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REVIEW

# Extracellular Matrix Remodeling During Palate Development

Xia Wang[a], Chunman Li[a], Zeyao Zhu[a], Li Yuan[b], Wood Yee Chan[c], and Ou Sha[a]

[a]Health Science Center, Shenzhen University, Shenzhen, China; [b]Department of Stomatology, Shenzhen People's Hospital, 2nd Clinical Medical College of Jinan University, Shenzhen, China; [c]School of Biomedical Sciences, The Chinese University of Hong Kong, Hong Kong, China

**ABSTRACT**
The morphogenesis of the mammalian secondary plate is a series of highly dynamic developmental process, including the palate shelves vertical outgrowth, elevation to the horizontal plane and complete fusion in the midline. Extracellular matrix (ECM) proteins not only form the basic infrastructure for palatal mesenchymal cells to adhere via integrins but also interact with cells to regulate their functions such as proliferation and differentiation. ECM remodeling is essential for palatal outgrowth, expansion, elevation, and fusion. Multiple signaling pathways important for palatogenesis such as FGF, TGF β, BMP, and SHH remodels ECM dynamics. Dysregulation of ECM such as HA synthesis or ECM breakdown enzymes MMPs or ADAMTS causes cleft palate in mouse models. A better understanding of ECM remodeling will contribute to revealing the pathogenesis of cleft palate.

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## Introduction

The morphogenesis of the mammalian secondary palate begins with the outgrow of two palatal shelves from the maxillary processes on both sides of the tongue on an embryonic day (E) 12.[1] The two vertically oriented palatal shelves soon elevate horizontally and opposite each other on E 14–15.[1] Then, the palatal shelves epithelia disintegrate in the midline and their mesenchymal compartment fuse completely to form an intact palatal roof.[1] Cells in the palatal shelves originate from three sources of embryonic tissue/structures: the superficial palatal epithelium is derived from the embryonic ectoderm, the underlying palatal mesenchyme mainly from the neural crest.[1,2] Supporting these cells is the infrastructure composed by complex extracellular matrix network.

The extracellular matrix (ECM) is a three-dimensional, highly dynamic non-cellular architectural scaffold present in all tissues. In mammals, the ECM is composed of a complex protein network including collagens (Col), proteoglycans (PGs), glycoproteins, and Proteoglycans (PGs).[3] ECM not only support the tissue integrity and elasticity but also control tissue homeostasis.[4] ECM remodeling is an important process in the morphogenesis of many organs such as lungs, intestine, and mammary glands.[5] During development, ECM is undergoing dynamic deposition, degradation by growth factors-controlled synthesis and proteolysis by matrix-degrading enzymes.[4] Abnormal ECM remodeling can lead to embryonic lethality or abnormal morphogenesis or pathological conditions such as fibrosis and cancer.[5]

In the palate, ECM not only forms the basic infrastructure where cells adhere via integrins but also play important roles in integrating and regulating growth factors network. They store and release growth factor, therefore controlling the bioavailability of active growth factors such as Tgf-βs, which in turn remodel ECM dynamics and palatal cell differentiation.[6-8] They accumulate water, bind other ECM molecules, mediating palatal shelf growth, expansion, and elevation.[9-14] Multiple signaling pathways important for palatogenesis such as FGF, TGF β, BMP, and SHH regulate ECM dynamics during palate development (Figure 1).[6-8,14-18] In this review, we will summarize the dynamic deposition and degradation of ECM during palate development.

**CONTACT** Ou Sha shaou@szu.edu.cn Shenzhen University School of Medicine, RM 433, A7 Building, No.1066 XuanYuan Road, Nanshan District, Shenzhen, Guangdong 518060, China

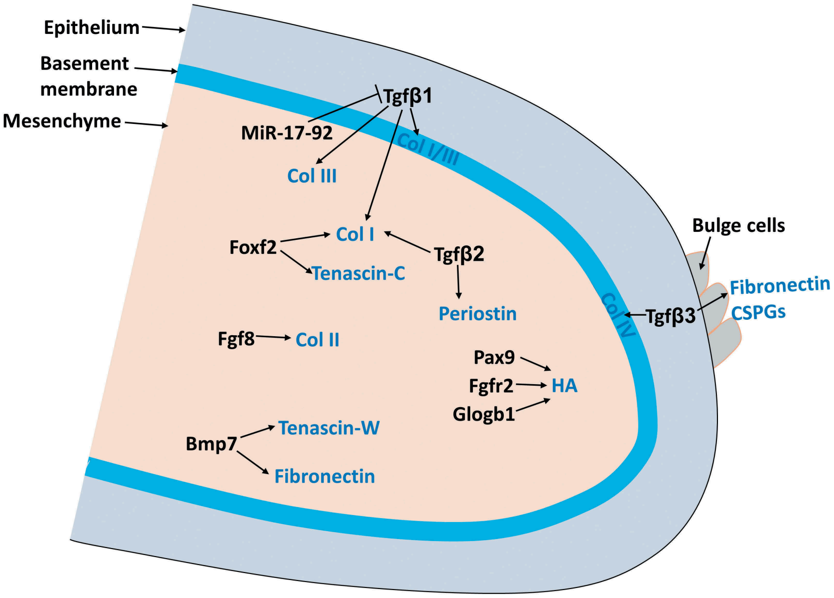

Figure 1. ECM remodeling during palate development.

## ECM molecules remodeling in the palate

### Collagens (Col)

Collagens are the major components of ECM in connective tissues. There are 28 distinct collagens composed of α1, α2, α3 subunits combination and classified into fibrillar collagens (Collagen I–III, V, and XI) and non-fibrillar forms (Collagen VI, IX, IV, etc.).[5,19] Fibrillar collagens form strong and stable fibrils and organize the fibrils into three-dimensional network, for example, Collagen I fibrils for bones and Collagen II fibrils for cartilages.[5] Non-fibrillar forms of collagens include Fibril-Associated Collagens and basement collagens. Fibril-Associated Collagens, such as Collagen IX, associate with collagen fibrils and bind them together to form thicker collagen fibers. Basement collagens are sheet-forming collagens such as Collagen IV, which form the two-dimensional network for all basal laminae.[5,19] A variety of collagens are highly expressed in the palate and dynamically remodeled during palatogenesis (Tables 1 and 2).

Col I and III are widely expressed in the palatal mesenchyme before and after palate shelf elevation.[7,20-25] During palatogenesis, their degradation is highly regulated. For example, Col I is downregulated in the palatal shelves of *Foxf2*$^{-/-}$ embryos which failed to elevate palate shelves.[15] Interestingly, in human palatal fibroblasts derived from orofacial cleft patients, COL I and III mRNA levels are strongly decreased, in contrast, the protein levels are increased compared to the control sample possibly contributing to decreased collagen degradation by MMPs and increased collagen cross-links.[24] COL I mRNA and protein levels in the palate are also downregulated in chondroitin sulfate proteoglycan defective mutants which have thinner palate due to abnormal bone and cartilage development.[14] TGF-β1, one of the most important growth factors during palate development, can induce palatal mesenchymal cells proliferation and Col I and III synthesis, which can be inhibited by MiR-17-92 clusters.[16]

Col IV is expressed in the epithelial cell basement membrane.[10,17,20,25] Col IV expression is reduced in the Tgf-β3 mutant in which palatal shelves failed to fuse.[17] Addition of Tgf-β3 in the palate culture increases Col IV deposition in the basement membrane of MEE cells.[17]

In human, mutation of COL2A1, COL11A1, COL11A2, COL9A1, and COL9A2 cause a group of hereditary conditions known as Stickler syndrome I–V, respectively, characterized by high myopia, retinal detachment, hearing loss, midfacial underdevelopment, and cleft palate is only described in Stickler syndrome I–III (Table 1).[26-32]

Type II collagen is the major extracellular matrix component of cartilage and essential for endochondral bone formation.[5] In the palate mesenchyme, only a few osteoblast precursors express Col II in the palatal mesenchyme.[8] Inactivate Type II collagen

**Table 1.** Cleft palate-related ECM gene mutation in human and mice.

| Gene | Protein | Human Disease | Cleft palate related Clinal features | References | Mouse mutation | Mouse mutation phenotypes | References |
|---|---|---|---|---|---|---|---|
| COL2A1 | Col II | Stickler syndrome type 1 (OMIM 108300) | Cleft palate or bifid uvula, | 26,109-111 | Transgenic Del1 mice carrying six copies of with COL2A1 (*Del*) mutation with a 150-bp deletion containing the 45-bp exon 7 and intro7: | Craniofacial ossification retarded; reduced cartilage and bone growth; cleft palate; | 38 |
| | | Kniest dysplasia (OMIM 156550) | Cleft palate | 111 | Transgenic mice with targeted inactivation of the COL2A1 gene | No endochondral bone or epiphyseal growth plate in long bones with normal membranous and periosteal skeleton | 34 |
| | | Platyspondyly lethal skeletal dysplasia (OMIM 151210) | No cleft palate | 111 | ENU induced mutation has a G to A transition at Chromosome 15:97815207, which causes a premature stop codon at amino acid 645 | Cleft palate; shortened nose | 112 |
| COL11A1 | Col XI | Stickler syndrome type 2 (OMIM 604841) | Cleft palate | 110 | Deletion of a cytidine residue about 570 nt downstream of the translation initiation codon in COL11A1 (*cho*) mRNA causes a reading frame shift and introduces a premature stop codon | Cleft palate, shortened head and mandible, short limbs, protruding tongue | 39,40 |
| | | Marshall syndrome (OMIM 154780) | Cleft palate | 26 | | | |
| COL11A2 | Col XI | Stickler syndrome type 3: (OMIM 184480) | Cleft palate | 110 | Full-length Col11a2 chain was unable to occur because of the presence of premature termination codons | NO cleft palate;<br>Hearing loss, smaller size, shorter snout, skeletal abnormalities including abnormal cranium morphology and long bone epiphyseal plate | 41,42 |
| | | Nance-Insley syndrome (OMIM 215150) | Cleft palate | 41 | | | |
| FN1 | Fibronectin | NR | | | Conditional knock out FN1 in cranial neural crest cells | Cleft palate, abnormal cardiac morphogenesis, thymus development defects | 48 |
| | | | | | Conditional knock out FN1 in mesoderm cells | Cleft palate, abnormal cardiac morphogenesis, thymus development defects, edema, etc. | 47 |
| TNXB | Tenascin-X | Tenascin-X deficiency (OMIM 606408) | Bifid uvula | 55,56,113,114 | NR | | |
| ACAN | Aggrecan | NR | | | Gene mutation in ACAN:<br>*cmd/cmd* (cartilage matrix defieciency), 7-bp deletion in exon 5; cmd-Bc, spontaneous mutation, complete loss of exons 2-18 | Cleft palate, short limbs, tail, and snout | 115,116 |
| VCAN | Versican | NR | | | Haploinsufficiendy of Versican in Adamts20 mutant mice (*Vcan*$^{hdf/+}$;*Adamts20*$^{bt/bt}$) | 65% mutant exhibited cleft palate | 12 |

NR, not reported.

**Table 2.** ECM molecules expression, function, and remodeling pattern during palatogenesis.

| | | Expression pattern in the palate | | | Functional roles during palatogenesis | | | Remodeling during palatogenesis | | |
|---|---|---|---|---|---|---|---|---|---|---|
| ECM component | Subtype | Location | Detection methods | Species/ References | Potential mechanism | Potential function | References | Abnormal remodeling | Detection methods | References |
| Collagens (Col) | Col I | Before palatal elevation: Epithelial basement membrane and palatal mesenchyme (stronger on the nasal side than the oral side in the middle and posterior palate) After palatal elevation: Mesenchyme around the bone | IHC | Mouse[7,20-23,117] | Main structure protein | Build up palate infrastructure | [7] | Col I is downregulated in the palatal shelves of *Foxf2*$^{-/-}$ mouse embryos | IHC | [15] |
| | | | | | | | | Col I and its degrading enzyme MMP 13 are downregulated in T1KO mice | qRT-PCR, WB, IHC | [14] |
| | | | qRT-PCR, dot blot | Human:[24] | | | | Col I synthesis by palatal mesenchymal cells can be induced by TGFβ1 and inhibited by MiR-17-92 clusters | qRT-PCR, WB | [16] |
| | | | | | | | | Col I synthesis by palatal mesenchymal cells can be induced by TGFβ2 | IHC | [7] |
| | Col II | After the palatal shelf elevation: A few palatal mesenchymal cells | IHC | Mouse:[8] | Main cartilage ECM | Palatal mesenchyme osteogenic/ chondrogenic fate determination | [8] | Enhanced FGF8 signaling causes strong expression of Col II in palatal mesenchymal cells | IHC | [8] |
| | Col III | Before palatal elevation: Epithelial basement membrane and palatal mesenchyme | IHC<br>qRT-PCR, dot blot | Mouse:[23,25]<br>Human:[24] | Main structure protein | Build up palate infrastructure | [23] | Col III synthesis by palatal mesenchymal cells can be induced by TGFβ1 and inhibited by MiR-17-92 clusters | qRT-PCR, WB | [16] |
| | Col IV | Epithelial basement membrane | IHC | [10,17,20,25] | NR | | | Col IV expression in the palate is reduced in the Tgf-β3 mutant and can be re-induced by Tgf-β3 | IHC | [17] |
| Fibronectin | | Before palate elevation: Palatal mesenchyme and around MEE, with strong expression around the bulging MEE cells After palate elevation: MES | IHC, ISH | [6,17,20-23] | NR | | | Absent in MEE cells and apical surface in the Tgf-β3 mutant palate and can be re-induced by Tgf-β3; Downregulated in BMP 7 homozygous palate; EDA domain reduced in *Foxf2*$^{-/-}$ palatal shelves; Inhibited by retinoid acid in a dose-dependent manner. | IHC, qRT-PCR, WB | [6,15,17,49] |
| Fibrillins | Fibrillin-1 | Weakly expressed in the palatal mesenchyme only before palatal shelf elevation | IHC | [6] | Compose microfibirls and activate Tgf-β | Palatal fusion | [6] | NR | | |
| | Fibrillin-2 | Before palatal shelf elevation: enriched in the nasal side of palatal mesenchyme After palatal elevation: increased and expanded around MEE cells and in the oral side of palatal mesenchyme | IHC | [6] | | | | Inhibited by retinoid acid in a dose-dependent manner | qRT-PCR | [49] |
| Tenascins | Tenascin-C | Before palatal elevation: in the mesenchyme close to the nasal and distal surface of the shelf; After palatal elevation: accumulated in the mesenchyme close to the MES | IHC, ISH | [1,6,53] | NR | | | Reduced in *Foxf2*$^{-/-}$ mutant palatal shelves⍰ Inhibited by retinoid acid in a dose-dependent manner | qRT-PCR | [15,49] |
| | Tenascin-W | Before palatal elevation: weekly expressed in the proximal-nasal quadrant of the vertical shelves; After palatal elevation: restricted to the dorsal mesenchyme around the MES, corresponding to the future osteogenic domains of hard palate | IHC, ISH | [1,6,53] | NR | | | Diminished in the palatal shelves of *BMP7*$^{-/-}$ palatal shelves; BMP7 induce Tenascin-W production in embryonic fibroblasts *in vitro* | ISH, WB | [6] |

(*Continued*)

## Table 2. (Continued).

| ECM component | Subtype | Expression pattern in the palate: Location | Expression pattern in the palate: Detection methods | Expression pattern in the palate: Species/ References | Functional roles during palatogenesis: Potential mechanism | Functional roles during palatogenesis: Potential function | Functional roles during palatogenesis: References | Remodeling during palatogenesis: Abnormal remodeling | Remodeling during palatogenesis: Detection methods | Remodeling during palatogenesis: References |
|---|---|---|---|---|---|---|---|---|---|---|
| Periostin | | In the anterior palate (hard palate), periostin is expressed in the mesenchyme on the oral side and part of basal membrane; Intensified around MEE cells in palate fusing process; highly expressed in the entire posterior palate (soft palate) | IHC, ISH | [7,53,62] | May help to determine soft palate formation | Soft palate formation | [7] | TGFβ2 induce periostin production in cultured palate shelves. | IHC | [7] |
| Laminin | Laminin 5 | Discontinuously in the basement membrane and intercellularly in MEE cells | IHC | [17,25] | NR | | | Becomes continuous in the basement membrane under MEE cells in the Tgf-β3 mutant palate | IHC | [17] |
| Proteoglycans | CSPGs | CS chains Before the palatal shelf elevation: palatal mesenchyme; During the palatal shelf elevation and the palatal shelf fusion: palatal mesenchyme, transiently upregulated on apical surface of MEE surface; After the palatal shelf fusion: Palatal mesenchyme around the bone | IHC | Mouse:[11,14,18] | Hold up water; Bind other ECM molecules; Modify TGFβ signaling | Palatal growth, expansion, elevation and adhesion | [12-14,118] | Reduced in *TGFβ3*$^{-/-}$ mutant the palatal shelf mesenchyme and disappeared in mutant MEE surface; TGFβ3 induce CSPGs production in cultured palate shelf MEE cells. | IHC | [18] |
| | | Versican Before the palatal shelf elevation: Palatal mesenchyme (stronger in nasal/medial and tip than oral/lateral palate) During the palatal shelf elevation and the palatal shelf fusion: Palatal mesenchyme (stronger in nasal/medial and tip than oral/lateral palate, although increased in oral/lateral part), transiently upregulated on apical surface of MEE surface; After the palatal shelf fusion: Decreased in palatal mesenchyme | IHC | [11,12] | | | | Cleaved versican decreased in *Adamts9;*$^{\pm}$*Adamts20*$^{bt/bt}$ palatal shelves | IHC | [12] |
| | | Biglycan/Decorin palatal mesenchymal cells at different palate development stages (Decorin restricted to the nasal side); peaked in MEE cells as the palatal shelf adhered | IHC, ISH, qRT-PCR | [13,76,77] | | | | Disappeared in the MES of TGFβrI kinase inhibitor (SB431542)–treated palates; Decorin are unable to downregulate in the mesenchyme when palatal shelves are elevating; Decorin are downregulated in ectopic Hh signaling palatal shelves | IHC, ISH | [13,76,77] |
| | HSPGs | Before the palatal shelf elevation: Epithelial basement membrane and anterior palatal mesenchyme; During the palatal shelf elevation and the palatal shelf fusion: Epithelial basement membrane (stronger in the oral/lateral side than the nasal/medial side) and the tip of the whole palatal mesenchyme; After the palatal shelf fusion: Epithelial basement membrane (stronger in the oral side than the nasal side); gradually disappear from the palatal mesenchyme; Around vessel basement membrane at all stages | IHC | [11,25] | | | | | | |
| | KSPGs | Lumican Restricted to the nasal mesenchyme of the palate | ISH | [77] | | | | Lumican are downregulated in ectopic Hh signaling palatal shelves | ISH | [77] |
| | HA | Before the palatal shelf elevation: palatal mesenchyme (anterior/mid-part stronger than anterior-most and posterior palate; stronger in the nasal/medial side than the oral/lateral side) | IHC | [9,11,78] | Retain water | Palatal shelf elevation | [1,9-11] | Reduced in *Fgfr2*$^{C342Y,\ Pax9}$ and *Golgb1* mutant palatal shelves; HA synthase Has 1, 2, and 3 disrupted in Tgf-β3 mutant shelves. | IHC | [9,49,88,89] |

in mouse develops short bones and cleft palate.[33-38] In these mutants, both chondrocyte differentiation and intramembranous ossification are disrupted. Augmented fibroblast growth factor 8 (FGF8) signaling in the anterior hard palate by using Shox2$^{Cre}$ causes a subset of palatal mesenchymal cells differentiating into Col II$^{+}$ chondrogenic cells at the expense of osteogenic cell fate.[8] These results indicate that appropriate level of Type II collagen is necessary for palate mesenchymal cell fate determination.

Mutation of COL11A1 in human causes Marshall syndrome and Stickler syndrome type. 2[26] Col11a1 homozygote mice have craniofacial abnormalities including cleft palate, shortened head, and mandible, short limbs, protruding tongue et.al.[39,40] The tongue protrusion is possible to obstruct palatal shelf elevation, contact, and fusion. However, the Col11a1 mutant palatal shelves can make contact and fuse when placed close to each other in organ culture.[39] It indicates that Col11a1 may play a role in palate growth. Mutation of COL11A2 in human cause Stickler syndrome type 3 and Nance-Insley syndrome.[26,41] However, inactivation of Col11a2 in mice does not lead to cleft palate, although other clinical features such as hearing loss and abnormal skeleton development replicate the human phenotypes.[41,42]

## Glycoproteins

### Fibronectins (FN)

Fibronectin (FN) is a glycoprotein with a high molecular weight of 230–270 KD. As one of the most widely expressed ECM in the vertebrate, FN is composed of types I, II, and III repeating units.[4,43] FN existing in multiple isoforms is encoded by a single FN gene, located in the human chromosome 2 and rodent chromosome 1.[44] Alternative splicing occurs at three regions, EIIIA/EDA and EIIIB/EDB and V region. FN has multiple sites for self-assembly and ligand binding for integrins, heparin, fibrin, collagen/gelatin, and growth factors, mediating biological processes such as cell adhesion, migration, differentiation.[4]

FN is one of the most abundant ECM components in the palate (Table 2). Although FN null embryos are embryonic lethal[45,46], conditional knockout FN in cranial neural crest cells and mesodermal cells leads to cleft palate.[47,48] Before palate shelve elevation, FN locates in the palatal mesenchyme[20-23] and around MEE, with strong expression around the bulging MEE cells.[17,20] FN expression is totally absent in MEE cells and apical surface in Tgf-β3 mutant, and the addition of Tgf-β3 in the palate culture increase FN deposition on the MEE apical surface (Martinez-Sanz et al., 2008). FN production by the human fetal palatal mesenchymal cells also can be inhibited by retinoid acid, a known cleft palate inducer, in a dose-dependent manner.[49] Strong *Fibronectin* mRNA expression is also observed at the midline epithelial seam (MES) in E14.5 wildtype or BMP heterozygous embryos.[6] While the same stage of BMP homozygous embryos has delayed palatal shelf elevation, only little *Fibronectin* mRNA expression was found in their still vertical palatal shelves. Besides, anti-fibronectin antibody can block palate shelve adhesion.[17] Recently, fibronectin splice-isoform ED-A domain, essential for Tgfβ latency complex formation, is shown reduced in the palatal shelves of *Foxf2*$^{-/-}$ embryos which failed to elevate.[15] These results indicate fibronectin is important during palate shelve elevation and fusion, downstream of Foxf2 and TGF/BMP signaling.

### Tenascins

Tenascins are a family of polymorphic glycoproteins with a molecular weight of 150–380 KD, including tenascin-C, -R, -W, -X and -Y.[43] Tenascins are composed of repeated domains including type III domains, EGF-like repeats, and a C-terminal globular domain.[43] Tenascin-R is mainly found in the central nervous system and Tenascin-X and -Y in skeletal muscles.[43] Tenascin-C and -W are widely expressed in developing tissues and play important roles in tissue morphogenesis and tumor growth.[43] The expression of tenascins is regulated by mechanical loading both during development and in adulthood.[50] Unlike most ECM proteins mediating cell adhesion and cytoskeletal organization, Tenascins modulate cell-matrix interactions and function as adaptors.[51,52]

*Tenascin-C* and *Tenascin-W* showed distinct spatial and temporal expression patterns during palatogenesis (Table 2).[1,6,53] At E13.5, before palatal shelves elevated, *Tenascin-C* expression was found in the mesenchyme close to the nasal and distal surface of the shelf; After elevation, *Tenascin-C* expression accumulated in the mesenchyme close to the MES. In contrast, before elevation, *Tenascin-W* weekly expressed in the proximal-nasal quadrant of the vertical shelves; After elevation, *Tenascin-W* was restricted to the dorsal mesenchyme around the MES, corresponding to the future osteogenic domains of hard palate. *Tenascin-W*, not *Tenascin-C* diminished in the palatal shelves of *BMP7*$^{-/-}$ embryos can be induced by Bmp7 in embryonic cranial fibroblasts in vitro.[6] While *Tenascin-C* expression is reduced in *Foxf2*$^{-/-}$ mutant palatal shelves[15] and retinoid acid overexpressed human palatal mesenchymal cells[49], it is possible that they are involved in different pathways regulating palate development.

Although Tenascin-X expression has not reported in the palate, Tenascin-X deficiency (CAH-X syndrome) exhibits a bifid uvula, a mildest form of cleft palate.[54,55] Interestingly, three proteins important in palate development, TGF-β2, TGF-β3, MMP13, are all increased in Tenascin-X deficiency patient fibroblast and tissues.[56]

### Periostin

Periostin is a secreted 90KD glycoprotein identified from a mouse MC3T3-E1 osteoblastic cell line and originally named as osteoblast-specific factor 2.[57] Periostin promote cell motility via integrin-dependent cell adhesion.[58] Periostin also plays an essential role in bone and tooth development.[59] Periostin null mice are growth retarded, showing incisor enamel defects indicating important roles for tooth and bone development.[59-61]

Periostin protein and mRNA are spatiotemporally expressed in the palate (Table 2).[7,53,62,63] In the anterior palate (hard palate), periostin is expressed by in the mesenchyme on the oral side and part of basal membrane.[7,62] In contrast, periostin is highly expressed in the entire posterior palate (soft palate).[7,62]

Periostin intensified around MEE when they are undergoing EMT and transdifferentiating into MES.[62] In contrast, laminin and Type IVcollagen, two major ECM in MEE basement membrane, are degraded earlier than periostin.[62] This indicates that periostin is involved regulating MEE fate during the palate fusion process.[62]

Both TGF-β2, Col I and periostin expression are detected in the palatine aponeurosis region of the soft palate.[7] Exogenous TGF-β2 can induce periostin and Col I expression in the palate tissue in organ culture which indicates that TGF signaling might regulate soft palate development by mediating periostin expression.[7]

### Laminins

Laminins are a group of heterotrimeric glycoproteins composed of α, β, γ polypeptide chains and contribute to the assembly of the basement membrane.[64] Laminins can bind to and interact with other ECMs such as Col IV and nidogen and activate cell receptors such as integrins, glycolipids, proteoglycans, and glycoproteins[65], therefore mediate cell adhesion, migration, and differentiation.[64] Before palatal shelve elevation, laminin is present discontinuously in the basement membrane and intercellularly in MEE cells (Table 2).[17,66] However, in the Tgf-β3 mutant which failed to fuse, laminins are upregulated and become continuous in the basement membrane under MEE cells.[17] It indicates that dynamic assembly of laminins in the basement membrane is regulated by the growth factors.

### Fibrillins

Fibrillins are a group of large extracellular glycoproteins including three isoforms, Fibrillin-1, −2, −3.[43] They compose the core microfibrils in the ECM of elastic and non-elastic tissues, and interact with integrins directly[43] or bind and activate Tgf-β.[67] *Fibrillin-1* mRNA is weakly expressed in the palatal mesenchyme only before palatal shelf elevation (Table 2).[6] In contrast, *Fibrillin-2* mRNA is enriched in the nasal side of palatal mesenchyme before palatal shelf elevation, then increased and expanded around *Tgf-β3*+MEE cells and in the oral side of palatal mesenchyme.[6] The close relationship of strong *Fibrillin-2*+mesenchyme cells and *Tgf-β3* +MEE cells indicates that Fibrillin-2 may be important for Tgf-β mediated palatal fusion. Retinoid acid, an important regulator during embryogenesis, dose-dependently inhibit fibrillin-2 production in human fetal palatal mesenchymal cells *in vitro*.[49]

### Proteoglycans (PGs)

Proteoglycans are a group of complex protein families characterized by anionic glycosaminoglycan (GAG) chains covalently bounding to core proteins.[68] GAG chains can be classified into the below 5 classes: chondroitin sulfate (CS), heparan sulfate (HS), keratan sulfate (KS), dermatan sulfate (DS), and hyaluronan (also called hyaluronic acid, HA). Hyaluronan is a non-sulfated glycosaminoglycan and not attached to a protein core. CS, HS, KS, and DS attaching to core proteins form CSPGs, HSPGs, KSPGs, and DSPGs, respectively. PGs are classified based on their cellular and subcellular location, overall gene/protein homology and protein modules within the respective protein cores.[68] Only pericellular and extracellular proteoglycans regarded as ECM proteins will be discussed in this review. Pericellular proteoglycans such as perlecan (HSPGs) located in the basement membrane interact with each other and participate in modulating growth factors.[68] Extracellular proteoglycans constitute the major structural complex, provide viscoelastic properties, retain water, and keep osmotic pressure and regulate cell migration, proliferation, apoptosis, and angiogenesis by interacting with several receptor tyrosine kinases.-[68] During palate development, CSPGs, HSPGs, KSPGs, and DSPGs are all enriched in palatal shelves (Table 2).[11,25,69,70] For a long time, GAGs accumulation and hydration were regarded as the main source of the intrinsic force for palatal shelf elevation, as cleft palate is induced after GAG biosynthesis suppression.[9,71,72] But evidence has emerged that proteoglycans are also essential for palatal adhesion and osteogenesis.

### CSPGs

CSPGs are highly expressed in the palatal mesenchyme during the palate development (Table 2).[11,14,18] Interestingly, CS chains are transiently upregulated on the apical surface of palatal medial edge epithelial (MEE) cells when they become closer and make contact.[11,18] Alteration of CS chain synthesis or its specific digestion disrupts palatal shelves adhesion *in vitro*, indicating CSPGs play a functional role at palatal adhesion.[18] The expression of CS chains is shown absent in the MEE cells of TGF-β3 null mutant mice, whose palatal shelves are unable to fuse in the midline.[18] However, the expression of CS chains, together with palatal shelf adhesion, can be re-induced by the addition of TGF-β3 in palate shelf organ culture.[18,73] Besides CS chains, core proteins such as biglycan, decorin, versican are also significantly increased in MEE cells as the palatal shelf adhering.[12,13] Inhibitor of TGFβ signaling with SB431542, a TGFβrI kinase inhibitor[74], caused the failure of palate shelf fusion together with the downregulation of biglycan and decorin protein from the MES. These studies indicate that biglycan and decorin are involved in palatal shelve adhesion downstream of TGFβ signaling. Although biglycan and decorin single or double knockout transgenic mice have no cleft palate[75], other factors might compensate their roles during palatal shelve adhesion.[13] All these results indicate that remodeling of proteoglycans by TGFβ signaling are important for the palatal adhesion process.

The protein and mRNA levels of biglycan and decorin are also found in the palatal mesenchymal cells at different palate development stages, although their expression is transiently downregulated when palatal shelves are elevating and closing.[13,76] Interestingly, in retinoic acid included mice cleft palate, decorin positive area, not biglycan, is unable to downregulate in the mesenchyme when palatal shelves are elevating, indicating that decorin is more important in palatal shelf elevation than biglycan during palatogenesis.[76]

Ectopic Hh signaling in the palatal mesenchyme leads to the defective palatine formation and fully penetrant cleft palate and defective osteogenesis.[77] In these mutants, significantly downregulation of the mRNA of decorin (*Dcn*) and lumican (*Lum*, a major KSPGs) in the palatal mesenchyme indicate that decorin and lumican also play roles in the palatal cell fate determination. Recently, another study showed that reducing half the abundance of CSPGs by knocking out a key CS biosynthesis glycosyltransferase caused malocclusion, skin hyperextension, severe intramembranous ossification, and cartilage formation defects in the craniofacial development.[14] These mutants exhibited significant thinner palate (5% mutant has a cleft palate), where Col I, Wnt3a, β-catenin are all downregulated.[14] Therefore, CSPGs in palatal mesenchyme probably mediate palatal mesenchyme osteogenesis by regulating the biosynthesis of collagen type 1 and deposition of CS-binding molecules Wnt3a during palate development.

### HSPGs

Heparan sulfate (HS), a sulfated GAG, is dynamically expressed in the developing palate (Table 2).[11,70,78,79]

Before palate shelf elevation, HS expressed in the basement membrane of the whole palate and in the mesenchyme of the anterior palate.[11] The expression of HS in the mesenchyme become evident in the mesenchyme at the tip of whole palatal shelf when palatal shelf is elevating and gradually disappears when palatal shelf fused together.[11] The expression of HS in the basement membrane is stronger in the oral/lateral side than the nasal/medial side after palatal shelf elevation.[11] HS directly or indirectly regulates SHH and FGF signaling[80-83], two key signaling pathways during palate development.[1,84] HSPGs bind both FGFs and FGFRs directly, stable their ternary complex, remain FGFs concentration in the local area, and regulate signaling activation.[81,82,85] HSPGs can act as Shh co-receptors activating Shh signaling and promote cell proliferation.[80] Genetically abolish heparan sulfate in the lung epithelial cells leads to reduced SHH production in the epithelial and expanded *Fgf10* expression in the underlying mesenchyme in the lung development.[83] Shh secreted by the palatal epithelium signals to the underlying palatal mesenchyme and regulate Fgfs expression.[1] Fgfs, in return, can either positively or negatively regulate SHH expression in the epithelium.[1,84,86,87] But additional studies need to clarify if HSPGs coordinate SHH and FGF signaling transduction in the epithelial–mesenchymal interactions during palate development.

*HA*

Hyaluronic acid (HA) is a high molecular mass GAG, which helps to retain a large amount of water in the mesenchyme. As a major component of palatal mesenchyme, HA is shown accumulating in the nasal side and in the hinge region of the palatal mesenchyme with higher levers in the anterior/mid-part than anterior-most and posterior palate (Table 2).[9,11,78] Regionally specific accumulation of extracellular GAGs, predominantly HA, is proposed to be the intrinsic force to drive palatal shelf elevation.[10,49] Mice homozygous for *Fgfr2*$^{C342}$, *Pax9* and *Golgb1* mutation, which have a palatal shelf elevation defect, exhibit reduced HA accumulation in the palatal shelves.-[9,88,89] HA synthase Has 1, 2, and 3, which synthesize HA at the plasma membrane, are disrupted in TGF-β3 mutant palatal shelves which failed to fuse in the midline, indicating that HA remodeling in palate is highly regulated by Tgf-β signaling pathways.[49]

## Integrin signaling

Integrins are a family of heterodimeric transmembrane receptors facilitating cell-ECM adhesion and signal transduction. By the combination of 18 α-subunits and 8 β-subunits, 24 distinct integrin heterodimers form and bind to different ECM proteins, such as α1β1, α2β1, α10β1, α11β1 for collagen; α3β4, α6β4, α7β4, α9β4 for laminin; α5β1, α8β1, αvβ1, αvβ6, etc., for RGD (a tripeptide sequence, present in ECM such as fibronectin and vitronectin).[90] Several integrin subunits are present in the palate. Integrins α5 is expressed by the palatal mesenchyme and apical side of MEE cells at E13.5, and its expression around MEE is absent in the Tgf-β3 mutant.[17] In contrast, Integrins β1 is absent from palatal mesenchymal cells but highly expressed by MEE cells at E13.5.[17,62] Although the expression of Integrins β1 is no change in the Tgf-β3 mutant.[17] The addition of Tgf-β3 in the palate culture induces both Integrins α5 and Integrins β1 expression on the MEE apical surface.[17] Anti-Integrins α5 antibody blocks palate shelve adhesion in organ culture.[17] Inactivation of Integrins α5 from either palatal neural crest cells with *TFAP2α*$^{IRESCre}$ or from mesodermal cells with *Mesp1*$^{Cre}$ can cause cleft palate.[47,48] But further studies are needed to clarify how Integrins α5 and Integrins β1 are involved in ECM remodeling and signaling transduction during palatal shelf elevation and adhesion. Integrin αV, β3, β5 are also highly expressed by MEE cells.[62] But their functions during palatal adhesion need to be further investigated. Loss of both Integrins α5 and αV from palatal neural crest cells with *Wnt1*$^{Cre}$ leads to cleft palate, where palatal shelves still remain small at E17.5[91], indicating that Integrins α5 and αV are essential for palatal shelve expansion. Only a small population of Integrin β8 heterozygous and homozygous embryos developed cleft palate[92], indicating other α subunits are required for cleft palate phenotype.

Talin (Tln) is one of the important intracellular proteins which activates integrins by binding to its β subunit.[93,94] Two *Tln* isoforms are present in most vertebrates[95] and three in zebrafish.[96] In zebrafish, *tln1* is required for the cranial neural crest cell proliferation during palate morphogenesis.[97] In mice, global loss of *Tln1* leads to embryonic lethality during gastrulation[98], while *Tln2* null mice are viable and fertile.[99] Conditional mouse models would provide more evidence on how Talin engages in Integrin signal transduction during palate development.

## ECM remodeling by Extracellular metalloproteinases

ECM is dynamically remodeled by extracellular metalloproteinases, including Matrix metalloproteinases (MMPs) and their endogenous tissue inhibitors (TIMPs), a disintegrin and metalloproteinases (ADAMs), and ADAMs with thrombospondin motifs (ADAMTS). During palate development, MMPs and TIMPs are spatiotemporally expressed in the mouse embryos, correlating to their ECM substrates (Table 3).[100]

MMPs 2, 3, 9, 13, 14, and 25 and TIMPs 1, 2, and 3 are spatiotemporally expressed in the mouse embryonic palate and MMP-9 and TIMP 4 are detected in the newborn human palate tissue suggesting ECM remodeling by MMPs and TIMPs are essential for palate development (Table 3).[100-104] More importantly, MMP-13 and TIMP-2 are transiently highly upregulated in the MEE while palatal shelves are elevating and fusing.[100-103] Their expression patterns preceded the decreases of their substrates, fibronectin, collagen I, and III.[100,101,103] In Tgf-β mutant mouse, which failed to fuse palate shelves in the midline, MMP-13 and TIMP-2 are significantly reduced or totally absent in the MEE.[105] And inhibition of MMP-13 synthesis and excessive TIMP-2 in palatal organ culture phenocopies Tgf-β mutant phenotype[105,106], indicating that ECM remodeling in the midline is essential for palatal fusion.

Almost 80% of double null of MMP-14 and −16 have a cleft palate.[107] Neither MMP-14 nor MMP-16 single mutant exhibits a cleft palate, indicating that MMP-14 and MMP-16 have overlapping roles on ECM remodeling during palate development.[107]

MMP-25 protein and mRNA are increased in the tips of palatal shelve while palatal growing, where it significantly decreased when neutralize TGF-β3[108] Knockdown of MMP-25 in palatal organ culture impairs palate shelf fusion and persistent MES, indicating MMP-25 is a direct transcriptional target for Tgf-β3 in the palate development.[108]

Adamts 20 was found universally in the palatal shelf mesenchyme, while Adamts 9 mainly in the palate microvascular endothelium.[12] Although Adamts 9 and 20 single mutant did not show cleft palate, haploinsufficiency of Adamts9 in Adamts 20 mutant mice (*Adamts 9*$^{\pm}$*;Adamts20*$^{bt/bt}$) showed a secondary cleft palate with 100% penetrance.[12] These mice had defects in palatal mesenchymal cell proliferation and versican cleaving.[12] Haploinsufficiency of versican (VCAN) in Adamts 20 mutant mice (*Vcan*$^{hdf/+}$*;Adamts20*$^{bt/bt}$) phenocopied the phenotype of *Adamts 9*$^{\pm}$*; Adamts20*$^{bt/bt}$ mice.[12] The collectively versican proteolysis by ADAMTS 9 and 20 in the palate are important for regulating palatal mesenchyme cell proliferation.

Taken together, these studies indicate an important role of ECM remodeling by extracellular metalloproteinases for palatal shelve expansion and fusion. But the corresponding ECM substrates of most extracellular metalloproteinases during palatogenesis are still unknown. It will be interesting to further explore how breakdown of other ECM such as collagen, proteoglycans, fibronectin, etc., by extracellular metalloproteinases facilitate palatogenesis.

## Conclusion and future direction

In summary, many ECM and related genes are found to be involved in the palate development. ECM not only form the basic infrastructure of palatal shelves, but also play pivotal roles regulating cell proliferation, adhesion, cell fate determination in the morphogenesis of the secondary palate. However, the complex ECM functions and remodeling for palatal shelf expansion, elevation, and fusion has not yet been identified. We still know only little about the interaction of ECM themselves, and ECM and growth factors at different stages of palate development. Although a variety of ECM proteins expressed temporospatial during palatogenesis, it is still unknown how their dynamical expression patter might contribute to the distinct anterior-posterior palatal shelf elevation behavior. Besides, multiple signaling pathway regulates palate ECM elasticity and stiffness. But the contribution of mechanical transduction of ECM stiffness to palate elevation remains largely unknown. Future genetic studies will help us further understand the function of ECM remodeling during palatogenesis.

**Table 3.** MMP associated with palate development.

| Extracellular metalloproteinases | Subtype/alternative name | Expression pattern in the palate | | | ECM substrates during palate development | Mouse mutant associated with cleft palate | | |
|---|---|---|---|---|---|---|---|---|
| | | Location | Detection methods | References | | Mouse mutants | Defects | References |
| MMPs | MMP-1/Collagenase-1 | Unknown | qRT-PCR | [24] | NR | NR | | |
| | MMP-2/Gelatinase A | Before palate shelf elevation: in the palatal mesenchyme and basement membrane, intensified gradually in the nasal-medial part.<br>During the palatal shelf elevation and the palatal shelf fusion: intensified gradually in the tip and nasal-medial part and MES<br>After the palatal shelf fusion: strong in palatal mesenchyme around MES. | qRT-PCR, IHC, ISH | [1-3,24,100,105] | Cleaves type I collagen | NR | | |
| | MMP-3 | Extensively expressed in palatal mesenchyme, transiently upregulated in a subset of nasal palatal epithelial cells. | IHC | [100,102,106] | NR | NR | | |
| | MMP-9/Gelatinase B | Extensively expressed in palatal mesenchyme, transiently upregulated while palatal shelves elevating and fusing. | qRT-PCR, IHC, ISH | Human and mouse:[24,102,104] | Cleaves laminin | NR | | |
| | MMP13 | Before palate shelf elevation: in the palatal mesenchyme and basement membrane, intensified gradually in the nasal-medial part.<br>During the palatal shelf elevation and the palatal shelf fusion: intensified gradually in the tip and nasal-medial part and MES<br>After the palatal shelf fusion: still strong in the nasal-medial mesenchyme and MES but decreased in other area. | IHC, ISH | Mouse[100,105,119] | NR | NR | | |
| | MMP-14/Membrane Type 1-MMP (MT1-MMP) | Highly in MEE | IHC, ISH | [105] | NR | Double knockout of MMP-14 and −16 | 80% of double null of MMP-14 and −16 have a cleft palate | [107] |
| | MMP-16/Membrane Type 3-MMP (MT3-MMP) | Unknown | | | NR | Double knockout of MMP-14 and −16 | 80% of double null of MMP-14 and −16 have a cleft palate | [107] |
| | MMP-25 | The tips of palatal epithelium and mesenchyme | IHC, ISH | [108] | NR | NR | | |

(*Continued*)

**Table 3.** (Continued).

| | | Expression pattern in the palate | | | | Mouse mutant associated with cleft palate | | |
|---|---|---|---|---|---|---|---|---|
| Extracellular metalloproteinases | Subtype/alternative name | Location | Detection methods | References | ECM substrates during palate development | Mouse mutants | Defects | References |
| TIMPs | TIMP-1 | Before palate shelf elevation: in the central and maxillary region of palatal mesenchyme<br>During the palatal shelf elevation and the palatal shelf fusion: intensified gradually in the tip and nasal-medial part and MES; palatal basement membrane<br>After the palatal shelf fusion: strong in the nasal-medial mesenchyme and MES but decreased in other area. | IHC | [100] | NR | NR | | |
| | TIMP-2 | Before palate shelf elevation: in the central and maxillary region of palatal mesenchyme<br>During the palatal shelf elevation and the palatal shelf fusion: intensified within palatal mesenchyme; palatal basement membrane<br>After the palatal shelf fusion: strong in the nasal-medial mesenchyme and future osteogenic sites. | IHC | [100] | NR | NR | | |
| | TIMP-3 | Palatal epithelium, transiently expressed in mid-oral and ventral-medial mesenchyme. | IHC | [100] | NR | NR | | |
| | TIMP-4 | Oral mucosa | IHC | [104] | NR | NR | | |
| ADAMTS | ADAMTS9 | Palatal capillary endothelium | ISH | [12] | Versican | Haploinsufficiendy of Adamts9 in Adamts20 mutant mice (*Adamts9*$^{\pm}$; *Adamts20*$^{bt/bt}$) | Complete cleft palate | [12] |
| | ADAMTS20 | Palatal mesenchyme | ISH | [12] | | | | |

NR, not reported.
IHC, immunohistochemical staining.
ISH, in situ hybridization.

## Disclosure of potential conflicts of interest

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article. The authors report no conflict interest.

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