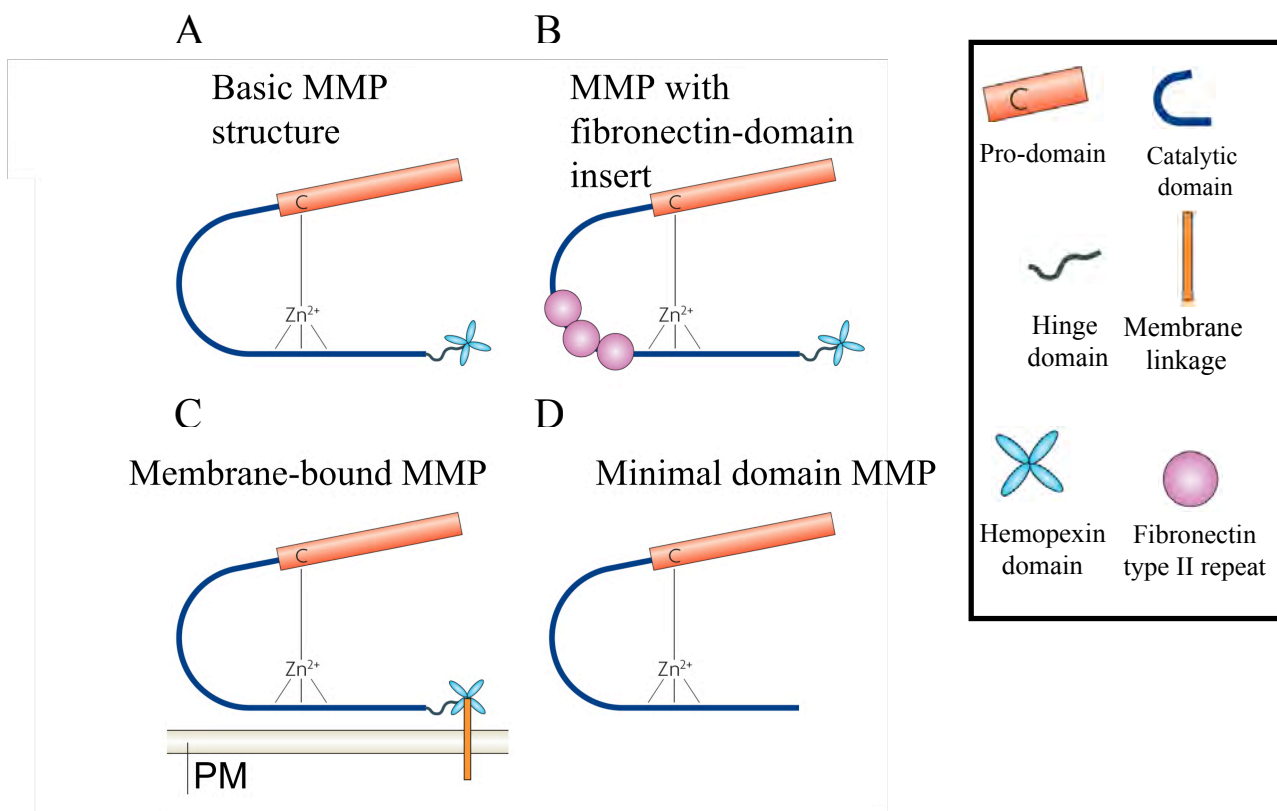
Figure 4. Schematic structure of matrix metalloproteinases. (A) Most MMPs have a conserved domain structure of a pro-domain, a catalytic domain, a hinge region and a hemopexin domain. (B) MMPs with fibronectin type II repeats. (C) Membrane type MMPs (MT-MMPs) are inserted in the plasma membrane. (D) Minimal MMPs lack the hinge and hemopexin domains. PM refers to plasma membrane. Figure is adopted and modified from Page-McCaw et al., 2007 [9].

In general, they consist of a catalytic domain containing a zinc-binding site with three highly conserved histidine residues, and an autoinhibitory pro-domain with conserved cysteine residue that coordinates the active-site zinc inhibiting catalysis [87]. Most of the MMPs contain at their C-termini a hemopexin domain attached by a flexible hinge. MMPs can degrade numerous

substrates at neutral pH [86]. Destabilization or removal of pro-domain allows the active site to cleave substrates. The hemopexin domain contains four-bladed β-propeller structure mediating protein-protein interactions contributing to enzyme activation, proper substrate recognition, and substrate degradation [9].

The MMP-mediated cleavage of ECM components generates fragments of different biological activities from their precursors with the ability to regulate tissue architecture through effects on the ECM and intracellular junctions. For example, MMP-mediated cleavage of ECM substrates removes physical barriers for cell migration. *In vitro* studies have shown that degradation of collagen I by MMP-1 is necessary for keratinocyte migration and wound healing [88] and that cleavage of collagen IV results in the exposure of cryptic sites which in turn promotes cell migration [89]. MMPs can also activate, deactivate or modify the activity of other proteinases, proteinase inhibitors, latent growth factors, chemotactic molecules, growth factor-binding proteins, cell surface receptors and cell-cell adhesion molecules [90]. These proteinases are up-regulated in diverse human diseases such as cancer and rheumatoid arthritis [9].

The MMPs are controlled at a number of steps including synthesis and secretion, activation of their pro-enzymatic forms, inhibition of already active forms and their clearance [86]. MMPs with furin recognition sequence are activated in the Golgi and secreted as active enzymes. MMP-14, one of the membrane-bound MMPs, is activated in this manner and can further activate pro-MMP-2 and pro-MMP-13 [86, 91]. MMP-14 deficient mice display a severe phenotype including craniofacial dysmorphism, dwarfism, retardation of postnatal growth, arthritis and death by 3-12 weeks of age due to inadequate collagen turnover in connective tissues [92]. Explant cultures derived from lungs and submandibular glands of MMP-14-*null* mice displayed reduced pro-MMP-2 activation [93, 94]. These findings indicate a central role for MMP-14 in regulation of a number of events involving specific cleavage and ECM reorganization. More recent studies have shown that activation of pro-MMP-2 requires dimerization of MMP-14 [95].

Tissue inhibitors of metalloproteinases (TIMPs) bind MMPs in a ratio of 1:1 and inhibit them [96]. All active MMPs can be non-specifically inhibited by α2-macroglobulin [97].

2.2. Matrix metalloproteinases cleaving native collagens

The MMPs with collagenolytic activity described in mammals are MMP-1 (collagenase -1), MMP-8 (collagenase-2) and MMP-13 (collagenase-3). MMP-1 and MMP-13 are synthesized by macrophages, fibroblasts and chondrocytes. MMP-8 is released predominantly from

neutrophiles and also from chondrocytes [86]. The degradation of collagen I requires specific collagenases because native, triple-helical molecules are resistant to cleavage by proteolytic enzymes at 37°C and neutral pH. The peptide bonds between residues Gly775 and Ile776 of the α1(I) chain and Gly775 and Leu776 of the α2(I) chain are the only sites in native collagen I molecules, which are known to be cleaved by collagenases. These cleavage sites are conserved in collagens from amphibians to mammals and are similar in collagens I-III [98, 99]. Cleavage of the collagens at this specific site by collagenases generates a three-quarter sized and a one-quarter sized helical fragments [100]. The collagenases show different specificity for different collagens. MMP-13 cleaves collagen II 10 times more efficiently than collagen I [101].

Other MMPs such as MMP-2 and MMP-14 can also cleave collagens [86, 102]. MMP-14 can degrade collagen I, II, III, laminins-111 and -332, fibronectin, vitronectin, fibrin and aggrecan [103]. The ability of MMPs to cleave the ECM components modulates focal adhesion stability and promotes cell migration and invasion [104-106].

2.2.1. MMP-13

MMP-13 (collagenase-3) was originally cloned from a human breast cancer cDNA library [107]. MMP-13, similarly to other MMPs is produced in the form of a pro-enzyme and needs to be cleaved to gain activity [9]. MMP-13 has wide substrate specificity but very restricted tissue expression [108]. Usually MMP-13 expression is limited to tissues with rapid remodeling of the ECM such as fetal bone. Elevated levels of MMP-13 have been identified in pathological conditions characterized by excessive ECM degradation such as rheumatoid arthritis, chronic cutaneous ulcers, malignant tumors and periodontal disease [9, 107, 109-112]. Over-expression of MMP-13 in human skin fibroblasts have demonstrated a role of MMP-13 in promoting survival and proliferation of cells and in activation of Akt and ERK-1/2 signaling in floating 3D collagen gels [113]. These findings suggest the existence of MMP-13-mediated survival mechanism for fibroblasts. A number of studies have focused on searching for mechanisms regulating MMP-13 expression. MMP-13 was shown to be up-regulated in human chondrocytes by IL-1β and TNFα [114]. IL-1β- and TNF-α-mediated MMP-13 induction requires activation of p38 MAPKinase in human chondrocytes [115, 116]. In contrast to these results, studies with mouse periodontal ligament (PDL) fibroblasts revealed that p38 MAPKinase negatively regulates IL-1β- and TNF-α-induced MMP-13 expression on both mRNA and protein level [117]. These finding suggest cell type-dependent regulatory

mechanism for MMP-13 expression. Another growth factor regulating MMP-13 expression is TGF-β. TGF-β-mediated induction of MMP-13 has been studied in several human cell types and the studies have revealed that induction occurs via activation of Smad and p38 pathways [118-120]. MMP-13 expression can be also regulated by other factors such as mechanical tension. It has been shown that in rheumatoid arthritis synovial cells MMP-13 levels were transiently decreased by mechanical stress [121]. The signaling pathways by which mechanical stimuli regulate MMP-13 expression are still unclear. *In vitro* studies indicated that MMP-13 expression is regulated when cells are embedded in 3D collagen lattices. Human skin fibroblasts or MC615 mouse chondrocytic cells placed inside 3D collagen gels up-regulate MMP-13 and α1β1 and α2β1 integrins. The observed up-regulation is the result of activation of two different signaling pathways (p38 in skin fibroblasts and ERK in MC615 cells) [122, 123]. *In vivo* studies, using orthodontic tooth movement model in rats, showed increased expression of MMP-13 on both compression and tension sides occurring very early following the application of a force in the PDL and alveolar bone [124, 125].

MMP-13-deficient mice show abnormal skeletal development with dwarfism [126]. The observed phenotype is similar to deformities seen in patients with spondylo-meta-epiphyseal dysplasia-Missouri type, which is a result of a missence mutation in the MMP-13 gene [86, 127]. Furthermore, MMP-13 knockout mice show an expansion of the hypertrophic chondrocyte zone and delay of apoptosis, which suggests that MMP-13 is needed for the transition from cartilage to bone at the growth plates of long bones [126]. The primary defect in MMP-13 deficient mice is the failure of chondrocytes to remodel the ECM rich in collagen II and aggrecan [126]. Another function for MMP-13 in long bone development occurs during ossification process. During this process, the cartilage ECM serves as a scaffold for mineralization and forms spicules or trabeculae. In MMP-13-deficient mice spicules of irregular shapes, were observed, indicating roles for MMP-13 in the initial remodeling. The abnormal increase in trabecular bone mass retained into adulthood in MMP-13-deficient mice, suggests a role for MMP-13 in bone remodeling.

2.3. Integrin-ECM-MMP interactions
Components of the ECM are substrate of MMPs, but the interactions of MMPs with the ECM is complex and includes influence of the 3D organization of the matrix on integrin-regulated MMP activity and synthesis.

2.3.1. Directed proteolysis via interactions with integrins

Several studies have demonstrated that integrins and MMPs interact directly and co-localize in different cell types. It has been shown that α2β1 co-localizes with MMP-1 on migrating keratinocytes via interaction between α2 I-domain and linker-hemopexin domains of MMP-1 [128]. Pro-MMP2 was reported to bind to α2β1 via fibronectin type II modules in the MMP [129]. It has also been shown that MMP-2 plays role in astrocyte motility and that MMP-2 and integrin β1 partially co-localize at the periphery of astrocyte. The MMP-2-β1 integrin interaction could thus act as a linker between pericellular proteolysis and the actin cytoskeleton [130].

In vitro studies with endothelial cells have demonstrated that integrin clustering also influences co-localization of MMP-14 to aggregate with integrin complexes, which supports a role for integrin-mediated redistribution of active enzyme to sites of cell-ECM contact [131]. It has been shown that MMP-14 co-localizes with β1 and αvβ3 integrins on human endothelial cells and participates in migration of cells on different ECM proteins in 2D [131].

2.3.2. Integrin-mediated synthesis of MMPs

The regulation of MMP synthesis by integrins is well documented. The collagen-binding integrin α1β1 has been shown to regulate MMP-7 and MMP-9 levels in mouse models of tumor growth [132], whereas α2β1 has been demonstrated to regulate MMP-1 and MMP-13 in normal human skin fibroblasts [55, 122] and in human osteosarcoma cells [133].

Skin explants from α1-deficient mice display increased activity of MMP-13 [45], MMP-2, MMP-7 and MMP-9 [132]. mRNA expression of MMP-2, MMP-9 and MMP-14 is significantly increased in glomeruli and cultured mesangial cells from α1-deficient mice and increased expression can be abrogated in these cells by blocking the activation of p38. Furthermore, increased activity of MMP-2 and MMP-9 has been observed in cultured α1-deficient mesangial cells [134]. α2-deficient keratinocytes showed elevated expression of MMP-2, MMP-3, MMP-8, MMP-9, MMP-13 and MMP-14 mRNA [65].

2.4. The MMPs during collagen remodeling

MMPs have can mediate collagen matrices remodeling. MMP-13 has been shown to enhance the contraction of 3D free-floating collagen gels by human skin fibroblasts [113]. During cancer cell migration through 3D collagen gel, the MMP-14-mediated collagenolysis in cooperation with β1 integrins was found to be crucial [135]. However, in the same studies,

blocking of the collagenolytic activity did not prevent cell migration of cancer cells in the low and high-density collagen gels. The results suggested protease-independent cell invasion, where the physical forces displace matrix fibrils and cells adopt amoeboid-like cell shape [135]. These studies have been performed using pepsin-extracted collagen I which is lacking non-helical telopeptides supporting cross-linking necessary for stabilization of collagen gel architecture. More recently, it has been shown that MMP-14 supports invasion of cancer cells within 3D collagen gels, prepared using telopeptide-intact collagen I, and within the stromal environment of the mammary gland [136]. The protease-independent mechanism of cell migration was not observed in these studies. This finding suggests that previously described protease-independent amoeboid activity might be the result of impaired structural integrity of collagen gels prepared from pepsin-extracted collagen I.

Recent studies have shown that not only the production of diffusible factors by tumor associated fibroblasts [137, 138] but also the physical matrix remodeling by these fibroblasts is indispensable for the invasion of carcinoma cells that retain their epithelial phenotype. Thus, the combination of proteolytic activity and force-mediated matrix remodeling by stromal fibroblasts is important to generate tracks through the matrix [139].

3. The periodontal ligament

3.1. The composition and the cells of the periodontal tissues

The periodontium can be considered as an organ composed of hard tissues (cementum and alveolar bone) and soft tissues (gingiva and PDL) **(Figure 5)**. The ECM of these tissues contains fibrous and non-fibrous elements such as collagens, fibronectin, elastin, laminins, osteopontin, bone sialoprotein (BSP), different growth factors, proteoglycans, lipids and minerals [140].

The PDL is the only ligament that connects two distinct hard tissues. It is a fibrous, complex, soft connective tissue, attaching the tooth root to the inner wall of alveolar bone. The width of the PDL in human ranges from 0.15 to 0.38 mm with the thinnest part around the middle third of the root. The PDL thickness decreases with age. It is functionally important for tooth support, and for allowing the teeth to withstand the forces generated during mastication.

Another important function is regulation of alveolar bone volume and serving as a cell reservoir for tissue homeostasis and regeneration [141]. The PDL also acts as a sensory organ necessary for the proper positioning of the jaws during mastication. The PDL has very high adaptability to rapid changes in applied forces and capacity to maintain its width [142]. This ability is an important measure of PDL homeostasis. The alveolar bone is a specialized bony

structure that supports the teeth and is constantly remodeled in response to tooth micromovements generated during mastication. Cementum is mineralized tissue limited to the surface of the root [143].
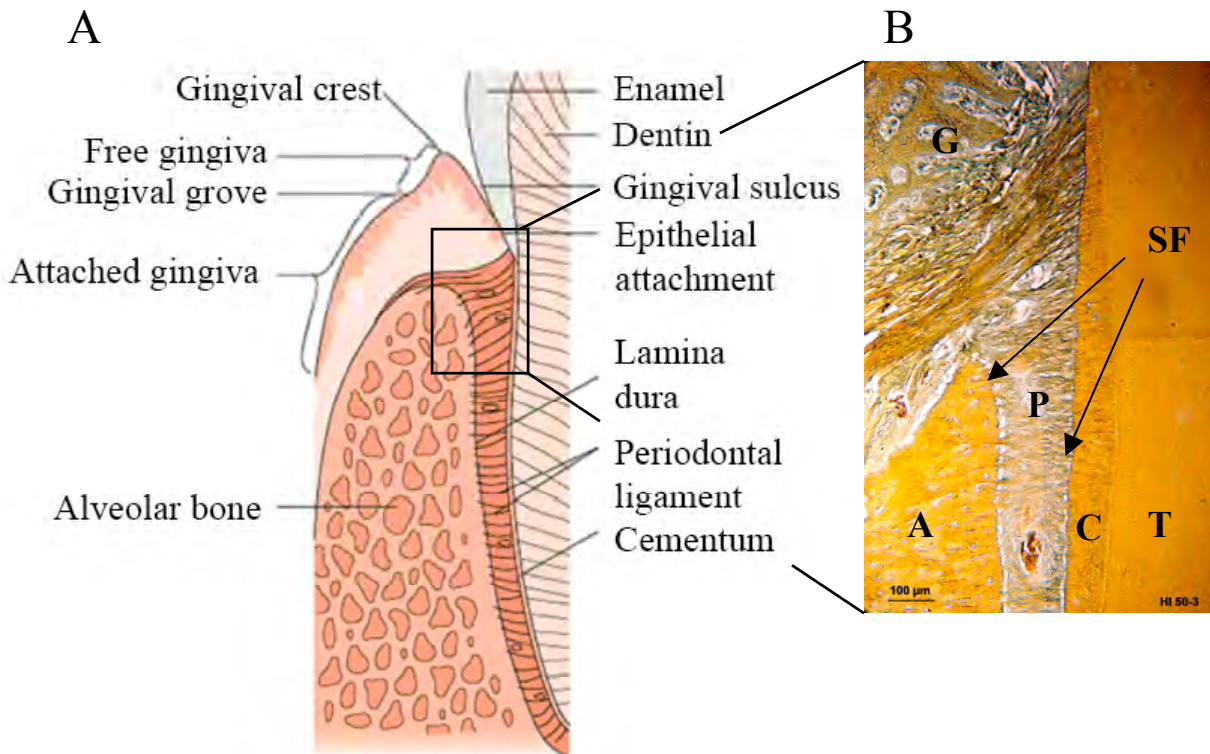
Figure 5. The periodontium. (A) Schematic illustration of the periodontium. Adopted and modified from Lindhe, 2003 [144]. (B) Histological section of the periodontium. A-alveolar bone, C-cementum, G-gingiva, P-peridontal ligament, SF-Sharpey's fibers, T-tooth. Courtesy of Knut A. Selvig.

The fibroblasts are the dominant cell population in the PDL. Other cells include osteoblasts, osteoclasts, epithelial cell rests of Malassez, monocytes, macrophages, cementoblasts, odontoblasts and progenitor cells.

The presence of the *stem cells* within the PDL has been reported in different species [145, 146]. The PDL stem cells are located closely to blood vessels and exhibit some of the typical cytological features of the stem cells, such as small size and responsiveness to stimulating factors. The stem cells isolated from adult human and sheep PDL have characteristics of *adult mesenchymal stem cells* and are a population distinct from bone marrow-derived mesenchymal stem cells [147]. The *PDL stem cells* originate from the *ectomesenchymal cranial neural crest cells* [147]. These progenitor cells can differentiate into cementoblasts, osteoblasts and PDL fibroblasts **(Figure 6)** [148].

The human PDL stem cells from adult PDL show the capacity to generate clonogenic adherent cell colonies [146] and express the stem cell marker STRO-1 similarly to bone marrow stromal stem cells [147]. The PDL stem cells also express mineralized tissue markers such as collagen
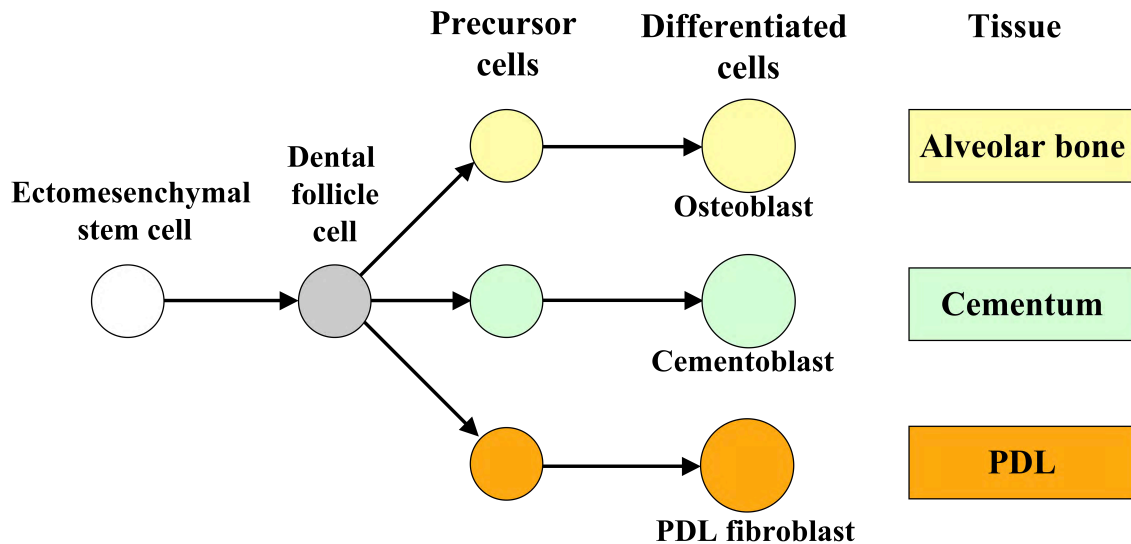
Figure 6. Cellular differentiation in the periodontal tissues.

I, collagen III, alkaline phosphatase (ALP), osteopontin (OP), osteocalcin (OC) and bone sialoprotein (BSP), and have capacity to form mineralized noduli *in vitro* under differentiation conditions.

Similarly to the PDL stem cells, the ***PDL fibroblasts*** originate from the ***ectomesenchyme***. The PDL fibroblasts are characterized by a high rate of collagen turnover in ECM [149], which occurs by simultaneous synthesis and degradation of collagen fibrils. The PDL fibroblasts are aligned along the general direction of the fiber bundles. They are large cells with a vast number of organelles associated with protein synthesis and secretion. The fibroblasts in the PDL are a heterogeneous population [150] with capacity to differentiate, depending on local microenvironment, into cementoblasts and osteoblasts [142, 151]. No PDL-specific marker is available, but due to common origin to cementoblasts and osteoblasts of the PDL, the osteoblast phenotype-related genes can be used as markers for identification of the PDL fibroblasts. The master regulatory transcription factor RunX2 is expressed by these cells, together with other genes encoding collagen I, ALP, OP and OC. The epithelial cell rests of Malassez are remnants from Hertwig's epithelial root sheath (HERS) and they are found close to cementum in the form of clusters. The exact function of these cells is not known but their

possible role in regeneration is suggested [152]. Cementoblasts and osteoblasts contribute to the production of cementum and the remodeling of alveolar bone, respectively.

The elements of gingival connective tissue originate from oral mucosa connective tissue. The **gingival fibroblasts** are of **mesenchymal** origin and are important for development, reorganization and regeneration of gingival connective tissue [153].

The ECM of the PDL contains collagenous and non-collagenous fibers. The majority of collagens in the PDL is organized in fiber bundles and these bundles are called principal fibers. The extremities of collagen fiber bundles are embedded in the alveolar bone and cementum of the tooth and form mineralized Sharpey's fibers. Individual fibrils are continuously remodeled by fibroblasts while the overall fibers mesh maintains its structure and function. Other collagens, except for collagen I and III, found in the PDL are collagens V, VI, XII, and XIV [154, 155]. Elastic oxytalan fibers were identified within PDL and described as a 3D meshwork surrounding the root and terminating in the apical complex of arteries, veins and lymphatic vessels. It is believed that oxytalan fibers regulate vascular flow to the tooth. A vast number of non-collagenous proteins including proteoglycans [156], tenascin-C (in attachment zones along cementum and bone), fibronectin [157], vitronectin (found on collagen fibrils), elastin [140] have been detected in human PDL. A cell-bound ALP have been identified in the incisor PDL of a rat [158].

3.2. Integrin-ECM interactions in the PDL

The regulation of the reorganization of ECM and the role of different cell types in the PDL is still poorly understood on the molecular level. The PDL fibroblasts interact with the ECM via integrins binding different collagenous and non-collagenous substrates. Integrins interacting with the ECM extracelullarly and with cytoskeletal components intracellularly are considered to be force transducing elements in fibroblasts [159].

A number of studies have characterized the integrin repertoire on molar PDL fibroblasts under different culture conditions. Cultured human PDL fibroblasts express mRNA encoding $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, αv, $\beta 1$, $\beta 3$ and $\beta 4$ integrin subunits. The same cells upon mechanical stimulation showed increased expression of $\alpha 6$ and $\beta 1$ integrins and decreased expression of $\alpha 5$ subunit [160]. Another studies by Palaiologou et al. have shown in human molar PDL fibroblasts the presence of mRNA for integrin chains $\alpha 7$, $\alpha 8$, $\alpha 10$, $\beta 5$, $\beta 6$ and $\beta 8$ but not $\alpha 11$ [161].

A very few functional studies of integrin-ECM interactions in PDL derived cells have been conducted. It has been reported that PDL fibroblasts migrate on collagen I, collagen III and collagen V but also on fibronectin and laminins. α1β1 integrin and α2β1 integrin have been detected on protein level in these cells [162]. α2β1 and α11β1 integrins are also known to enhance polymerization of collagens I and III, which indicates a role in matrix assembly for these integrins [58]. This property might have implications for the PDL reorganization and regeneration. Both collagen I and collagen III are up-regulated during wound healing and regeneration [163, 164] and during orthodontic movement (collagen I, collagen III and collagen V) [165].

It has been shown that human molar PDL fibroblasts interact with collagen V via αvβ3 integrin, but possibly other RGD-recognizing integrins also can mediate interactions with collagen V [166]. αvβ3 integrin, in addition, regulates extracellular assembly of fibrilin-1, thereby modulating cell-mediated homeostasis of microfibrils. It is also suggested that oxytalan fibers, may be controlled by PDL fibroblasts [167].

Recently, it has been reported that αvβ6 integrin-mediated TGF-β1 activation in the junctional epithelium plays protective role in inflammatory periodontal disease. β6-deficient mice develop classic symptoms of chronic periodontal disease. In a rat model, αvβ6 blocking led to appearance of initial signs of periodontitis. αvβ6 integrin is also down-regulated in human periodontal disease [168].

3.3. Matrix metalloproteinases in the PDL

Tooth eruption involves bone and PDL reorganization [169]. This process requires degradation and reorganization of ECM components and MMPs play a central role in this event [9]. It has been demonstrated that MMP-2 is expressed constitutively *in vitro* and *in vivo* by human PDL fibroblasts [170, 171]. In experiments where media were collected from free-floating collagen gels containing human PDL fibroblasts, an increased collagenase activity was noted and the collagen content within collagen lattices was reduced. Active MMP-2 and pro-MMP9 were also detected in the conditioned media. In attached collagen gels such changes were not observed until gels were detached and tension released, suggesting that tension relaxation enhances collagenolytic activity [172].

The PDL fibroblasts normally express MMP-13 and MMP-13 expression in these cells can be stimulated by IL-1α [173] or TNF-α [174]. Studies with mouse molar PDL fibroblasts revealed that p38 MAPKinase negatively regulates IL-1β and TNF-α-induced MMP-13

expression on both mRNA and protein level which is in contrary to results obtained from human skin fibroblasts [117].

The PDL tissue is a reservoir of mineralized matrix-forming cells [175]. It has been shown in the osteoblastic MC3T3-E1 cells, that MMPs can regulate osteoblastic differentiation [176]. Studies with bacterial collagenases [177] added to cells, and induction of endogenous collagenases by IL-1β [178] showed decreased osteoblastic differentiation in MC3T3-E1 cells and human PDL fibroblasts, respectively. The exact mechanism for how this occurs is unknown, but most likely involves degradation of collagenous molecules needed for integrin-dependent differentiation signals [179-181]. Integrin-mediated adhesion to collagen has been shown to enhance expression of osteoblastic markers such as ALP, OC and BSP in bone marrow cells [182]. Increased MMP-dependent bioavialability of latent growth factors stored in the ECM might also be involved [183]. The MMP-dependent changes in osteoblastic differentiation are reflected in varying levels of differentiation markers and such correlation has been shown in human molar PDL fibroblasts. One of the characteristics of the periodontal disease are increased MMP levels [184], thus the cell differentiation in the PDL might be inhibited which might result in a decreased pool of cells capable of bone regeneration and replacement [185].

3.4. Periodontal disease

Periodontal disease is initiated by bacteria colonizing the dentogingival region of the tooth. The series of infections in the PDL tissue of a susceptible host may lead to soft and hard tissues destruction and loss of tooth attachment.

Gingivitis is a prerequisite for the periodontal disease development. Microorganisms forming the dental biofilm release the factors triggering host immune response and inflammation. Inflammatory factors, such as IL-1 or TGF-β which are released during immune response cause up-regulation and/or activation of MMPs produced by PDL fibroblasts, leading to breakdown and loss of the ECM of the PDL [186]. Such interference with the fibroblast function by periodontal disease results in destruction and finally, loss of tooth supporting tissue [187]. Interestingly, not all individuals diagnosed with gingivitis will develop periodontal disease. As already mentioned, the microorganisms are crucial for the initiation of the inflammatory periodontal disease but the progression of the disease is dependent on host-related risk factors such as genetic polymorphisms, systemic diseases and on environment-related factors such as smoking [188]. It is estimated that there are more than 600 different

bacteria species residing in the oral cavity. The shift of balance from commensal gram-positive bacteria to pathogenic gram-negative, proteolytic organisms has been associated with PDL tissue breakdown [189]. Several of the pathogenic bacteria forming dental plaque have been associated with periodontal disease. The disease is not triggered by one organism but is a result of mixed infections. *Aggregatibacter actinomycetemcomitans* has been associated with aggressive periodontitis while *Porphyromonas gingivalis*, *A. actinomycetemcomitans*, *Tannerella forsythia*, *Treponema denticola* and *Eikenella corrodens* have been associated with chronic periodontitis. The colonization of host tissues starts with adhesion of bacteria to them. *P. gingivalis* adhesion to host cells requires formation of fimbriae and fimbriae have been found to bind epithelial cells, fibronectin and fibrinogen. The fimbriae of *P. gingivalis* were reported to compete with the ECM proteins to bind over-expressed $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins in Chinese hamster ovary (CHO) cells. It is suggested that such properties might affect normal ECM proteins turnover and the ECM repair, thus adding to damage to the gingival tissue [190]. In *A. actinomycetemcomitans* outer membrane, a protein named extracellular matrix adhesion protein A (EmaA) was identified as a direct mediator of adhesion to collagen V [191]. The adhesion of *T. denticola* to fibronectin and collagen I is followed by the degradation of the ECM proteins [189]. Increased levels of cytokines and chemokines are observed during inflammation and immune response in periodontal disease. In *P. gingivalis,* type II fimbriae, predominant fimbrial phenotype associated with periodontal disease, have been shown to induce expression of IL-1β, IL-8, IL-12 and TNF-α in macrophages-like U937 cells. The lipopolysaccharide (LPS) of *P. gingivalis* has been shown to induce levels of prostaglandin E2 and IL-6 in human PDL fibroblasts. *A. actinomycetemcomitans* can stimulate gingival epithelial cells to express IL-8 and human PDL cells to express IL-6. *P. gingivalis* and *A. actinomycetemcomitans* can also induce bone-resorptive cytokines like TNF-α, IL-1β and IL-6 when injected subcutaneously [189].

Under normal conditions, the synthesis and degradation of connective tissue is tightly regulated and balanced. Disturbed balance between MMPs and their inhibitors resulting in connective tissue matrix breakdown is observed during periodontal disease. Highly increased expression of MMP-13 is observed in the PDL of patients diagnosed with periodontitis [184]. MMP-8 and MMP-9 are major collagenase and gelatinase, respectively, detected in gingival cervical fluid of chronic periodontitis patients while MMP-1 is a major collagenase found in aggressive periodontitis [189]. Low levels of TIMPs have been detected in gingival cervical fluid of chronic periodontal disease [192]. An activation of different latent MMPs by

periodontopathogenic bacteria has been demonstrated. MMP-1 from gingival fibroblasts and MMP-8 from polymorphonuclear leukocytes can be activated by *P. gingivalis* trypsin-like protease and *T. denticola* chemotrypsin-like protease [193]. MMP-1 and MMP-3 from gingival fibroblasts and MMP-9 from medium of HT1080 cells can be activated by *P. gingivalis* thiol-protease [194]. Periodontopathogenic proteases have also been reported to inactivate inhibitors of MMPs. *P. gingivalis* cysteine proteinase, periodontanin, can inactivate α1-protease inhibitor. In general, high levels of MMPs and decreased levels of TIMPs are characteristic for severe periodontal disease [195].

The host response to infectious pathogens depends on nature and virulence of pathogens, but also on genetic factors. Several genetic polymorphisms have been associated with periodontal disease. Most of research focused on possible polymorphisms of genes involved in inflammation and immune response. It has been observed that polymorphic genes regulating the production of IL-1 in response to bacterial LPS may have an impact on susceptibility of host to severe periodontitis. Polymorphisms within leukocyte Fc receptors, affecting affinity of the interaction with immunoglobulins, have been shown to associate with periodontitis severity. Low affinity receptors are more common in patients with more severe and rapidly progressing periodontal disease [196]. Certain chromosomal or genetic disorders such as Down's syndrome, leukocyte adhesion deficiency syndrome, Papillon-Lefévre syndrome and the Ehlers-Danlos syndrome have been reported as predisposing to periodontal disease. Systemic disorders such as diabetes can increase the risk of periodontitis [188]. Smoking is one of the environmental factors strongly predisposing to periodontal disease. The relationship between periodontitis and smoking appears to be dose-dependent. It has been shown that more severe loss of tooth attachment apparatus are observed in heavy smokers compared to light smokers. The length of exposure to tobacco products is also a significant factor. The prevalence for periodontal disease increases with age [188].

Aims of the present study

The overall aim of this study was to increase our understanding for the role of α11β1 integrin *in vivo* and *in vitro* using an α11-deficient mouse model and cells isolated from such animals. The potential role of α11 in human tooth supporting apparatus was also evaluated. The specific aims of the present studies were:

Paper I

- To analyze the α11-deficient mouse phenotype *in vivo*.
- To investigate the role of α11β1 integrin in cell attachment, cell spreading, cell proliferation and reorganization of 3D collagen lattices using MEFs isolated from α11-deficient animals.

Paper II

- To isolate incisor PDL fibroblasts.
- To characterize the repertoire of collagen-binding integrin in cultured mouse PDL fibroblasts.
- To identify molecular mechanisms underlying α11-deficient phenotype *in vitro,* which most likely has consequences for the phenotype observed *in vivo*.

Paper III

- To examine the repertoire of collagen-binding integrins in human PDL fibroblasts and human PDL tissue.
- To explore a possible contribution of α11β1 to reorganization of collagen gels by PDL fibroblasts.
- To investigate the α11 expression pattern in the PDL tissue of healthy and periodontally diseased individuals.
- To search for single nucleotide polymorphisms in the proximal promoter of *ITGA11*.

Materials and Methods

The experimental procedures and materials are described in Papers I-III.

Results

1. α11β1 integrin-dependent regulation of periodontal ligament function in the erupting mouse incisor (Paper I)

α1β1, α2β1, α10β1 and α11β1 integrins constitute the family of collagen-binding integrins, acting as primary receptors for native collagens. Data from *in vitro* studies of C2C12 cells expressing α11β1 as the only collagen-binding integrin have shown that α11β1 integrin prefers collagen I to collagen IV [81]. It was previously demonstrated that α11β1 is the only detectable collagen-binding integrin in the incisor PDL fibroblasts in moue [82]. The most abundant protein in the PDL is collagen I. In the **continuously erupting** rodent incisor, the PDL has been shown to play a central role during tooth eruption [197].

In Paper I we analyzed the phenotype of the α11-deficient mouse and characterized cells isolated from α11-deficient and α11-expressing mouse embryos.

1.1. Generation of an integrin α11-deficient mouse strain

The α11-deficient mouse was generated using gene targeting techniques. In order to introduce a null mutation in *Itga11*, parts of exon 3 and intron 3 were replaced with an internal ribosome entry site, a bacterial reporter LacZ, and PGK *neo* cassette. The targeting construct was introduced into R1 ES cells and colonies resistant to G418 were selected. A total of 325 clones were screened by Southern blotting and two clones (95 and 215) were selected for further work. These clones were injected into blastocyst of C57BL/6J mice. Obtained chimeric males were mated with C57BL/6J females, and the offspring were screened with Southern blotting for the presence of the targeted allele. Intercrossing of heterozygous F1 mice gave rise to live homozygous offspring with expected Mendelian ratios and no phenotypic defects at birth. Both homozygous males and females were fertile.

1.2. The skeletal system of the α11-deficient mice

When animals were 3 weeks old, it was noted that each litter contained mice smaller then their littermates. Genotyping showed that these smaller animals were homozygous mice and both males and females demonstrated 20-30% reduction in weight persisting through adulthood. X-rays showed that overall skeleton size was smaller and compatible with proportional dwarfism. To rule out possible effect from the neo cassette, flanked by loxP sites, the neo cassette was deleted by crossing with Cre-deleter mice. The described phenotype persisted after the neo cassette removal, confirming that the observed weight difference was due to inactivation of

α11 integrin alleles. The neo-deleted mice were used for further analysis. Since previous reports showed that α11 is highly expressed in perichondrium [81, 82], initial studies focused on analysis of skeletal system. However no structural defects of the forming cartilage or bone at 1-8 weeks were observed. No differences in chondrocyte proliferation between control and mutant mice were detected.

1.3. Tooth phenotype of α11-deficient mice

The α11-deficient mice displayed increased mortality starting form 1 year of age, and 10 such mice died between 12-19 months compared to only one control mouse. Necropsy of the α11-deficient mice revealed severe malnutrition, bringing attention to the digestive system. Even though α11 is co-expressed with α2 integrin in villus cluster fibroblasts, no defects were found in this area. In the older mice a incisor phenotype was clearly noticeable. Mutant mice showed delay in the time of incisor eruption and altered tooth shape. In the older animals the incisal part of the upper incisors was often missing, whereas intraalveolar part was still present. Analysis of tooth eruption showed that it was reduced at 3-6 weeks and was stopped at 6-7 months. The smaller size of animals and tooth phenotype were present in both strains of independently generated *Itga11*-deficient mice. Micro-CT of incisor showed increased thickness of dentin layers in the apical region resulting in the pulp closure.

Histological analysis of incisor PDL showed increased thickness due to increased amount of collagen as determined by Sirius red staining. Fibroblast density, but not the cell number, was reduced when compared with an even distribution in the control PDL. The acellular cementum was increased in thickness and increased number of cell rests of Malassez was a characteristic feature of the PDL in animals older than 6 months. Electron microscopy showed normal collagen fibrils and normal collagen network. The PDL of the molars did not display any abnormalities at any age with regard to tooth eruption or morphology. In order to examine whether defective incisors were the underlying cause of the proportional dwarfism, animals were fed a soft food diet. This regimen partially rescued the reduced weight phenotype.

1.4. *In situ* localization of α11 RNA and immunohistochemical analysis of α11 protein in the PDL

The expression of α11 RNA and protein was analyzed during embryonic day (E) 14-17 and postnatally. Immunohistochemistry detected α11 in the dental follicular mesenchyme that forms the PDL and in the preodontoblasts of developing molar and incisors. α1 and α2 integrin

proteins were detected only in capillaries in the PDL while α10 protein was completely lacking. α11 protein levels were significantly higher in adult incisor PDL compared to molar PDL. No compensatory regulation of collagen-binding integrins in molar or incisor PDL was observed when α11 was not detected in these young ages. Collagens III and XII and CD-31-positive cells were detected in both control and mutant incisor PDL but no differences in expression between mutant and control animals were observed. Due to denaturating conditions of the decalcification protocols, performance of *in situ* MMP activity assay was not possible but instead PCR analysis was performed. In semiquantitative PCR increased mRNA levels of MMP-9 and decreased levels of MMP-14 were observed in the α11-deficient incisor PDL tissue. Reduced levels of MMP-13 were also noted.

1.5. *In vitro* phenotype of α11-deficient mouse embryonic fibroblasts

The MEFs isolated from α11-deficient embryos showed significantly reduced adhesion to collagen I compared to control cells, whereas adhesion to collagen IV was changed only marginally. Adhesion to fibronectin was not affected by the absence of integrin α11. The proportion of α11-deficient cells that had spread on collagen I was reduced by 50% compared to control cells. No differences were observed in the case of spreading on fibronectin. Fewer vinculin-positive focal contacts were formed in α11-deficient MEFs seeded on collagen I. Mutant cells also displayed reduced capacity to reorganize collagen gels. Incubation of control cells with a mixture of α1 and α2 integrin-blocking antibodies did not alter their ability to contract collagen gels whereas α11-deficient cells reduced lattices to 60% of their original size. The simian virus 40 large T antigen (SV40)-immortalized α11-deficient MEFs retransfected with human α11 cDNA were able to contract collagen gels as efficiently as α11-expressing SV40 MEFs. Analysis of MMP expression and activity showed a reproducible decrease in MMP-13 and MMP-14 mRNA levels in mutant MEFs cultured in 3D collagen gels whereas the activities of MMP-2 and MMP-9 were not affected by the lack of α11 integrin.

2. α11β1 integrin-mediated collagen lattice contraction by incisor periodontal ligament fibroblasts requires MMP-13 (Paper II)

Results obtained from studies of α11-deficient mice **(Paper I)** allowed us to identify a specific population of cells affected by the lack of α11 integrin. The disturbed functions of mutant incisor PDL fibroblasts most probably have direct connection to the α11 phenotype.

2.1. Isolation of incisor PDL fibroblasts

To study the mechanism underlying molecular defect at cellular level incisor PDL fibroblasts were isolated from mutant and control animals. Due to the small amount of tissue, the attempts to establish cultures from pooled isolated incisor PDLs or from extracted incisors, failed. To overcome this problem α11-deficient mice were crossed with immortomice carrying SV40 large T under the control of the temperature-sensitive H-2Kb-tsA58 promoter [198] and cells were isolated by explant cultures from extracted incisors. Two control and two α11-deficient incisor PDL fibroblast isolates were established in this way **(Figure 7)**.

***Itga11* ko**   **H-2K^b^tsA58**   **α11^+/-^/tsA58^+/-^**   **α11^+/-^/tsA58^+/-^**

Tissue explant

α11 ^-/-^/ tsA58^+/+^
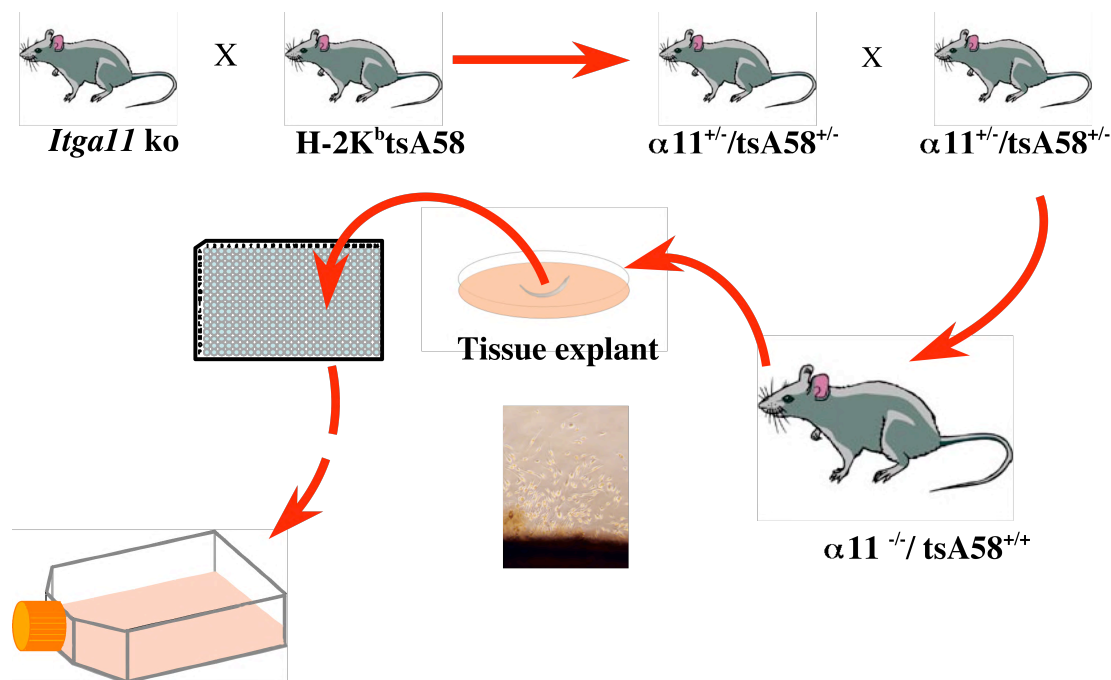
Figure 7. Isolation of immortalized incisor periodontal ligament fibroblasts.

2.2. Characterization of incisor PDL fibroblasts

Metabolic labeling and immunoprecipitation experiments with integrin antibodies revealed high levels of α11 integrin derived from control teeth and lack of protein in cell explants of α11-deficient PDL. α11 was not detected in a cloned molar PDL fibroblast cell line mPDL-L2 isolated in a similar manner from immortomouse molar PDL [199]. Incisor PDL fibroblasts *in vivo* exist in a 3D microenvironment. To mimic this condition, cells were placed in a 3D collagen gel. Incisor PDL fibroblasts in attached collagen gels, display increased levels of α11 protein at 48h demonstrating that mechanical strain supports high α11 integrin levels. α11 levels decreased in cells cultured in free-floating collagen lattices. A characteristic feature of

PDL fibroblasts is their ability to express osteogenic markers and form osteogenic noduli when induced with differentiation medium containing BMP-2 [199]. When α11-deficient and control cells were subjected to such treatment they showed similar capacity to differentiate and form noduli. Semiquantitative PCR revealed that cells upon differentiation treatment expressed collagen α2(I), collagen α1(XII) and periostin, which are known PDL fibroblast markers. Incisor PDL fibroblasts also expressed mRNA for osteogenic markers such as the transcription factor RunX2, OP (bone ECM protein) and low levels of OC (detected in only one mutant and one control isolate). Based on this finding we concluded that the isolated cells had properties of PDL fibroblasts.

2.3. Functional analysis of α11β1 integrin in incisor PDL fibroblasts

To examine the effect of α11-deficiency on the ability of incisor PDL fibroblasts to interact with collagen I, cells were tested in cell adhesion, cell migration and collagen gel contraction assays. In adhesion assays, a reduced ability of α11-deficient cells to attach to collagen I was observed. A combination of antibodies to α1 and α2 integrin reduced cell attachment of control cells by 50-60%. In order to evaluate the ability of the incisor PDL fibroblasts to remodel 3D collagen lattices, cells were incorporated into attached and free-floating gels. α11-deficient cells showed reduced ability to reorganize both types of matrices. In floating gels α11-deficient cells contracted gels only to 80% of the initial area whereas control cells contracted gels even down to 20% of their initial area. The need for α11 integrin in reorganization of collagen gels was verified in an experiment with siRNA to α11 mRNA. Reduced levels of α11 protein to approximately 40% in the α11-expressing cells, reduced the ability of cells to contract collagen gels by 20%. The effect of α11 expression on cell ability to migrate on collagen I was tested in chemotaxis assay. In absence of chemoattractant no migration was observed and presence of 10% serum only marginally stimulated cell migration. A very effective chemoattractant was PDGF-BB, which strongly stimulated migration of control cells, but migration of α11-deficient cells was strongly attenuated.

In summary, α11β1 integrin is a major collagen receptor in incisor PDL fibroblasts mediating cell adhesion, migration and collagen lattice remodeling.

2.4. α11β1 regulates MMP-13 levels inside a 3D collagen gel

It has been shown in human skin fibroblasts and human osteosarcoma cell lines that fibroblasts within a 3D collagen gel induce MMP levels in a collagen-binding integrin-dependent manner

[122, 133]. Analysis of MMPs mRNA levels indicated dysregulated MMP-13 and MMP-14 levels in α11 deficient MEFs **(Paper I)**.

To examine possible connection between α11 expression and regulation of MMP levels in incisor PDL fibroblasts, the cells were cultured in free-floating or attached gels and MMP protein levels and collagenase activity were tested. Western blot analysis of cells revealed that MMP-2 levels were strongly induced in floating and attached gels in an α11 integrin-independent manner. MMP-13 protein levels were induced inside of 3D collagen gels only when α11β1 integrin was expressed by the cells. No consistent changes were seen in MMP-9 and MMP-14 protein levels. Surprisingly, the analysis of conditioned media did not show increased collagenase activity in α11-expressing cells when placed inside 3D collagen gels. The possible explanation is that the collagenase partially might be trapped within the collagen gel, and hence is only partially detected in the media. A decreased collagenase activity in media collected from α11-deficient cells was observed in all conditions. In another type of assay, a clear reduction of collagenase activity in α11-deficient cells was shown in monolayers where cells were plated in a drop of collagen I and cultured for 48h. Cells were removed and to monitor collagense activity the remaining collagen was stained. α11-expressing cells produced clear zones in the area where the cells had been placed while α11-deficient cells showed only marginal effects on the collagen layer.

2.5. α11β1-mediated collagen reorganization in part depends on MMP-13

As already mentioned it has been shown before that fibroblast-mediated collagen gel contraction is dependent on MMP-13 [122]. To test if part of this activity is mediated by α11β1 integrin, the effects of a broad MMP inhibitor (GM6001) and MMP-13 specific inhibitor (CL-82198) were tested in collagen gel contraction assays. The contraction of collagen gels by α11-expressing cells was inhibited by 20% when GM6001 was used whereas CL-82198 reduced contraction somewhat less. These experiment were performed using pepsin-extracted collagen I. When collagen I containing non-helical telopeptides at N- and C-terminal ends, allowing the collagen cross-linking formation [200] was used, the effects of both inhibitors were even greater **(data not shown)**. Addition of exogenous MMP-13 to collagen gels containing α11-expressing incisor PDL fibroblasts increased the contraction of collagen gels by approximately 10%.

3. A role for $\alpha 11\beta 1$ integrin in the human periodontal ligament (Paper III)

Our earlier data demonstrated that $\alpha 11\beta 1$ integrin is indispensable for the PDL-driven tooth eruption in mouse **(Paper I)**. To explore a possible role of $\alpha 11\beta 1$ integrin in human periodontium, the expression of $\alpha 11$ in human PDL tissue, in cultured human PDL fibroblasts and in human gingival fibroblasts, was characterized. A report by Lallier et al. suggested that in addition to the integrin chains $\alpha 1$ and $\alpha 2$, additional collagen-binding integrins are expressed by human PDL fibroblasts, which contribute to cell adhesion and cell migration on collagen I [162].

3.1. The repertoire of collagen-binding integrins in human PDL and gingival fibroblasts

Analyses of collagen-binding integrins on mRNA and protein level were performed on three different primary fibroblast cultures, established from human PDL and gingival tissues. Using quantitative PCR, $\alpha 1$, $\alpha 2$ and $\alpha 11$ integrin mRNA were detected in all PDL fibroblast and gingival fibroblast isolates. $\alpha 11$ mRNA levels were higher in all PDL fibroblast isolates compared to gingival fibroblast isolates. $\alpha 10$ integrin mRNA was only detected in PDL fibroblasts, but expression was very weak. Further analysis was performed on selected PDL and gingival cells isolates (hPDLF1 and hGF1). To analyze collagen-binding integrins protein levels, immunoprecipitation experiments and immunofluorescence analysis were performed. Immunoprecipitation of metabolically labeled cells detected $\alpha 1$, $\alpha 2$ and $\alpha 11$ integrin chains, but not $\alpha 10$. The levels of $\alpha 11$ seemed to be higher in the PDL fibroblasts, which was confirmed by western blotting. Indirect immunofluorescence of PDL fibroblasts and gingival fibroblasts showed that $\alpha 1$, $\alpha 2$ and $\alpha 11$ integrins each localized in focal contact-like pattern when cells were plated on collagen I.

3.2. $\alpha 11\beta 1$-mediated collagen gel contraction is enhanced by IGF-II

The ability of cells to reorganize 3D collagen lattices reflects the contractile activity of the cells. Addition of anti-$\beta 1$ integrin antibody almost completely prevented collagen gel contraction, whereas addition of anti-$\alpha 1$ and anti-$\alpha 2$ antibodies mix was not enough to completely block contraction, indicating that other integrin(s) contributed to collagen gel contraction. Since function-blocking antibodies to $\alpha 11$ are not available, $\alpha 11$ mRNA was downregulated with siRNA, resulting in decreased $\alpha 11$ protein levels. $\alpha 11$ siRNA blocked 10-20 % of the contraction of gingival fibroblasts and PDL fibroblasts and the combined use of $\alpha 11$ siRNA and an $\alpha 2$ function-blocking antibody almost completely abolished the ability of

both cell types to contract collagen lattices. In the present study the influence of IGF-II and PDGF-BB on collagen remodeling properties of gingival fibroblasts and PDL fibroblasts was tested. Addition of IGF-II or PDGF-BB increased contraction of both cell types. A stronger contraction of lattices containing PDL fibroblasts was observed. Western blot analysis showed that in gingival fibroblasts expressing moderate levels of α11 and IGF-II stimulated synthesis of α11 protein in a time-dependent manner. In PDL fibroblasts expressing high levels of α11 to start with, the effect of IGF-II was not so obvious. To determine if PDGF-BB and IGF-II stimulated collagen gel contraction was preferentially α1, α2, or α11 integrin-mediated, we used C2C12 cells stably transfected with comparable levels of individual collagen-binding integrins. Under the conditions used, none of the C2C12-cell types displayed preference for any particular growth factor.

3.3. The repertoire of collagen-binding integrins in healthy and diseased PDL tissue

In a limited number of samples, mRNA and protein levels of collagen-binding integrins were analyzed. Reverse transcriptase PCR analysis showed the presence of mRNA encoding α1, α2, α10 and α11 integrins in both healthy and diseased PDL tissue. The expression levels of integrins differed within healthy and diseased groups themselves. Western blot analysis did not show any consistent difference in α11 levels in healthy or periodontitis-positive samples.

3.4. Sequence analysis of the *ITGA11* promoter

Sequence analysis of the 223 bp upstream nucleotide region of *ITGA11* promoter did not reveal any single nucleotide polymorphisms within the SBS1, SBS2 or EBS regions of the DNA, neither in patients, nor in controls.

Discussion and future perspectives

1. α11β1 integrin-dependent regulation of periodontal ligament function in the erupting mouse incisor (Paper I)

The α11 integrin is most closely related to the α10 integrin. In mouse embryos both subunits display expression patterns restricted to different interstitial connective tissue cells. Comparison with the expression pattern of other collagen-binding integrins has shown that the expression of α11 integrin partially overlaps with expression pattern of α2 and is complementary to expression of α10 integrin [82]. Analysis of mice deficient in α1, α2 or α10 integrin subunits have revealed only mild phenotypes in unchallenged mutant animals [61, 62, 69, 201]. The mild phenotypes of mice deficient in individual collagen-binding integrins suggest compensation in cell-collagen interactions in normal physiological conditions or possible overlapping roles of individual integrins. Both α2 and α11 integrins are co-expressed in the intestine [82] but no phenotype was identified in the intestine in α11-deficient animals by standard histological analysis. The existence of redundancy mechanism for these two integrins is one possible explanation for the lack of phenotype in the α11-*null* intestine.

Even though α11 is expressed in perichondrium, α11-deficient mice did not display any cartilage defects. Other tissues normally expressing α11 integrin such as cornea, intervertebral disc, intestine and skin did not show any obvious phenotypes in corresponding unchallenged α11-deficient mice. Instead, the malnutrition and reduced body mass of α11-deficient mice correlated with the observed tooth phenotype. Supplementing the diet with the soft food prevented malnutrition and increased mortality but mutant mice still remained smaller. This indicates that other defects exist in addition to the tooth phenotype.

It is interesting that α11 tooth phenotype is selective for incisors, which in contrast to molars erupt continuously in rodents. α11 is expressed in the dental follicle mesenchyme of both molars and incisors. But in adult teeth α11 is only expressed in the incisors, which most likely explains the restriction of the tooth phenotype to the incisors. Since molar eruption occurs by different mechanism, the role for collagen-binding integrins in this process is unclear. It is possible that receptors other than collagen-binding integrins can participate in attachment of molar PDL cells to collagen. Such a possibility has already been suggested for the dermal fibroblasts in the skin of α1-deficient animals [45]. These mice do not show any developmental and structural abnormalities and no α2 integrin up-regulation in embryos was observed. This was also true in adult smooth muscle cells and hepatocytes, which normally

express high levels of α1. Instead, it has been suggested that direct α1-mediated integrin-collagen interactions are not required in these tissue, and indirect binding to collagen I might be mediated by proteins such as fibronectin [201]. This might also be the case for molar PDL tissue where no collagen-binding integrins are expressed and the tooth eruption process occurs normally.

Except for α11β1 integrin, there are other molecular mechanisms underlying differences between molars and incisors. It has been reported that certain transcription factors expressed during embryonic development, growth factors or MMPs needed during different stages of the tooth eruption process, are differently expressed in molar and incisor PDL. For example, EGF affects only incisor eruption whereas CSF-1 preferentially affects the osteoclasts-dependent stage of molar eruption [202]. In the absence of FGF-10, which supports the stem cell compartment and is indispensable for incisor growth, only incisors, but not molars, are missing [203].

Mice deficient in MMP-14 display affected molar root development and tooth eruption [204-206]. The role of the PDL in rodent incisor eruption is still controversial. It has been suggested that PDL fibroblasts migrate occlusally through the PDL space and create the tractional force pulling the tooth towards the surface of the oral mucosa [207]. Alternatively the eruptive force has been suggested to be provided by the hydrostatic tissue pressure within vascular tissue of the PDL [208]. The α11-deficient mouse is the first genetic model supporting the tractional force model.

Only partial inhibition of cell adhesion to collagen I has been observed in α1-*null* MEFs [201] and α2-*null* keratinocytes and dermal fibroblasts [56], indicating the presence of another collagen-binding integrin . *In vitro* studies of α11-deficient MEFs have shown that the absence of α11 significantly reduces cell attachment to collagen I, spreading on collagen I, and reorganization of collagen I lattices. These findings indicate that α11β1 integrin is a major collagen I receptor on MEFs. Our *in vitro* data imply that incisor PDL fibroblasts in the α11-deficient mice bind collagen with lower affinity, resulting in weakened tractional force in turn affecting the ***continuos incisor eruption***.

The analysis of the PDL tissue showed increased thickness in α11-deficient animals, the presence of wide acellular cementum and increased number of cell rests of Malassez. Maintaining the correct width of the PDL is an essential function of PDL fibroblasts and such property of these cells might be lost in the absence of α11β1 integrin. Sirius red staining showed that increase in thickness of the PDL occurs due to collagen accumulation in α11-

deficient mice. Collagen I and MMP synthesis have been shown to be regulated by integrins [122, 133]. α1-deficient mice display increased collagen synthesis in granulation tissue of the skin wounds but surprisingly no increase in skin thickness was observed due to up-regulation of collagenase (MMP-13) [45]. In the PDL tissue of the α11-null mice no changes in mRNA levels of collagen I and collagen III were detected by semiquantitative PCR. In the α11-deficient mice the observed increase in collagen might also occur due to increased RNA stability or due to posttranslational mechanisms. Another explanation for the increased collagen amount might be reduced MMP activity in α11-*null* PDL tissue. mRNA analysis of the PDL tissue showed decreased levels of MMP-14 mRNA but increased levels of MMP-9. The observed phenotype in the α11-deficient mouse is manifested only postnatally when ossification has occurred. Since sectioning of PDL tissue requires lengthy decalcification protocols it does not allow for reliable *in situ* zymography to detect possible changes in MMP activity. Instead, the PCR analysis of MMPs in MEFs cultured within 3D collagen lattices was performed which revealed reduced expression of MMP-13 and MMP-14 mRNA in α11-deficient cells. Based on the obtained results, we suggest that changed MMP levels are major factors that lead to the observed disturbed collagen turnover in the α11-*null* PDL.

In summary, this data indicate a role for α11β1 integrin in incisor tooth eruption. Our data suggest that α11β1 is needed for the PDL fibroblasts to generate tractional forces needed for tooth eruption. *In vitro* studies showing reduced ability of α11-deficient MEFs to interact with collagen support the phenotype of impaired incisor eruption. In order to verify if re-expression of α11 integrin in the PDL of α11-deficient mouse can rescue the observed phenotype, α11-over-expressing transgenic mice have been generated recently. The transgenic animals will be bred with wild type and α11-deficient mice in order to obtain α11$^{+/+}$ and α11$^{-/-}$ offspring carrying transgenic α11 integrin. *In vivo* and *in vitro* analyses similar to those presented in this report will be performed to validate if the α11-transgene can rescue the α11-phenotype. It will also be interesting to determine the effect on tissue and cellular level when α11-transgene is over-expressed in wild type animals in tissues normally not expressing α11.

2. α11β1 integrin-mediated collagen lattice contraction by incisor periodontal ligament fibroblasts requires MMP-13 (Paper II)

The major protein in the PDL is collagen I, and the most abundant cell population is the fibroblast. Finding the cellular collagen attachment mechanism in the PDL can help to understand physiology and pathophysiology of this specific tissue. Cell-collagen interactions

can be mediated by direct binding to collagen via collagen binding integrins [27] and indirect binding via $\alpha v \beta 3$ integrin-mediated binding bridged via collagen-binding proteins such as fibronectin [209], osteopontin [210], periostin [211] or bigH3 [212]. Even though integrins are well characterized, a clear role for these receptors in developing and mature molar and incisor PDL has not been established yet. The molar PDL fibroblast cell line mPDL-L2 has been characterized before [199] but it still remains unclear how the attachment mechanism works in mouse molar PDL cells. The analysis of mPDL-L2 cells showed lack of all collagen-binding integrins in these cells. This finding suggests that indirect binding to the periostin matrix is involved in adhesion of these cells to the ECM. Periostin is a ligand for integrin $\alpha v \beta 1$ and $\alpha v \beta 3$ [211] and it has been reported to bind collagen I [213]. Periostin-deficient mice demonstrate a disturbed structure of both molars and incisors and the phenotype is described as periodontal-disease like. The phenotype develops within three months after birth, suggesting a critical role for periostin in the maintenance of the PDL structure [214]. Interestingly, the tooth phenotype of $\alpha 11$-deficient animals is manifested at similar age of the animals.

Tooth development is a complex process involving molar and incisor specific transcriptional events. Although molar and incisor PDL fibroblasts have the same ectomesenchymal origin, FGF-10 [203] and $\alpha 11 \beta 1$ integrin **(Paper I)** is only present in the adult incisor PDL tissue. Periostin is also detected in incisor PDL tissue, but the mechanism of incisor PDL fibroblast cell attachment seems to be more complex and clearly involves direct cell attachment to collagen I via $\alpha 11 \beta 1$ integrin **(Paper I)**.

Part of $\alpha 11$-deficient mouse phenotype is a thickened PDL with increased collagen accumulation. In order to examine the molecular mechanism underlying observed phenotype, it was essential to isolate fibroblasts from incisor PDL. Since we failed to isolate primary cells due to limited amount of PDL tissue, the cells were isolated from $\alpha 11$-deficient animals carrying temperature sensitive SV40 large T [198]. Isolation of immortalized PDL cells from rodents has already been described before [199, 215, 216]. Molar PDL cell lines from rats and mice carrying temperature sensitive SV40 large T were isolated and characterized [199, 215]. Mouse dental follicle cells obtained from mouse incisor tooth germ, were immortalized by infection with mutant human papiloma virus type 16 E6 [216] resulting in a cell line with presumably incisor-like characteristics. To our knowledge, the incisor PDL cell isolates are the first incisor PDL cells to be isolated from already differentiated adult mouse incisor PDL tissue. The progenitor cells in the dental follicle are thought to contribute to the formation of all periodontal tissues like cementum, PDL and alveolar bone [217]. The PDL cells have been

suggested to have potential to differentiate to odontoblasts or cementoblasts [142, 151]. It has been demonstrated that PDL cells have osteoblast-like properties, including ALP activity [218] and display an ability to form mineralized nodules [219, 220] like osteoblasts. There is no marker typical for the PDL cells but a number of tendon/ligament phenotype- and osteoblastic phenotype-related genes are thought to be involved in the differentiation of the PDL cells. Initial analysis of incisor PDL fibroblasts confirmed expression of such markers like collagens I and XII, periostin, RunX2 and osteopontin. Furthermore, incisor PDL fibroblasts showed increased ALP activity and ability to form mineralized noduli. Based on these findings, we concluded that the cells we isolated have properties of PDL cells. Another important issue regarding the isolated incisor PDL fibroblasts concerns possible side effects resulting from immortalization or clonal effects following isolation. Previously, it has been shown that the immortalization process itself can influence and dysregulate expression of random genes. Infection of keratinocytes with oncogenic mutant of Ras (Ras12) causes switch in $\alpha3\beta1$ integrin function, which in turn induced MMP-9 expression in the cells [221]. To exclude clonal effects, at least three independent isolates of control and $\alpha11$-deficient incisor PDL fibroblasts were generated and initially screened for expected expression of collagen-binding integrins. Two isolates from each group, with similar integrin expression patterns, were chosen for further analysis. Both independent isolates within each group responded similarly in functional assays such as cell attachment or collagen gel contraction. Analysis of MMP levels indicated similar expression pattern of MMP-13 and similar collagenase activity in both isolates within the group. In the PDL tissue of $\alpha11$-deficient animals MMP-9 mRNA was strongly up-regulated **(Paper I)**. In our incisor PDL fibroblasts the levels and the activity of MMP-9 differed between isolates from the same group thus suggesting that observed inconsistencies might be side effect of immortalization or clonal effect.

The PDL is a tissue characterized by high mechanical tension. In adult molar and incisor PDL tissue proteins are synthesized and degraded constantly maintaining the tissue homeostasis. 3D collagen lattices are model systems that have been used to mimic the normal microenvironment of tissues [222]. It has been reported that 3D collagen gels induce expression of MMP-13 human skin fibroblasts [122] and addition of exogenous MMP-13 to collagen gels containing skin fibroblasts increased the ability of cells to reorganize collagen matrices [113]. The addition of the MMP inhibitor GM6001 decreased the ability of fibroblasts to contract collagen gel by human osteosarcoma cells [223]. It has also been reported that MMP-13 is a major

MMP regulated by integrins in fibroblasts [113, 122]. ECM proteins like collagen I, collagen XII and periostin can be regulated by mechanical tension in the PDL [224-227]. The regulation of collagen α1(I) and periostin synthesis by mechanical stress involves autocrine TGF-β loops [226, 227]. TGF-β latent complexes are activated by mechanical stretching [228] and MMP-13 gene expression has been reported to be enhanced by TGF-β [119]. Thus it is possible that the tractional force reduction, due to absence of α11-integrin, results in decreased levels of active TGF-β causing the drop in MMP-13 levels. In the future it will be important to clarify if such regulation occurs in incisor PDL fibroblasts *in vitro* and *in vivo*. In α11-deficient PDL, collagen is accumulated and this report suggest that impaired collagen turnover is in part due to disturbed MMP synthesis. Matrix remodeling requires presence of MMPs secreted by fibroblasts embedded in the ECM. Interestingly, western blot analysis of MMP-13 levels showed induction only in α11-expressing incisor PDL fibroblasts placed in attached collagen gels. Since human skin fibroblasts or MC615 mouse chondrocytic cells placed inside 3D collagen gels up-regulate MMP-13 via two different signaling pathways, resulting in the up-regulation of p38 in skin fibroblasts and ERK in MC615 cells [122, 123], it appears that regulation mechanism of MMP-13 might be cell type specific. It is interesting to note that MMP-13-deficient mice have a bone phenotype but no tooth phenotype. The most trivial explanation is that MMP-13 shows a limited expression in the normal PDL tissue, and only partially contributes to normal tissue homeostasis. Other possible explanations include the existence of redundancy or molecular compensation mechanism. MMP-13 deficient mice show up-regulated MMP-8 during wound healing, suggesting compensation within the MMPs gene family [229]. Another possibility is compensation by MMP-14, which has properties of collagenase. MMP-14 has a clear molar PDL phenotype in a MMP-14-deficient mouse model indicating that MMP-14 is indispensable for the tooth eruption process and collagen turnover [205]. Its role in incisor remains to be defined. In addition to molecular compensation mechanisms, the lack of tooth phenotype of MMP-13-deficient mice might be due to physiological adaptation or compensation mechanism of unknown nature.

Another interesting issue is a clarification of MMP-13 regulation in the context of fibroblast involved in stromal regulation of cancer. MMP-13 is expressed in breast carcinoma, chondrosarcomas, basal cell carcinomas and squamous cell carcinomas of the head and neck [230]. Several proteins are known to be substrates for MMP-13 *in vitro*, including TGF-β and collagen I. TGF-β secreted by carcinoma-associated fibroblasts (CAFs) and acting on the epithelium promotes carcinogenesis [231]. MMP-13 degrades components of basement

membranes promoting tumor invasion and progression of human squamous cell carcinoma and human fibrosarcoma HT1080 cells [230]. MMP-13 is expressed by CAFs cells in human breast cancer, and *in vitro*, breast cancer cells can stimulate fibroblasts to secrete MMP-13. α11 is also expressed by CAFs in lung cancer [232]. It will be interesting to determine if α11β1 mediates part of the MMP-13 synthesis in CAFs.

In summary, the present data suggest that in the incisor PDL fibroblast α11β1 is regulated by mechanical strain. In a future it might be interesting to study the regulation of α11 during molar and incisor tooth movement. Since α11β1 integrin is a major collagen receptor of fibroblasts, its potential role in downstream events in various connective tissue disorders remains to be defined. The expression and activity of MMP-13 in incisor PDL fibroblast is regulated in α11β1-dependent manner. It remains to be confirmed if MMP-13 is involved in collagen remodeling by incisor PDL fibroblasts *in vivo*.

3. A role for α11β1 integrin in the human periodontal ligament (Paper III)

Since α11β1 integrin is needed during the PDL-driven tooth eruption in mouse and α11-deficient mice display a PDL phenotype **(Paper I)**, it was important to explore a possible role of this integrin in human PDL. The integrin repertoire on human PDL cells has been partially characterized previously [161, 162, 233, 234] but a very few functional analyses of collagen-binding integrins in PDL cells have been performed. Studies by Lallier et al. using antibodies to α1 and α2 to investigate PDL cells interactions with collagen, suggested that other integrins, like α10β1 and α11β1 might be functional and mediate such interactions [162]. Since 3D collagen gel contraction assay has been suggested to mimic tooth eruption [235] and is a convenient assay to analyze collagen receptor function, we used it to evaluate the role of α11 on human PDL fibroblasts.

Analysis of human gingival fibroblasts and PDL fibroblasts showed that three collagen-binding integrins α1β1, α2β1 and α11β1 are expressed at the protein level. Similarly to the functional studies by Lallier et al. only partial inhibition of collagen gel contraction occurred when α1 and α2 integrin-blocking antibodies were used. In our studies the inhibitory effect was most pronounced in gingival fibroblasts expressing lower levels of α11 protein than PDL fibroblasts. Downregulation of α11 by siRNA to α11 mRNA, caused a strongly reduced ability of both cell types to contract collagen lattices. A combination of α11 siRNA treatment with a function-blocking antibody to α2 almost completely abolished the ability of the cells to

contract collagen gels. The obtained results stand in line with previous studies of MEFs indicating α11β1 as a major integrin mediating collagen gel contraction [81]. Based on these results it was concluded that on fibroblasts α2β1 and α11β1 are the major collagen-binding integrins mediating collagen lattice remodeling in human gingival and PDL fibroblasts. *It is interesting that in adult mice, α11 expression is limited only to incisor PDL while in human α11 was detected also in adult molar PDL.*

Further detailed analyses are required to investigate if α11 levels are increased in inflamed PDL tissue. Growth factors such as PDGF-BB or IGF-II are involved in PDL tissue regeneration after injury [236-238]. In the present study, the contraction of collagen lattices by human gingival fibroblasts and human PDL fibroblasts was stimulated by PDGF-BB and IFG-II. Comparison of the ability of gingival fibroblasts (expressing low α11 levels) and PDL fibroblasts (expressing high levels of α11) to reorganize collagen gels implied that the strength of contractile activity of cells is governed by the relative levels of collagen-binding integrins. IGF-II appeared to stimulate α11 levels in gingival fibroblasts cells whereas such effect was not seen in PDL fibroblasts cells, expressing high levels of α11 to start with. The stronger contractile activity of PDL fibroblasts cells in response to IGF-II might reflect a higher level of α11β1 integrin in these cells. Based on the obtained results, it seems that in both cells types, α11β1 integrin mediates collagen gel contraction in an IGF-II-dependent manner. A stimulation of α11β1 integrin during regeneration of PDL might be important in the contraction phase of the granulation tissue. This mechanism might allow more efficient replacement with new non-fibrotic PDL tissue.

In conditions afflicting connective tissues, such as Ehlers-Danlos syndrome or Marfan's syndrome, severe defects of the PDL have been observed [239, 240]. These findings suggested that defects in the PDL might be a contributing factor for increased susceptibility to periodontal disease [239, 240]. More recently, it has been shown that αvβ6 can be of importance in preventing of periodontal disease. The αvβ6 integrin levels are downregulated in patients suffering from periodontal disease [168]. Recently, it has been shown in a pig model, that β6 integrin levels are increased in both skin and gingiva during wound healing [241]. Based on these results, it seems that decreased levels of β6 integrin in periodontal disease might prevent PDL tissue regeneration. In general, genetic background influences susceptibility to different diseases including chronic inflammatory periodontal disease. Most of the gene polymorphism studies in the PDL have focused on cytokines (IL and TNF genes), immune receptors and MMPs since these factors are suggested to contribute to development of

periodontal disease [242]. Genetic polymorphisms within MMP-1, MMP-2 and MMP-3 promoters have been studied in the context of periodontal disease but no associations with increased susceptibility to periodontal disease were found [243, 244]. In mice deficient in $\alpha 11$ integrin no direct PDL fibroblasts-collagen interaction occurs due to lack of any collagen-binding integrin and results in severely disturbed tooth eruption due to decrease of tractional force. The synthesis of collagen is increased in the PDL of such animals. The promoter region is crucial for binding of RNA polymerase and initiation of RNA transcription, and existence of polymorphisms might lead to dysregulated $\alpha 11$ integrin levels, potentially predisposing to periodontal disease. We assumed that decreased expression of integrin $\alpha 11$ in human might result in weakened PDL fibroblast-collagen interactions potentially predisposing to periodontal disease. Thus, it seemed relevant to clarify if there were any polymorphisms within the *ITGA11* promoter. However, no polymorphisms were identified by sequencing 223 bp upstream nucleotide region representing the proximal promoter of *ITGA11*, containing SBS1, SBS2 and EBS regions. The comparison of mRNA and protein expression of $\alpha 11$ in healthy and inflamed PDL tissues showed presence of the $\alpha 11$ integrin in both tissue types but the levels varied within the control and inflamed tissues.

Further studies of human PDL might include comparison of integrin repertoire of primary and permanent teeth to clarify which integrins are involved in cell-collagen interactions and with possible implications for tooth eruption and PDL integrity.

Concluding remarks

The studies included in this thesis attempted to investigate the function of α11β1 integrin *in vivo* and *in vitro*.

First of all, α11-deficient mice have been generated and the α11-*null* phenotype has been described. Our data support a model in which tractional force is needed for tooth eruption to occur, and that α11β1 integrin by interaction with the collagen matrix of the PDL provides the "tool" for generating this force. Thus, α11-deficient mouse is the first genetic model supporting a role for tractional force in the late phase of the incisor eruption process. The presented data also indicate that α11β1 integrin in incisor PDL fibroblasts is a regulator of the PDL width.

Secondly, α11-deficient immortomouse was generated which enabled the isolation of fibroblasts from adult incisor PDL. Our data show that α11β1 integrin is a major collagen-binding integrin expressed by these cells, while α11 was not detected on fibroblasts derived from adult molar PDL. Mechanical strain was identified as a factor regulating α11 expression. Furthermore, we could show that α11β1 integrin regulated MMP-13 protein levels. Since MMP-13 is expressed in a number of cancers, it might be important to clarify the molecular mechanism of α11-mediated regulation of MMP-13.

A final issue addressed in the present study was the role of α11β1 integrin in the human tooth apparatus. For the first time, all collagen-binding integrins have been characterized on the protein level in PDL and gingival fibroblasts. In contrast to mouse PDL, α11 was detected in both incisor and molar adult human PDL tissue. Comparative analysis of healthy and inflamed PDL tissue did not reveal any differences in α11 levels. Based on the α11-*null* phenotype *in vivo* it was suggested that decreased levels of α11 might predispose to periodontal disease. However, within a limited part of the proximal *ITGA11* promoter no polymorphisms were found in DNA collected from a limited cohort of patients suffering from chronic periodontitis.

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