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# Fibulins and matrilins are novel structural components of the periodontium in the mouse

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ABSTRACT

Periodontitis refers to inflammatory disease of the periodontal structures (the gingiva, dental cementum, periodontal ligament (PDL) and alveolar bone) that ultimately leads to their destruction. Whereas collagens are well-examined main components of the periodontium, little is known about the other structural proteins that make up this tissue. The aim of this study was to identify new extracellular matrix (ECM) components, including fibulins and matrilins, in the periodontium of mice.

After sacrificing 14 mice (Sv/129 strain), jaws were prepared. Each tissue sample contained a molar and its surrounding alveolar bone. Immunohistochemistry was carried out on paraffin-embedded sections.

Our results show that mice exhibit fibulin-3, -4 and -5 and matrilin-1, -2, -3 and -4 in PDL and in blood vessels of alveolar bone and PDL as well as in the pericellular matrix of osteocytes and cementocytes. In dental cementum, only fibulin-4 is expressed.

For the first time, we show that fibulin-3, -4 and -5 and matrilin-1, -2, -3 and -4 are essential components of the periodontal tissues. Our findings indicate an association of these proteins with collagens and oxytalan fibers that might be of future interest in regenerative periodontitis therapy.

## 1. Introduction

Periodontitis is an inflammatory and destructive disease that affects the periodontal tissues, including the gingiva, dental cementum, periodontal ligament (PDL) and alveolar bone (Bartold & Narayanan, 2006; Page, Offenbacher, Schroeder, Seymour, & Kornman, 1997). The PDL is the central structure that dynamically attaches the tooth to the surrounding bone via a complex system of collagenous fibers (Hassell, 1993; Nanci & Bosshardt, 2006). This fiber apparatus consists primarily of collagens I and III (Huang, Ohsaki, & Kurisu, 1991) and smaller amounts of collagens V, XII and XIV (Becker et al., 1991; Karimbux, Rosenblum, & Nishimura, 1992; Zhang, Schuppan, Becker, Reichart, & Gelderblom, 1993). Although this network has been well studied, little is known about other structural proteins that might be important in the pathogenesis of periodontitis and that, consequently, might represent potential targets for periodontitis therapy.

Fibulin-3, -4 and -5 belong to a family of extracellular glycoproteins that can be found in elastic tissues such as aorta, lung and skin as well as in blood vessel walls and basement membranes (Kobayashi et al., 2007; Miosge et al., 1996; Yanagisawa et al., 2002). Unlike the larger proteins fibulin-1 and -2, which seem to be dispensable for elastogenesis (Argraves, Tran, Burgess, & Dickerson, 1990; Sicot et al., 2008), fibulin-3, -4 and -5 play a crucial role during this process (Choudhury et al., 2009). Although mature elastic fibers have not been identified in the periodontium, immature elastic fibers, the so-called oxytalan fibers, have been detected (Fullmer & Lillie, 1958). The observation that mechanical stress induces the synthesis of these fibers suggests a crucial role for oxytalan fibers in maintaining the stability of the PDL (Jonas & Riede, 1980; Strydom, Maltha, Kuijpers-Jagtman, & Von den Hoff, 2012).

Matrilin-1, -2, -3 and -4 are non-collagenous glycoproteins of the ECM (Deak, Piecha, Bachrati, Paulsson, & Kiss, 1997; Paulsson & Heinegard, 1981; Wagener, Kobbe, & Paulsson, 1997; Wagener et al., 1998) that are capable of forming filamentous networks (Chen, Johnson, Haudenschild, Tondravi, & Goetinck, 1995), binding to fibrillar collagens (Pareti, Niiya, McPherson, & Ruggeri, 1987) and modulating collagen fibrillogenesis (Nicolae et al., 2007). These matrilins are widely expressed in embryonic tissues, including skin, lung and kidney (Klatt, Paulsson, & Wagener, 2002). In postnatal tissues, they are detectable in cartilage (Paulsson & Heinegard, 1981; Wagener

*Abbreviations:* ECM, extracellular matrix; PDL, periodontal ligament; TBS-T, Tris-buffered saline with Tween 20

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et al., 1997).

In this study, we localize matrilins and fibulins in the mouse periodontium for the first time and introduce them as two novel structural components of this tissue.

## 2. Materials & methods

### 2.1. Tissue sources and preparation

The study was performed on 14 mice (Sv/129 strain) of both sexes between 18 and 47 weeks of age. The animals were obtained according to the regulations of the Animal Welfare Act of the County of Lower Saxony, Germany. After sacrificing the animals, the molar regions of the mandibles and maxillae were detached from the attached gingiva and dissected into specimens each comprising a molar and its surrounding PDL and alveolar bone. The specimens were decalcified in 20% ethylenediaminetetraacetic acid (E5134, Sigma-Aldrich, Saint Louis, Missouri, USA) for 5 weeks, dehydrated using an ascending alcohol series and embedded in paraffin. Then, they were cut into tissue sections of 6–7 µm thickness.

### 2.2. Antibodies

Affinity-purified polyclonal rabbit-anti-fibulin-3, -anti-fibulin-4 and -anti-fibulin-5 antibodies were generously provided by Dr. Timpl (Max Planck Institute, Martinsried, Germany). The antibodies have previously been shown to be specific for the individual fibulin subtypes (Kobayashi et al., 2007). The anti-matrilin-2, anti-matrilin-3 and anti-matrilin-4 antibodies used in this study are polyclonal, affinity-purified rabbit antibodies provided by Prof. Wagener (University of Köln, Germany). They have been demonstrated to be specific for the individual matrilin subtypes (Hauser & Paulsson, 1994; Klatt et al., 2000, 2001; Piecha et al., 1999).

### 2.3. Immunohistochemistry

Immunoperoxidase staining of decalcified paraffin-embedded tissue sections was performed as follows. First, the tissues were deparaffinized, rehydrated and rinsed for 10 min in Tris-buffered saline with Tween 20 (TBS-T). To unmask the antigens, the samples were treated with citrate buffer for 20 min; endogenous peroxidase was then blocked by a 20-min treatment with a universal blocking solution and with a solution of methanol and 3% $H_2O_2$ in the dark. The sections were pretreated for 2 min with 10 µg/ml protease XXIV (P8038; Sigma, Deisenhofen, Germany). Each of the steps in the reaction was followed by rinsing for 10 min in TBS-T. The sections were incubated with antibodies at a dilution of 1:100 in Antibody Diluent® (Cell Marque, Rocklin, California, USA) for 12 h at room temperature. Antibody detection was performed using a two-step horseradish peroxidase-based polymer detection system (HiDef Detection™, Cell Marque). The color reaction utilized a diaminobenzidine substrate. Tissue sections without antibody treatment served as negative controls. Color reactions were observed using a light microscope (PrimoStar, Zeiss, Göttingen, Germany).

## 3. Results

Fibulin-3 was detectable in lacunae of cementocytes but not in the surrounding ECM (Fig. S1a). In the PDL, fibulin-3 was located in the ECM and in blood vessels (Fig. 1b). In the alveolar bone, fibulin-3 was found in the lacunae of osteocytes and blood vessels, but not in the ECM (Fig. 1c).

Fibulin-4 was located in the lacunae of cementocytes (Fig. S1b), in the ECM of dental cementum, in the PDL and in blood vessels (Fig. 2b). Fibulin-4 was not located in the ECM of the alveolar bone, but it was found in the lacunae of osteocytes and in bone blood vessels (Fig. 2c).

Fibulin-5 was detectable in the lacunae of cementocytes (Fig. S1c) but not in the ECM of cementum. In the PDL, it was found in ECM and in blood vessels (Fig. 3c). It was located in the lacunae of osteocytes and in blood vessels of the bone, but not in the ECM (Fig. 3b).

Matrilin-1 was not detectable in the ECM of the cementum, but in the lacunae of cementocytes. In the PDL, matrilin-1 was located in the ECM as well as in blood vessels (Fig. 4b). In blood vessels of bone and in the lacunae of osteocytes, matrilin-1 was present, but not in the bone ECM (Fig. 4c).

Matrilin-2 was not detectable in the ECM of cementum, but in the lacunae of cementocytes (Fig. S1d). In the PDL, both the ECM and blood vessels expressed matrilin-2 (Fig. 5d). Matrilin-2 was not found in the ECM of the alveolar bone but in the blood vessels and in lacunae of osteocytes (Fig. 5b).

Matrilin-3 was not located in the ECM of cementum, but in the lacunae of cementocytes. In the PDL, matrilin-3 was found in the ECM as well as in blood vessels (Fig. 6b). In the ECM and in lacunae of osteocytes in alveolar bone, matrilin-3 was not expressed. In the blood vessels of bone, however, matrilin-3 was found (Fig. 6c).

Matrilin-4 was not located in the ECM or in the lacunae of cementum, but in the ECM of the PDL as well as in blood vessels of the PDL (Fig. 7b). Matrilin-4 was not expressed in the ECM and in lacunae of osteocytes in alveolar bone. However, it was located in blood vessels in bone (Fig. 7c). A summary of the immunohistochemistry results is given in Tables 1 and 2.

## 4. Discussion

In this work, we demonstrate for the first time the localization of fibulins and matrilins in the adult periodontium of mice. In elastic tissues, fibulins are closely associated with elastogenesis (Choudhury et al., 2009); because mature elastic fibers have not been described in the periodontium, we assume an association of fibulins with oxytalan fibers. These early stages of elastic fiber development build a dense three-dimensional network between collagenous fibers in the PDL (Carmichael & Fullmer, 1966; Fullmer & Lillie, 1958; Sims, 1975). This distribution is consistent with our results that indicate homogenous expression of fibulins in all regions of the PDL. Moreover, all fibulins were located in blood vessel walls. This finding strengthens the assumption that fibulins co-localize with oxytalan fibers because they are closely associated with the vascular system (Strydom et al., 2012). In addition to their role in elastogenesis, fibulins have recently been described as ECM proteins involved in collagen maturation; fibulin-4, in particular, seems to function as a scaffolding protein during this process (Papke et al., 2015). Our results indicate that the expression of fibulins in periodontal tissues, which are very rich in collagen, can be ascribed to their role in collagen maturation. The PDL's uniquely high collagen turnover (Sodek, 1977) further substantiates this assumption. Remarkably, fibulin-4 was the only fibulin expressed in the ECM of cementum. This finding is consistent with Papke et al. (2015), who suggest a unique and very particular role for fibulin-4 in ECM organization. Interestingly, a study on the tooth root development in mouse incisors and molars reveals that all members of the fibulin family except for fibulin-4 are expressed in cementoblasts of developing teeth (Sun et al., 2012). The present study was performed on adult mouse molars; supposedly, there is a switch in the expression pattern of fibulins during tooth root development that needs to be clarified by future studies. In the alveolar bone, all fibulins and matrilin-1 and -2 were expressed in pericellular regions of osteocytes suggesting a participation of the proteins in the mineralization process of the bone ECM. For fibulin-1, an involvement in the craniofacial bone formation has been described (Cooley et al., 2014); fibulin-5 is indispensible for the regulation of mesenchymal cell proliferation in premaxillary bone sutures (Noda, Nakamura, & Komatsu, 2015). The present results indicate a role for fibulins in the adult murine bone beyond craniofacial development.

Matrilins are widely expressed in embryonic tissues (Deak et al.,