UHASSELT
KNOWLEDGE IN ACTION

Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Masterthesis

The role of FAPalpha in dental stem cells and root development

Ana Amaya Garrido

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Environmental Health Sciences

SUPERVISOR :
Prof. dr. Ivo LAMBRICHTS

CO-SUPERVISOR :
Dr. Ronald DRIESEN

2017
2018

Transnational University Limburg is a unique collaboration of two universities in two countries: the University of Hasselt and Maastricht University.

UHASSELT
KNOWLEDGE IN ACTION

www.uhasselt.be
Universiteit Hasselt
Campus Hasselt:
Martelarenlaan 42 | 3500 Hasselt
Campus Diepenbeek:
Agoralaan Gebouw D | 3590 Diepenbeek

UHASSELT
KNOWLEDGE IN ACTION

Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Masterthesis

The role of FAPalpha in dental stem cells and root development

Ana Amaya Garrido

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Environmental Health Sciences

SUPERVISOR :
Prof. dr. Ivo LAMBRICHTS

CO-SUPERVISOR :
Dr. Ronald DRIESEN

Content

Acknowledgments

My greatest and most sincere gratitude undoubtedly goes to my daily supervisor Ronald Driesen. I can't thank him enough for his kindness and trust, which helped me to grow as a researcher. I would also like to thank professor Ivo Lambrichts for the great opportunity he provided me with this interesting topic of study.

My warm thanks go to all my friends and colleagues, for making me feel like home, I couldn't be more grateful to all the funny coffee breaks. Especially I must remark Joël Beaumont and Sam Vanherle for all their help and consultations. I would also like to express thanks to the entire morphology group, I value all the time we spent together in the lab.

Further, I would like to thank my friend Michal Gulka, one of the smartest scientists I've ever met and my great support here in Hasselt. Lastly, but most important, I would like to thank my family and friends. Especially my parents for supporting me from the distance and my sister for encouraging me to achieve my goals.

Abbreviations

BM-MSCs: bone marrow-mesenchymal stem cells

CXCR4: CXC chemokine receptor type 4

DFSCs: dental follicle stem cells

DPP-IV: dipeptidyl-peptidase IV

DPSCs: dental pulp stem cells

ECM: extracellular matrix

EMT: epithelial-mesenchymal transition

ERM: epithelial rests of Malassez

ESCs: embryonic stem cells

FAPα: fibroblast activation protein-alpha

HERS: Hertwig's epithelial root sheath

IL-1b: interleukin-1b

MMP: matrix metalloproteinase

MSCs: mesenchymal stem cells

PBM: photobiomodulation

PSCs: perinatal stem cells

ROS: reactive oxygen species

SA: solid/multicystic ameloblastoma

SCAPs: stem cells from the apical papilla

SHEDs: exfoliated deciduous teeth

SDF-1: stromal cell-derived factor 1

TAMs: tumour-associated macrophages

TGF-β1: transforming growth factor-beta 1

UA: unicystic ameloblastoma

Summary

Fibroblast activation protein-alpha (FAPα) is an integral membrane protein with dipeptidyl-peptidase and type I collagenase activity predominantly expressed during embryogenesis. Reaching adolescence, FAPα expression is strongly reduced, only increasing during pathological conditions including wound healing, tumour growth and myocardial infarction. FAPα expression was recently observed in bone-marrow mesenchymal stem cells (MSCs) contributing to stem cell migration. Ongoing research from our group revealed the presence of FAPα in MSCs from the dental tissue. In this project, we hypothesized that FAPα plays an important role in dental root development. For that we aimed to study FAPα expression in dental stem cells directly involved in root development. First, in dental follicle stem cells (DFSCs) and Hertwig's Epithelial Root Sheath (HERS), a bilayer of epithelial cells that is disrupted during development acquiring stem cell features. Then, on Stem Cells from the Apical Papilla (SCAPs), providing new information about this less studied tissue which is lost after dental root is matured. At last, the expression of FAPα was studied in ameloblastoma, an odontogenic tumour provoked by impaired tooth development. To achieve this, a characterization through immunohistochemistry and immunofluorescence analysis was performed in tissue sections and in culture.

In this study we report for the first time the presence of FAPα on DFSCs and SCAPs. In the dental follicle, a high positivity for FAPα was either found in the extracellular matrix or in the epithelial remnants of HERS, also highly stained for E-cadherin. We also provided novel histological insights about apical papilla structure identifying two regions: cortex fibrosa, located at the periphery with dense aligned collagen type I, and medulla, in the core of the tissue possessing loose cross-wise organized collagen. A decrease in apical papilla size was found during maturation, ranging from 0,3148 to 0.090 cm^2 in patients from 16 to 20 years old respectively. In young patients a fraction FAPα-vimentin+ was found in the cortex fibrosa whereas in older patients this region is predominantly FAPα+vimentin+. The medulla of young patients possessed a very positive fraction of cells FAPα+vimentin+ compared to the older ones presenting FAPα+vimentin- cells. Immunohistochemistry showed a marked staining of TGF-β1 in the cortex fibrosa where the higher density of collagen was found. SDF-1 was also found at the periphery of the tissue co-located with FAPα, suggesting a possible migration towards the cortex fibrosa, what was corroborated with a transwell assay in which migration rate of SCAPs was increased in response to this factor. FAPα was also identified in ameloblastoma, both in the invasive epithelium as well as in the fibrous stroma. These results suggest a role of FAPα in the migration of dental stem cells during root maturation related to its collagenase function.

Introduction

1. Fibroblast Activation Protein-α (FAPα)

Fibroblast Activation Protein-α (FAPα) or "seprase" is an integral membrane protein belonging to the serine protease family capable of cleaving the bond between proline and other amino acids of proteins (3). The human FAPα gene is highly conserved among species and is located on 2q24.2 (4). The serine protease family is composed of a variety of enzymes including dipeptidyl peptidase IV (DPP-IV), which shares 50% of homology in amino acids with FAPα. Despite that, FAPα presents an additional enzymatic function that DPP-IV is lacking, the cleavage of collagen type I through collagenase activity (4). Furthermore, gelatin zymography proved that FAPα can cleave extracellular matrix (ECM) proteins, including collagen I, collagen IV, fibronectin, laminin and gelatin (5).

1.1 FAPα expression

Since its discovery, the presence of FAPα has been intensively studied and reported overall at four main sites: the embryogenic mesenchyme, mesenchymal stem cells, wound healing tissues and stromal fibroblasts of epithelial cancers.

Normally, when reaching the age of adolescence, FAPα expression is nearly lost in healthy tissues. However, FAPα was first detected in activated fibroblasts responding to cancers and in granulation tissue (6). After that, others reported its presence in several types of epithelial cancers like colorectal, breast, ovarian, lung and pancreas (7), and diseases such as arthritis (8) or fibrosis (4).

Diverse studies suggested that FAPα contributes to tumour cell invasion through tissue remodeling. Tissue remodeling is a crucial process in development, wound healing, chronic inflammation, fibrosis and cancer (9). It mainly requires the degradation of the extracellular matrix (ECM), composed mainly by proteoglycans and fibrous proteins like collagen (10). This degradation is mainly executed by metalloproteinases (MMPs) and dipeptidyl-peptidases (DPPs). Due to its enzymatic activities, FAPα was added to this group of enzymes as a candidate of tissue remodeling through dipeptidyl-peptidase and collagenase type I activity (11). This assumption was corroborated in human ovarian carcinoma cells where FAPα appeared to increase proliferation, invasion and migration (12). Moreover, the collagenase activity was further confirmed in cell culture with the degradation of type I collagen (13). Finally, a study in pancreatic cancer cells provided an excellent insight of these evidences where through a 3-D matrix system, FAPα enzymatic activity appeared to modify locally ECM facilitating tumour invasion (14).

Nevertheless, an increase in the presence of FAPα was revealed in mouse during tissue remodeling in early mesenchymal development (15). Given this, it may be postulated that FAPα shares a similar functional role in embryogenic development and tumour microenvironment related to the remodeling of ECM.

What's more, a positive role of this protein was outlined in scar tissues after myocardial infarction, where FAPα participated in wound healing and matrix turnover. In this case, FAPα was activated by Transforming Growth Factor-B1 (TGF-β1), specifically through smad1/2 pathway (16). This

effect of FAPα on fibroblast migration and collagenase activity indicated an emerging role in cardiac wound healing.

The last site where FAPα expression was surprisingly detected was bone-marrow derived mesenchymal stem cells (BM-MSCs) from healthy patients (17). In fact, it was demonstrated that the presence of this protein was associated with enhanced migration of these cells. BM-MSCS are released in response to injury (18), and the depletion of FAPα inhibited the migration of these cells. However, this process could be rescued by re-expressing the protein. Interestingly, FAPα levels were upregulated by cytokines interleukin-1b (IL-1b) and again TGF-β1 in bone marrow-MSCS, also contributing to migration (13). Nevertheless, the model illustrates that the activity of FAPα was not responsible for the enhancement of the migration, but it exerts its effect by modulation of RhoA GTPase, an enzyme that regulates the organization of actin cytoskeleton (19) and in consequence, cell migration and adhesion. These findings lead to suggest that non-enzymatic function of FAPα is also linked to the migration ability of MSCs, one of the key processes that is needed to enable stem cells therapy.

For this reason, we will study the role of FAPα in MSCs obtained from the dental tissue. Nonetheless, to fully understand the functionality of this protein we first must define the morphology and development of the dental tissue.

2. Tooth development and root formation

Teeth are composed of two layers of mineral, enamel and dentine, which are encasing the dental pulp. Dental pulp is mainly fibroblastic but also contains blood vessels and nerves that are entering the tooth by the apical papilla (20). The roots are attached to the alveolar bone through the periodontal ligament and the dentin from the root is surrounded by cementum rather than enamel as in the crown (21) (Figure 1).

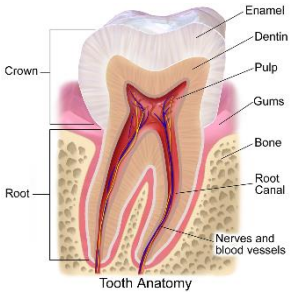

Figure 1: Schematic representation of tooth anatomy (1). Root is formed by the canal crossed by nerves and blood vessels. Pulp is encapsulated by dentin and enamel in the crown.

Before describing tooth development, it is important to remark that the interactions between mesenchyme and epithelium controlled by plenty cytokines and transcription factors, are crucial for a successful process. The formation of the tooth starts with the thickening of the oral epithelium (Figure 2). This invagination forms a bud and the mesenchyme underneath starts to condensate. Next, the epithelium starts to fold forming a cap, where the primary enamel knot is visible surrounded by the mesenchyme previously condensed. When the epithelium is extended into the mesenchyme, the bell stage is formed. The inner enamel epithelium encapsulates the dental papilla and the outer dental epithelium is surrounded by the dental follicle. By this stage, first enamel knot is replaced by secondary ones. In the new-born mammalian, the adjacent odontoblasts and ameloblasts have differentiated, and begin to produce dentin and enamel respectively (22).

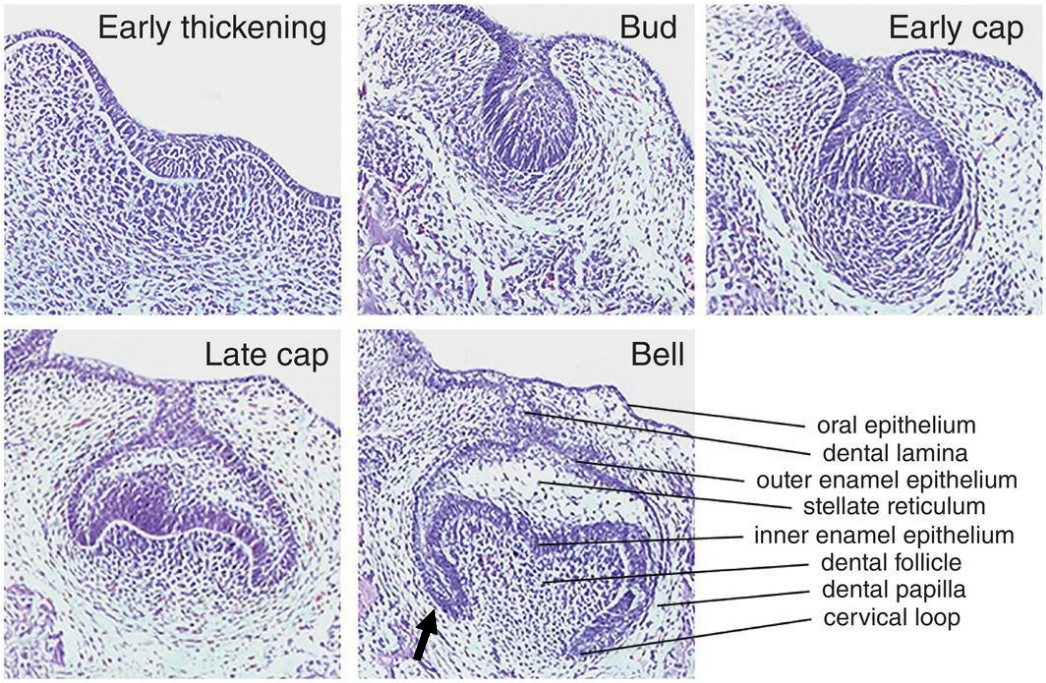

Figure 2: Representation of mammalian tooth development (1). After the early thickening of the oral epithelium, the bud is formed, followed by the early and late cap phase. At last, bell stage is reached, consisting of the cervical loop, dental papilla, dental follicle, inner and outer enamel epithelium, stellate reticulum, dental lamina and oral epithelium. Black arrow →HERS,

After the crown is completed, the formation of the root starts as the epithelial cells from the cervical loop proliferate apically and influence the differentiation of odontoblasts from undifferentiated mesenchymal cells, and cementoblasts from follicle mesenchyme. This apically bilayered sheath of the inner and outer enamel epithelium forms Hertwig's epithelial root sheath (HERS). HERS (Figure 2 black arrow) is believed to be responsible for determining the shape of the roots and participate in cementum formation (23). Subsequently, it should possess stem cell characteristics to be able to differentiate into other cell linages. Hence, it was confirmed that HERS cells undergo Epithelial Mesenchymal Transition (EMT) during the formation of acellular cementum (24). EMT is a process in which epithelial cells are converted into mesenchymal cells (25) necessary for embryogenesis and tissue development. When this occurs, there is a progressive loss of epithelial markers including a switch from E-cadherin expression to N-cadherin and a gain of MSCs markers like vimentin (26). Specifically, TGF-β1 stimulated the differentiation of HERS into periodontal ligament fibroblasts and cementoblasts-like cells (27).

Backwards to tooth development, when the first radicular dentin is formed, HERS is fenestrated and individual cells migrate away from the root into the region of the future periodontal ligament, where they form epithelial rests of Mallasez (ERM) (28). Nevertheless, after the fragmentation of HERS into isolated clusters of ERM, cell number is decreased (29). The underlying explanation of this remains unknown, but several factors could be involved, including apoptosis at the beginning of the fenestration of HERS or migration to other sites of the teeth (30).

It is clear that HERS plays a role in root formation, but it is the interaction with the apical papilla what drives the force of root elongation and maturation of the root (31). The apical papilla refers to the dental papilla located at the apices of developing permanent teeth derived from the ectomesenchyme (32). There is little information available in literature about this loose tissue (33). However, until now, the apical papilla is believed to be important for tooth development and possesses a population of early mesenchymal stem cells, named SCAPs (34).

3. Mesenchymal stem cells

Stem cells are non-specialized cells that possess the ability to differentiate into a particular cell type under the right conditions. They have gained considerable attention from the medical community as a possible source for regenerative therapeutic applications. There are three types of stem cells: embryogenic stem cells (ESCs), perinatal stem cells (PSCs) and adult or mesenchymal stem cells (MSCs). MSCs are adult multipotent stem cells found in specialised tissues with the ability of migration and replacement after injury (35). These cells can differentiate into three specific linages: osteogenic, chondrogenic and adipogenic. This feature differentiates them from ESCs, which can be specialized into the three great different lineages (ectoderm, endoderm and mesoderm) and extra-embryonic tissues. In addition, PSCs also show pluripotency differentiating into the three different lineages compared to the limited potential of MSCs. Nevertheless, MSCs have been outlined as exceptional in cell therapy due to an easy isolation from almost all tissues, such as skin, bone marrow, blood,… (36) and dental tissue, where we will focus our project.

3.1 Dental stem cells

Dental stem cells are MSCs similar to BM-MSCs which can differentiate into cells that generate mineral and play an important role in tooth homeostasis (2). Specifically, dentin repair and maintenance in postnatal organisms occurs through the differentiation of these cells into odontoblasts during reparative tissue formation (37). In 2000, Gronthos et al. were the first to isolate dental stem cells from human pulp tissue (DPSCs) (38), and after that, four more types of dental MSCS were characterized, including stem cells from exfoliated deciduous teeth (SHED) (39), periodontal ligament stem cells (PDLSCs) (40), stem cells from apical papilla (SCAPs) (32), and dental follicle precursor cells (DFPCs) (41).

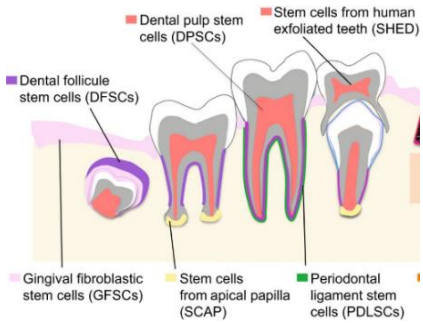

Figure 3: Dental stem cells classification (2). There are five types of dental stem cells: dental follicle stem cells (DFSCs), stem cells form the apical papilla (SCAPs), dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs) and stem cells from human exfoliated teeth (SHED).

Besides root formation, it is not clear either which type of dental stem cells is involved in repair after teeth is damaged. It has been suggested that during tooth development, odontoblasts derived from the apical papilla form primary and secondary dentin and are therefore named "primary odontoblasts". On the other hand, "replacement odontoblasts", derived from the pulp, replace mainly these primary odontoblasts and make tertiary or reparative dentin (32).

In this study, we hypothesize that FAPα plays an important role in dental root development. To assess this hypothesis, we will first study FAPα expression in culture on dental stem cells directly involved in root formation, DFSCs and SCAPs. The corresponding loose connective tissues, dental follicle and apical papilla, will be described as well based on FAPα expression. Next, we will define the apical papilla structure among age observing growth and inductor factors such as TGF- β1 and Stromal cell-derived factor-1 (SDF-1) in relation with FAPα. After this characterization is achieved by intensive immunohistochemical and immunofluorescence analysis, a transwell assay will be performed to test FAPα+SCAPs migration ability.

4. Ameloblastoma

Once we have obtained information about the role of FAPα in dental stem cells, we aim to report a new insight of this protein in disrupted tooth development. Specifically, we will study further its function in a type of tumour where its presence hasn't been reported yet, named ameloblastoma. Ameloblastoma is an odontogenic neoplasm of the jaws and is considered a benign tumour (42) with heterogeneous histological subtypes. It shows negative levels of recovery after surgery, which results in eating and speaking disorders (43). The cellular and molecular mechanisms that provoke it are not clear yet, but it has been suggested that its origin resides in an impaired development of the epithelium in the tooth, including cells of the enamel organ and dental lamina (44).

Several classifications have been reported according to the histological features of ameloblastoma, but most of the distinctions lead to two main subtypes; follicular and plexiform. The most common ameloblastoma is the follicular subtype, which possesses a fibrous stroma with epithelial islands surrounded by cuboidal/columnar cells (45). Plexiform would be the second most common, composed of strands of epithelium organized as a network between the fibrous stroma. Among follicular and plexiform, it is possible to define two more clinical variants recognized; solid/multycistic (SA) or unicystic (UA), much less aggressive than the first one (45). In all subtypes, epithelial cells invade into the surrounding tissues without losing cell-cell attachments (46). After bell stage, in the follicular ameloblastoma, the number of cells resembling stellate reticulum are reported to be higher compared to the plexiform type (44). However, the exact mechanism of invasion has not been clarified.

Regarding these evidences and together with the role of FAPα in the tumour previously discussed, we hypothesize that FAPα enhances epithelial invasion in ameloblastoma throughout its collagenase function. To assess this hypothesis, we aim to study the expression of FAPα in various subtypes of ameloblastoma and report information about collagen distribution.

Materials and methods

-Isolation of SCAPs via the explant method

Human third molars were collected from different patients (14-19 years old) after informed consent in collaboration with the Hospital Ziekenhuis Oost-Limburg (ZOL). The apical papilla was separated from the dental pulp and all tissues were collected in culture medium consisting of α-Minimal Essential Medium (α-MEM) (GIBCO Invitrogen Corp, Paisly, Scotland, UK) supplemented with 10% heat inactivated fetal calf serum (FCS) (Biochrom AG Berlin, Germany), 2 mM L-Glutamine, 100 U/ml Penicillin and 100 µg/ml Streptomycin (GIBCO Invitrogen Corp). Stem cells were isolated via the explant method: one-mm^3 pieces were placed into a 6-well plate containing culture medium. Explants were cultured for 14 days allowing stem cells to grow out of the tissue at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed twice a week. After 10 to 14 days, 80% to 90% confluency was reached, and cells were sub-cultured. For all experiments, cells from passage one to three were used.

-Stimulation of SCAPs with TGF- β1

For the stimulation of SCAPs with TGF-β1, in a 24-well plate 10.000 cells/well were seeded with α-MEM and supplements described before, and 20 ng/ml of TGF-β1 was added. TGF-β1 was omitted from control and cells were incubated at 37ºC in a humidified atmosphere containing 5% CO_2 for 48 hours. After that, cells were fixed in 4% paraformaldehyde 20 min and stained with antibodies shown in Table 1.

-Tissue preparation for histological analysis

Tissue samples from apical papillae were fixed in 4% paraformaldehyde overnight and routinely embedded in paraffin. After deparaffinization in xylol and rehydration in graded series of ethanol, sections were washed in distilled water and stained for Hematoxylin-eosine /Masson's trichrome/Sirius red.

-Characterization of FAPα expression on DFSCs and SCAPs

DFSCs and SCAPs phenotypes were quantified following different classification: FAPα+vimentin+, FAPα+vimentin-, FAPα-vimentin+ and FAPα-vimentin-. >150 cells were classified after staining in n=5 patients. Quantification was performed through ImageJ software using the cell counter tool. Positivity was considered when >90% of the cell surface was stained.

-Immunohistochemistry

Antigen retrieval was performed in deparaffinised tissue sections using citrate buffer (Dako) heated in the microwave oven (3x5 min cyclic). After cooling down for 30 min, sections were washed in Phosphate Buffered Saline (PBS) and used for either diaminobenzidine (DAB) or fluorescent immunostaining. For DAB immunostaining, sections were treated with peroxidase block (Dako) for 20 min. Afterwards, sections were washed with PBS and incubated with protein block (Dako) for 30 min, to limit background staining. Consequently, sections were incubated with the primary antibodies shown in Table 1. As negative control, the primary antibody was omitted

from a section. Peroxidase-conjugated secondary antibodies diluted in PBS were applied for 45 min at room temperature followed by 3 washes in PBS. The chromogenic substrate DAB was used to visualize peroxidase (DAB kit, Dako). Cells were counterstained with Mayer's hematoxylin and mounted using DPX mounting medium (Leika Biosystems, Diegen, Belgium). The immune-reactivity was determined using a photomicroscope equipped with an automated camera (Nikon Eclipse 80i, Nikon Co., Japan).

For immunofluorescent staining, sections were treated with protein block for 20 min, followed by a wash in PBS and incubation with the primary antibodies shown in Table 1 overnight in a humidified atmosphere. As negative control, the primary antibody was omitted from a section. The next day, sections were washed with PBS and incubated with fluorochrome conjugated secondary antibodies (Life technologies, Eugene, OR, USA) for 1 hour. After 3 washes in PBS, nuclei were counterstained with DAPI for 10 min and sections were mounted in fluorescent embedding medium (Dako).

-Immunocytochemistry

Stem cells were seeded onto glass coverslips ($5x10^3$ cells/cm^2) and stained with the peroxidase-based EnVision System (DakoCytomation, Glostrup, Denmark). When cultures were 80% to 90% confluent, cells were fixed with paraformaldehyde 4% for 20 min and washed with PBS. When necessary, cells were permeabilized with 0.05% Triton-X100 for 30 min at 4°C and washed with PBS. To block non-specific binding sites, cells were incubated with protein block (Dako) at room temperature for 20 min. After washing with PBS, cells were incubated overnight with primary antibodies shown in Table 1. After rinsing in PBS, horseradish peroxidase-conjugated secondary antibodies were applied for 30 min at room temperature. For immunofluorescent staining, cells were incubated with fluorochrome conjugated secondary antibodies and mounted in fluorescent mounting medium (Dako). Fluorescent signal was determined using a Leika fluorescence microscope.

Table 1: List of primary antibodies used, dilution and source.

Primary antibody	Dilution	Company
FAPα	1:200 (immunostaining) 1:500 (western blot)	Abcam
Vimentin	1:100	Abcam
CD31	1:100	Santa Cruz Biotechnology
TGF-β1	1:200	Santa Cruz Biotechnology
SDF-1	1:200	Santa Cruz Biotechnology
E-cadherin	1:200	Abcam

-Collagen visualization through Second Harmonic Generation (SHG) and confocal microscopy

Confocal microscopy and SHG imaging was performed with an inverted Zeiss LSM510 META (Carl Zeiss, Jena, Germany) mounted on an Axiovert 200 M. The SHG signal and autofluorescence were recorded using an analogue photomultiplier tube. 3-D volume rendering was calculated with ImageJ through the 3-D viewer tool.

-Apical papilla and cell size quantification

Apical papilla area and root length were measured manually during isolation process. Average from different subunits of apical papilla/root was calculated per patient (n=4). Cell size was measured through ImageJ software. >300 cells were measured per region of apical papilla (cortex fibrosa and medulla) and patient (n=4). After changing units from pixels to µm, images were converted into 8 bit, threshold was adjusted and particles were analysed. Young patients were considered from 14 to 16 years old and old patients from 17 to 20 years old.

-Migration assay by transwell

A transwell assay was used to investigate *in vitro* the migration ability of SCAPs. SCAPs were added to the upper wells of 8 µm pore size transwell inserts in α-MEM containing 2 mM L-glutamine and 100 U/ml of penicillin in a 24 well culture plate. 500 ul of α-MEM without (control) or with 20 and 50 ng/mL SDF-1 (migration inductor) were added to the lower chambers. After 24 hours of incubation in 5% CO_2 at 37ºC, SCAPs remaining on the top of the membrane were removed with a cotton swab and those migrated to the bottom of the membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Quantification of migrating cells was performed through ImageJ software as previously described.

-Western blot for FAPα presence

To determine the protein level of SCAPs, cells were lysed with RIPA buffer (0.5 M Tris-HCl, pH 8.0, 150 mM NaCl, Sodium deoxycholate 0.5% and SDS 0.1% and Triton-X100 1%) and protease inhibitor cocktail (Roche), and then centrifuged at 4000 rpm 5 minutes at 4ºC. Supernatants were discarded, and solutions were centrifugated 15 minutes at 4000 rpm and 4ºC. Supernatants were collected, and protein concentrations were determined using a Bradford assay (Thermo Fisher Scientific). Total cell lysates (30 µg) were fractionated by sodium dodecyl sulphate-polyacrylamide gel (10%) electrophoresis (SDS-PAGE), transferred to nitrocellulose, and immunoblotted with primary FAPα antibody shown in Table 1. After detection using a horseradish peroxidase (HRP)-conjugated secondary antibody (Dako), visualization was performed with chemiluminescence (ECL) detection system (Thermo Fisher Scientific). β-actine was used as loading control (Santa Cruz Biotechnology, 1:1000).

-Ameloblastoma sections

Ameloblastoma sections were obtained from patients collected in Nigeria, in collaboration with the department of oral pathology/medicine in University of Ibadan after informed consent.

-Statistical analysis

Statistics was performed using GraphPad Prism software version 5.0b. For the different quantifications, t-test was performed for comparisons of two means and two-way ANOVA for repeated measurements for more than two groups with a Bonferroni post hoc test for multiple comparisons. Data were presented as mean standard error of the mean (SEM). The statistical analysis of the results was performed by taking a significance level of 5% ($p<0.05$).

Results

In this study, we hypothesized that FAPα plays an important role in dental root development. To assess this hypothesis, we first studied FAPα expression in culture on dental stem cells directly involved in root formation, DFSCs and SCAPs showing a high positivity both on the plasma membrane and on the nucleus of these cells. Dental follicle was described showing positivity for FAPα at the matrix and at the epithelial remnants. In the apical papilla a heterogeneous expression of FAPα was identified in two defined histological regions: cortex fibrosa and medulla. The presence of growth factors like TGF-β1 and SDF-1 was found in the apical papilla, mostly at the periphery of the tissue. At last, transwell assay showed an increase in migration rate of FAPα+SCAPs in response to SDF-1.

-FAPα is expressed in DFSCs and in the dental follicle.

To study the presence of FAPα in DFSCs involved in root formation, we performed an immunofluorescent staining of FAPα and the stem cell marker vimentin (Figure 1A) on DFSCs in culture. The results of the quantification of different phenotypes based on the positivity for these two markers revealed the following cell populations i.e. 83.85% FAPα+Vimentin+, 10.22% FAPα+vimentin-, 0.86% FAPα-vimentin+ and 5.062% FAPα-vimentin- (Figure 1B). Significant differences between FAPα+vimentin+ and the rest of populations (***), and between FAPα+vimentin- and FAPα-vimentin+ were found (***) ($n=5$ *$P \leq 0.05$, ***$P \leq 0.001$).

Due to an unexpected location of FAPα at the region of the nucleus (Figure 1D) we also performed a quantification of DFSCs positive either for FAPα on the plasma membrane (75±18.58%) or both on the plasma membrane and nucleus (24.84±18.58%) (Figure 1D-G). No significant differences were found between these two populations (Figure 1C) ($n=5$, $p<0.05$).

To extrapolate the FAPα quantification of DFSCs in culture, we performed an immunofluorescent staining for E-cadherin, a cell adhesion and epithelial cell marker, together with FAPα in dental follicle tissue sections (Figure 2A). We found a high concentration of FAPα-expressing cells within the extracellular matrix of the dental follicle and in the epithelial remnants, where FAPα appears to be positive both on the plasma membrane and on the nucleus (Figure 2B,C) corroborating 3-D volume rendering (Figure 1G). Inside the clusters of epithelial cells originated from the breakdown of HERS, cells were also strongly positive for E-cadherin (Figure 2B). In the epithelial rest there is also absence of vimentin indicating epithelial cells except from one isolated cell which also results positive for FAPα, indicating a different phenotype from the rest of the epithelial cluster (Figure 2C*). However, stromal cells are extremely positive for vimentin (white arrows Figure 2).

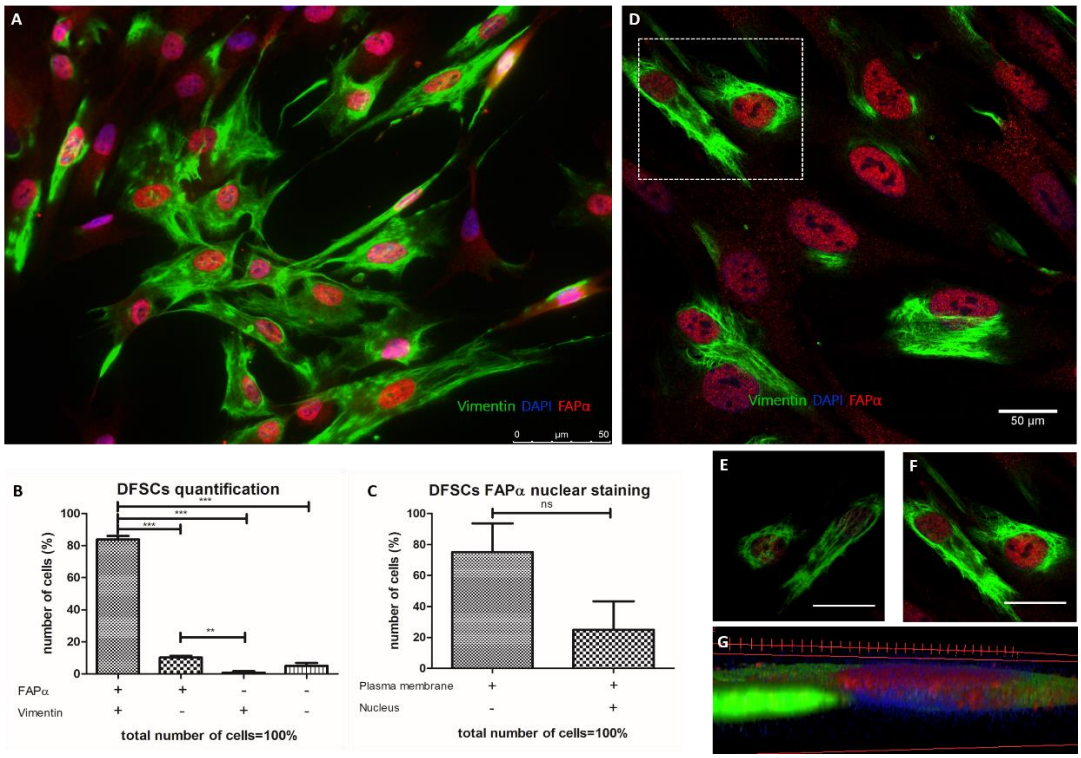

Figure 1: **FAPα is highly expressed on the plasma membrane and nucleus of DFSCs. (A)** Triple immunofluorescent staining of FAPα (red), vimentin (green) and DAPI (blue) in cultured DFSCs. **(B)** Quantification of DFSCs phenotypes based on FAPα and vimentin expression. **(C)** Characterization of FAPα location on DFSCs on the plasma membrane or/ nucleus. **(D)** 3-D volume rendering of the triple immunofluorescent staining of DFSCs through confocal microscopy. Higher magnification of box in image D **(E,F,G)** where FAPα appears to be located on the plasma membrane and more precisely, on one side of the nucleus. Image processing including changes in brightness and contrast was applied equally in all images. Data are represented as mean±SEM. (n=5 *P≤0.05, ***P≤0.001). Scale bars represent 50 µm (A, D, E, F).

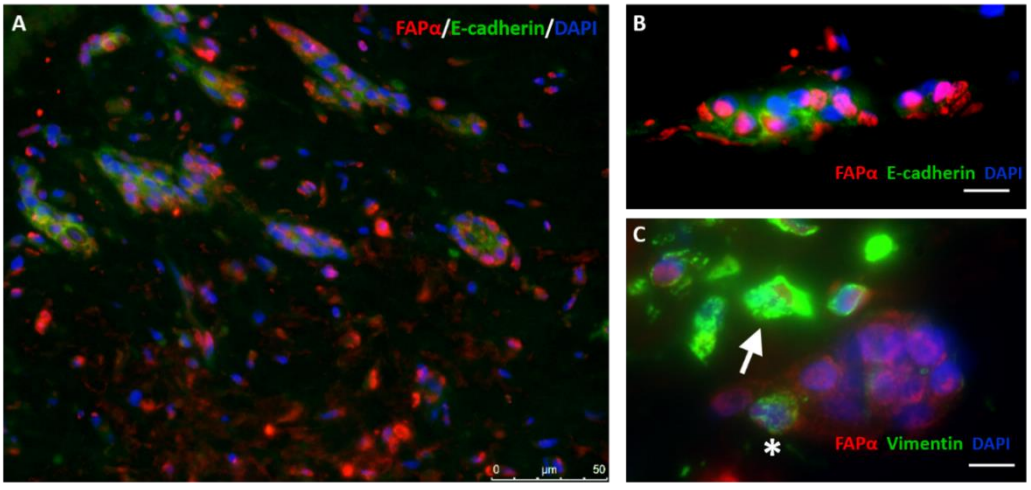

Figure 2: **FAPα expression is positive in the dental follicle. (A)** Triple immunofluorescent staining of FAPα (red), E-cadherin (green) and DAPI (blue) in a semi-thin section of the dental follicle. White arrow indicates epithelial rests of HERS. **(B)** Higher magnification of an epithelial rest highly positive for E-cadherin and FAPα. **(C)** Higher magnification of a triple immunofluorescent staining of FAPα (red), vimentin (green) and DAPI (blue) showing an epithelial rest with vimentin negative epithelial cells (absence of green). Note the presence of one vimentin positive cell at the periphery of the epithelial rest (*). Stromal cells are highly positive for vimentin (white arrow). Scale bars represent 50 µm (A,B,C).

-FAPα is highly expressed in SCAPs and differs in multiple passages.

Besides the dental follicle, several studies report SCAPs as a driving force for root maturation. SCAPs were used for immunofluorescent staining of FAPα and the stem cell marker vimentin (Figure 3A) and a characterization was performed. The results of the quantification of different phenotypes based on the positivity for these two markers revealed the following cell populations i.e 70.07% of FAPα+vimentin+ cells, 25.84% FAPα+vimentin-, 0.44% FAPα-vimentin+ and 3.65% FAPα-vimentin- (Figure 3B). Significant differences between FAPα+vimentin+ and the rest of populations, and between FAPα+vimentin- and FAPα-vimentin- were found (***) (n=5 *P≤0.05, ***P≤0.001). The presence of FAPα on SCAPs was further demonstrated by western blot analysis (Figure 3F). We also quantified the positivity for FAPα in SCAPs only on the plasma membrane or both on the plasma membrane and nucleus, showing a percentage of 71.55±13.36 and 13.61±2.582 respectively with no significant differences (n=5, p<0.05) (Figure 3C).

To observe the changes in vimentin and FAPα expression in SCAPs cultured during multiples passages, we performed repeated the triple immunofluorescence staining at passage 13 (Figure 3D) and performed a new quantification compared to passage 1 (Figure 3E). The following variations in the populations at passage 1 compared to 13 were found i.e. FAPα+vimentin+ from 66.21% to 74.25%, FAPα+vimentin- from 31.09% to 18.24%, FAPα-vimentin+ from 0% to 1.505% and FAPα-vimentin- from 2.69% to 5.98%.

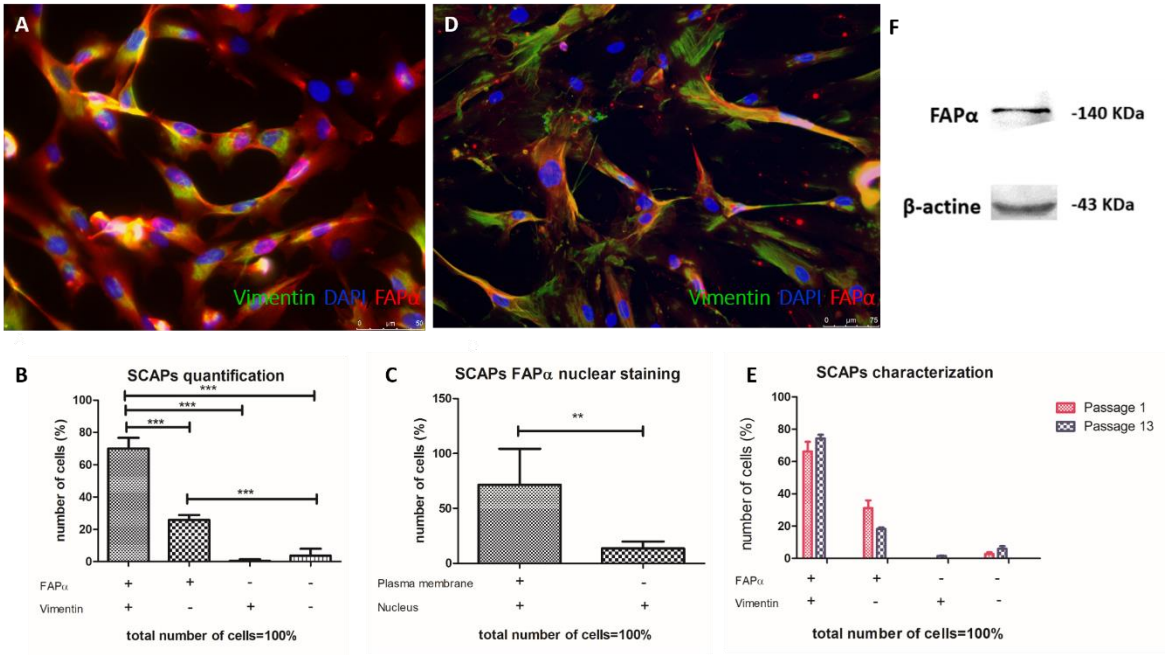

Figure 3: **FAPα is highly expressed on the plasma membrane and nucleus of SCAPs. (A)** Triple immunofluorescent staining of FAPα (red), vimentin (green) and DAPI (blue) in cultured SCAPs at passage 1. **(B)** Quantification of different SCAPs phenotypes based of FAPα and vimentin expression. **(C)** Characterization of FAPα location on SCAPs on the plasma membrane or/ nucleus. **(D)** Triple immunofluorescent staining of FAPα (red), vimentin (green) and DAPI (blue) in cultured SCAPs at passage 13. **(E)** Comparative quantification of different SCAPs phenotypes based on FAPα and vimentin expression at passage 1 and 13. **(F)** Western blot analysis showing the presence of FAPα in SCAPs and β-actine as a loading control. Data are represented as mean±SEM (n=5 *P≤0.05, ***P≤0.001). Scale bars represent 50 μm (A, D).

-New histological findings in the apical papillae based on FAPα.

There is scarce information available on the histological composition of the apical papilla, perhaps due to its disappearance after completed root formation. Due to differences observed in the size of this tissue in different patients, area and root length per subunit were measured from four different patients of 14 to 20 years old (Figure 4A-D). Results showed a decrease in root length from 1.229, 1.096, 1.034, to 0.9747 cm and in apical papilla area from 0.3148, 0.1448, 0.1550 to 0.09017 cm^2 in patients of 16, 17, 20 and 20 years old respectively (Figure 4E).

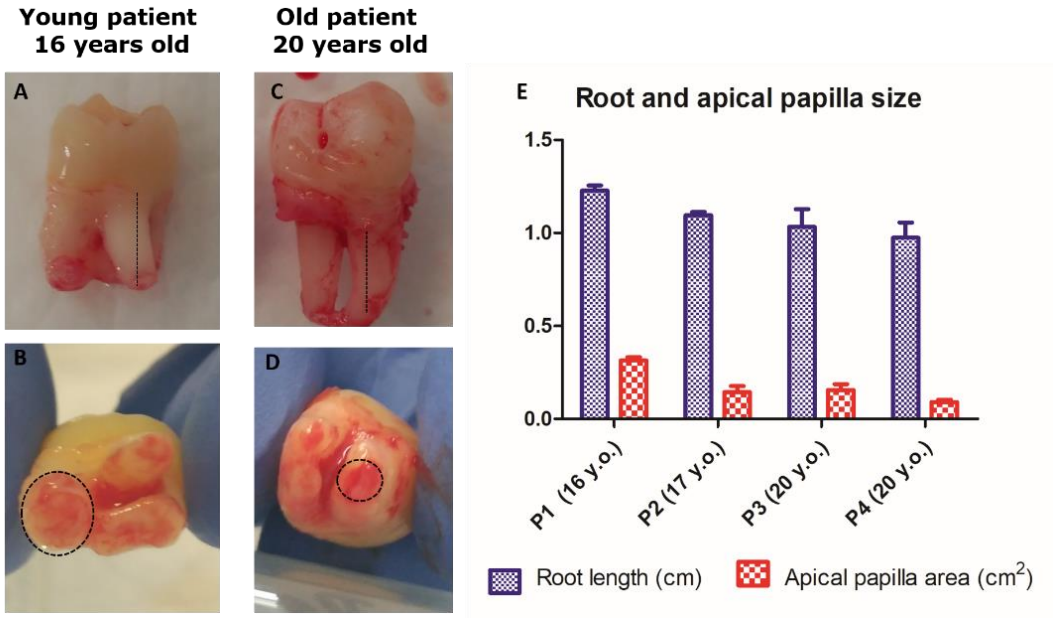

Figure 4: **Apical papilla size decreases among age.** Third molar extracted from a 16-year old patient **(A,B)** and from a 20-year old patient **(C,D)** used to measure root length (cm) and apical papilla area (cm^2). Inter-lineated circles indicate apical papilla subunits and interlineated lines indicate root length. **(E)** Comparison between root length and apical papilla size in patients from different ages calculated by average between apical papilla subunits and teeth per patient.

However, we here report some new histological insights of this tissue. After an extensive characterization, we defined two different regions in apical papilla of young patients: cortex fibrosa and medulla. In the cortex fibrosa (periphery) there is a highly dense quantity of compacted collagen type I (Figure 5B,D) compared to the medulla (core of the tissue), where the ECM is showing a loose organization and spindle-shaped cells are present (Figure 5C,E).

After establishing the structure of the apical papilla, staining analysis for FAPα in a 15-year old patient revealed a heterogeneous expression at the cortex fibrosa of the tissue (Figure 5F) with two different cell populations; FAPα+vimentin+ or FAPα-vimentin+ (Figure 5H). In the medulla a higher positivity for FAPα was found (Figure 5I) with both positive and negative expression of vimentin. In older patients these differences were reduced to a single region presenting only FAPα+vimentin+ cells at the cortex fibrosa and FAPα+vimentin- cells at the medulla (data not shown).

To complement these histological findings, the number of cells in the apical papilla was calculated in the two defined regions from different patients, but no significant differences were found (Figure 5J). However, the cellular orientation appeared to be different as well presenting in the cortex fibrosa an epithelium simple cuboidal and underneath a cell rich region with perpendicular orientation (Figure 5B). In contrast, the medulla in old patients is composed by cells with smaller area than in the cortex fibrosa (Figure 5C). To confirm this, cell area average was calculated from different patients showing a decrease from 0.019 to 0.0179 μm^2 in the cortex fibrosa and medulla of young patients and from 0.0181 to 0.0075 μm^2 of old patients. Significant differences were found between the medulla cell area from young and old patients and the cortex fibrosa and medulla from old patients ($n=5$ $^*P \leq 0.05$, $^{***}P \leq 0.001$). Second harmonic generation reported a higher concentration of collagen at the cortex fibrosa of old patients (Figure 6B) compared to young (Figure 6A).

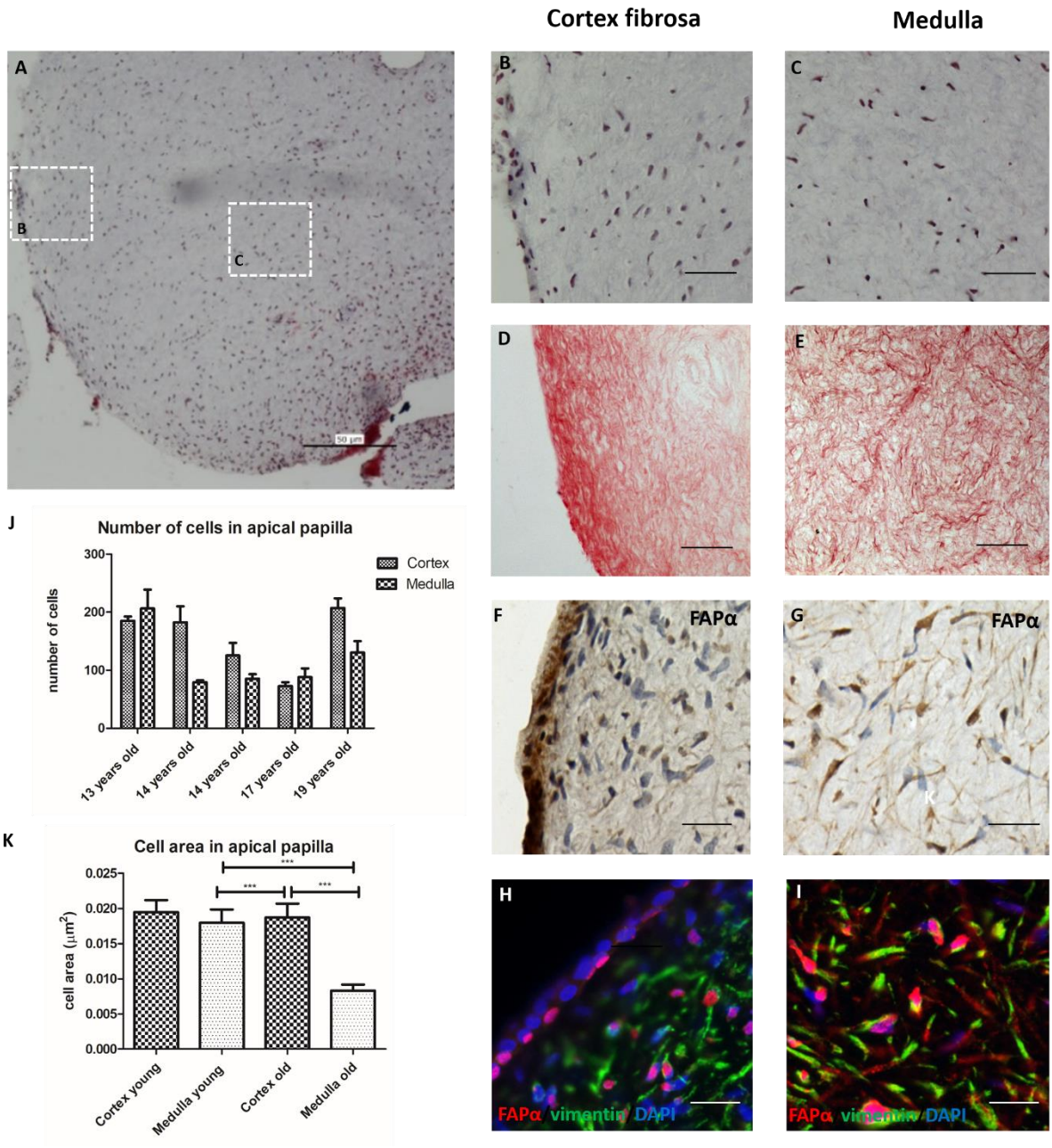

Figure 5: Apical papilla is defined by two histological regions positive for FAPα: cortex fibrosa and medulla, presenting differences in cell area. (A) Paraffin section of apical papilla from a 15-year-old healthy patient stained with Masson's Trichrome. Higher magnification of image A showing two different histological regions in apical papilla; cortex fibrosa **(B)** and medulla **(C)**. Sirius red staining showing collagen concentration in the cortex fibrosa **(D)** and medulla **(E)**. DAB staining for FAPα revealing a heterogeneous expression at the cortex fibrosa **(F)** and a high expression level in the medulla **(G)**. Triple immunofluorescent staining for stem cell marker vimentin (green), FAPα (red) and DAPI (blue) showing heterogeneity for FAPα at the cortex fibrosa **(H)** and high expression in the medulla **(I)**. Number of cells present in the apical papilla in the cortex fibrosa and medulla of five different patients **(J)**. Average of cell area calculated in cortex fibrosa and medulla from young and old patients **(K)**. Scale bars represent 50 μm (A,B,C,D,E,F,G) and 75 μm (H,I).

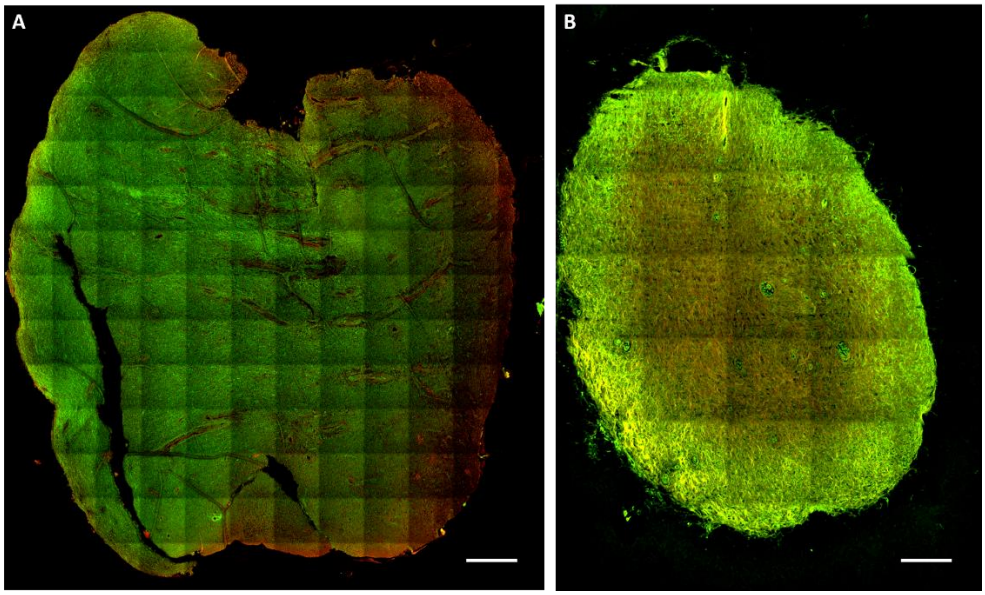

Figure 6: **Structural differences in apical papilla patients among age.** Visualization through Second Harmonic Generation of the apical papilla from a 14-year-old patient **(A)** and a 17-year-old patient **(B)** showing a difference in size and in collagen distribution within the cortex fibrosa and the medulla. Scale bars represent 100 μm.

To observe the location of blood vessels we performed an immunofluorescent staining for CD31, an endothelial cell adhesion molecule (Figure 7A-D). Quantification revealed a location of blood vessels mostly at the periphery of the tissue (Figure 7F) observing a ratio of number of blood vessels/area of 1.440 and 1.705 in the cortex fibrosa and 0.7150 and 0.8100 in the medulla for young and old patients respectively. Moreover, the total number of blood vessels resulted to be higher for old patients compared to young, increasing from 20 to 39 (Figure 7E).

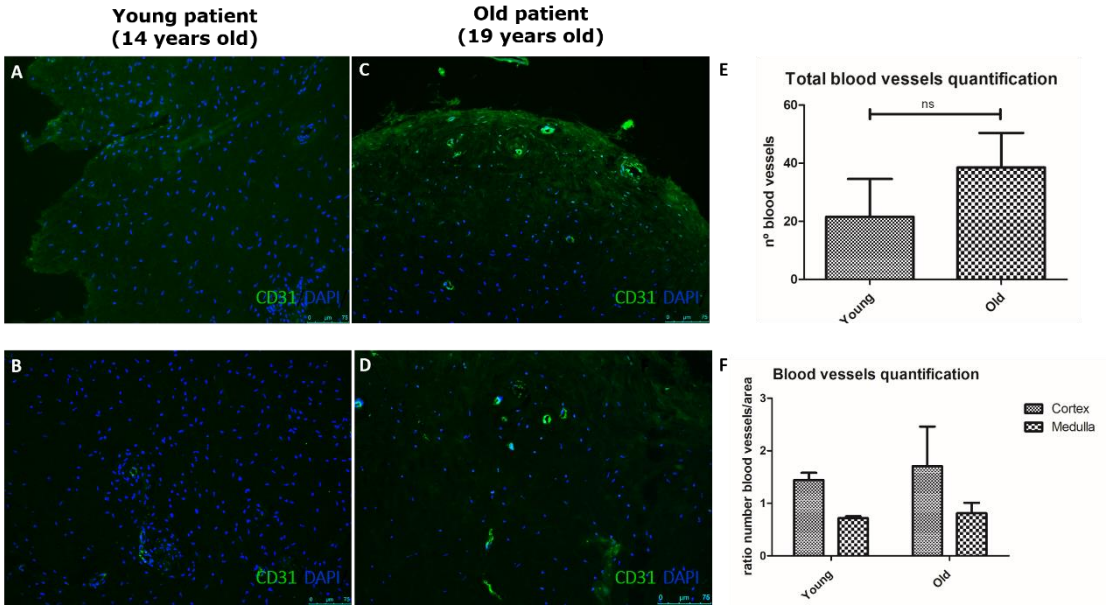

Figure 7: Number of blood vessels is higher in old patients and tend to be located at the cortex fibrosa of apical papilla. Immunofluorescent staining for endothelial marker CD31 (green) and DAPI (blue) of a 14 year old patient **(A,B)** and 19 year old patient **(C,D)**. **(E)** Total number in blood vessels in young and old patients. **(D)** Blood vessels quantification/area in cortex fibrosa and medulla from old and young patients (no significant differences, $p<0.01$ n=4). Scale bars represent 75 μm (A, B, C, D).

-SCAPs develop a migrating-phenotype positive for FAPα after stimulation with TGF-ß1

TGF-β1 is an important modulator in cell proliferation and differentiation. Recent literature stresses that in BM-MSCs, an increase of FAPα expression is observed after TGF-β1 stimulation (13). To study this effect, we stimulated SCAPs with 20 ng/ml of TGF-ß1 during two days in culture, and after that, immunofluorescent staining was performed for FAPα and vimentin (Figure 8). Vimentin filaments were clearly visible as well as FAPα expression (Figure 8E-H). Moreover, in comparison with control (non-stimulated cells) (Figure 8A-D), SCAPs showed a migrating-like phenotype presenting pseudopodia (white arrows). Interestingly, these extensions appeared to be positive for FAPα (Figure 8I). To observe TGF-β1 expression in the apical papilla, immunostaining analysis was performed showing a higher positivity in the ECM of the cortex fibrosa (Figure 8J,K).

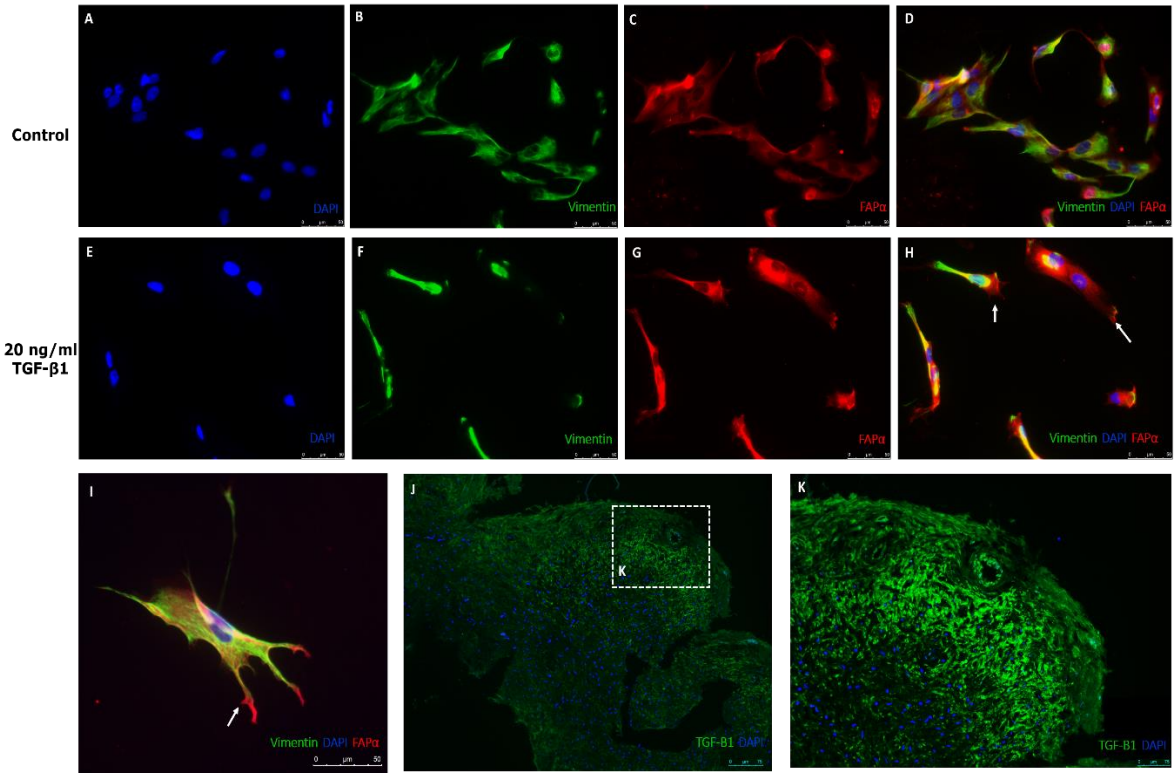

Figure 8: TGF- β1 induce a migrating-phenotype in SCAPs and is located at the periphery of the apical papilla. Triple immunofluorescence staining for vimentin (green), FAPα (red) and DAPI (blue) of SCAPs in culture after two days without any stimulation **(A,B,C,D)** and after two days of stimulation with 20 ng/ml of TGF-β1 **(E,F,G,H,I)**. **(J)** Immunofluorescent staining of TGF-β1 (green) and DAPI (blue) of the apical papilla from a 17-year-old patient. **(K)** Higher magnification of box in J. Note the presence of pseudopodia in white arrows. Scale bars represent 75 µm (J,K) and 50 µm (A-I).

-Migration of SCAPs is enhanced by SDF-1.

Due to the disappearance of the apical papilla after permanent tooth have been developed, some biological processes could explain the fate of the resident SCAPs. We aimed to determine whether SCAPs were able to migrate in response to the migration-inductor SDF-1. To study that, a transwell assay was performed (Figure 9). Migration rate was increased from 0.70 to 0.90 with 20 ng/ml to 50 ng/ml SDF-1 stimulation in a well with 20.000 cells seeded and from 1.3 to 2.1 in a well with 50.000 cells (Figure 9I). The control migration rate was 0.50 and 1.5 for 20.000 (Figure 9A,B,C) and 50.000 cells respectively (Figure 9D,E,F). These findings show that migration rate of SCAPs is enhanced by SDF-1 stimulation. To observe in which part of the apical papilla SDF-1 was situated, immunostaining was performed showing a higher expression at the periphery of the tissue, underneath the cortex fibrosa (Figure 10E,F,G). Moreover, a co-location of FAPα and SDF-1 was found (Figure 10D).

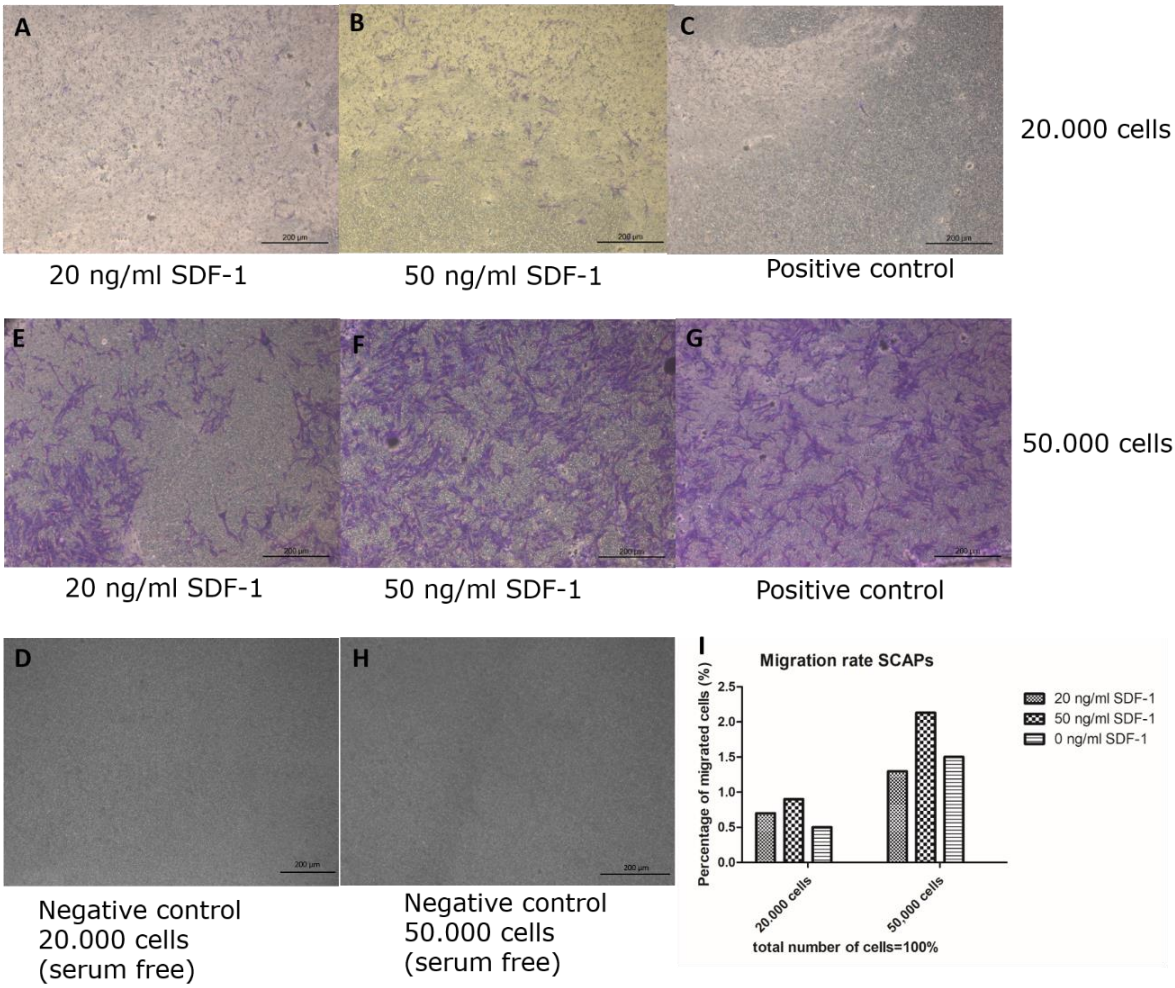

Figure 9: **Migration of SCAPs is induced by SDF-1.** Crystal violet staining of transwell inserts in 20.000 SCAPs **(A-C)** and 50.000 **(E-G)** stimulated with 20 ng/ml **(A,E)** and 50 ng/ml of SDF-1 **(B,F)**, compared to positive control **(C,G)** and serum free medium as a negative control **(D,H)** during 24 hours.

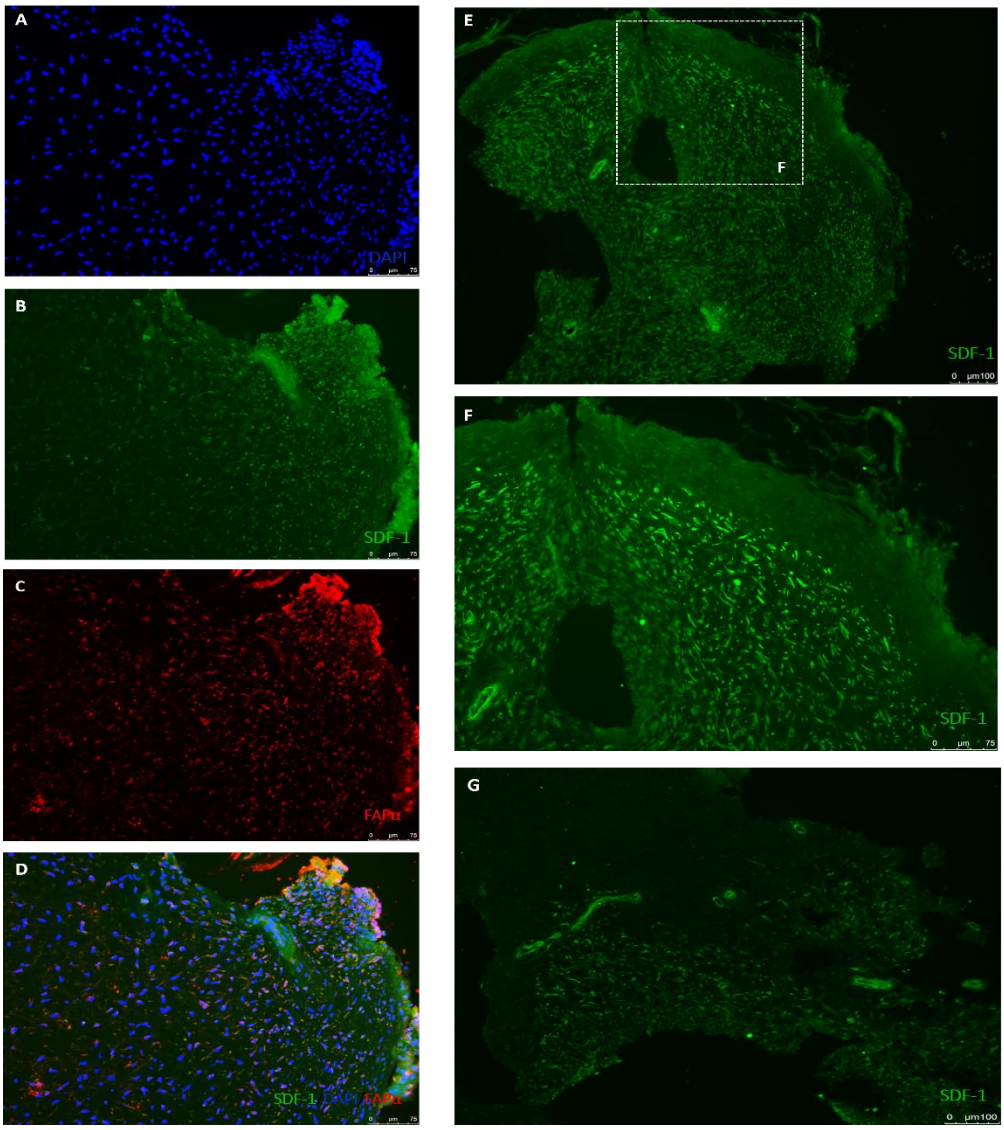

Figure 10: SDF-1 is mostly located at the periphery of the apical papilla. Immunofluorescent staining of DAPI (blue; **A**), SDF-1 (green; **B**) and FAPα (red; **C**) and overlap **(D)** of the periphery of a subunit of the apical papilla from a 17 year-old patient showing a co-location with FAPα. **(E,G)** Whole subunit of apical papilla from the same patient showing a higher positivity of SDF-1 at the periphery of the tissue. **(F)** Higher magnification of Image E. Scale bars indicate 75 µm (A, B, C, D, F) and 100 µm (E,G).

-FAPα is expressed within two subtypes of ameloblastoma: follicular and plexiform

To observe if FAPα was also involved in disrupted tooth development, we studied its presence in the odontogenic tumour ameloblastoma. Visualization of DAB staining showed a strong expression of FAPα in both subtypes of ameloblastoma (Figure 11, 12). In the follicular one, the positivity was visible at the epithelium of the follicles (Figure 11*) as well and the stroma (Figure 11**). Moreover, big circle-shaped cells were located inside of some of the follicles, which we named "giant cells". Some of these "giant cells" appeared to be positive for FAPα (Figure 11 black arrows). The plexiform subtype showed a strong positivity for FAPα in the connected epithelium clusters (Figure 12*), as well as in the matrix (Figure 12**). Collagen amount was higher in follicular subtype (Figure 11B) compared to plexiform (Figure 12B) and heterogeneous in both subtypes.

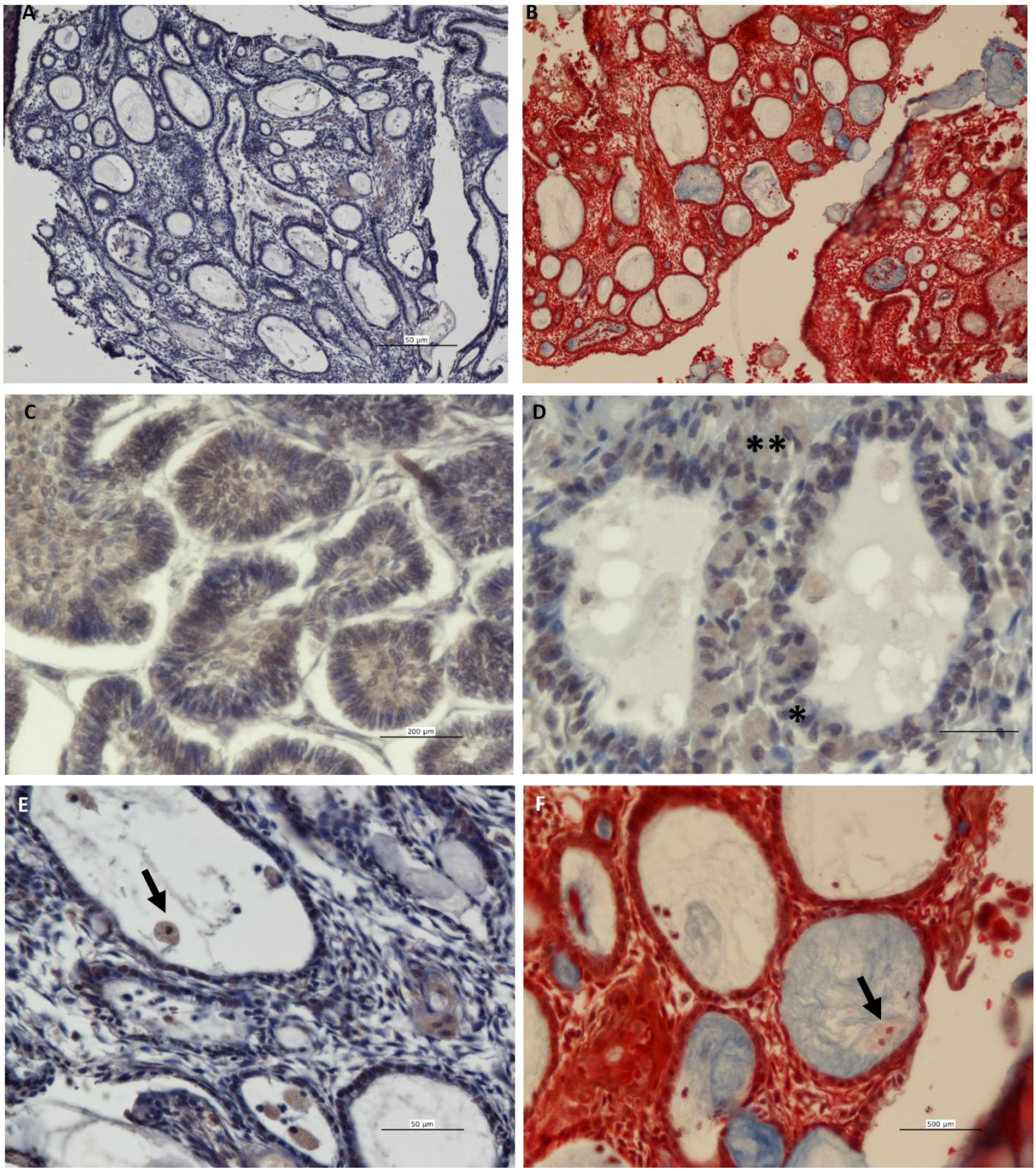

Figure 11: FAPα is expressed in the follicular subtype of human ameloblastoma. Paraffin sections of a patient of ameloblastoma stained with DAB for FAPα **(A, C, D, E)** showing positivity both in the epithelium clusters (*) and in the matrix (**). Masson's Trichrome staining showing collagen concentration inside follicles **(B, F)**. Black arrows indicate giant cells. Scale bars indicate 50 µm (A, B), 200 µm (C), 500 µm (F), 50 µm (D, E).

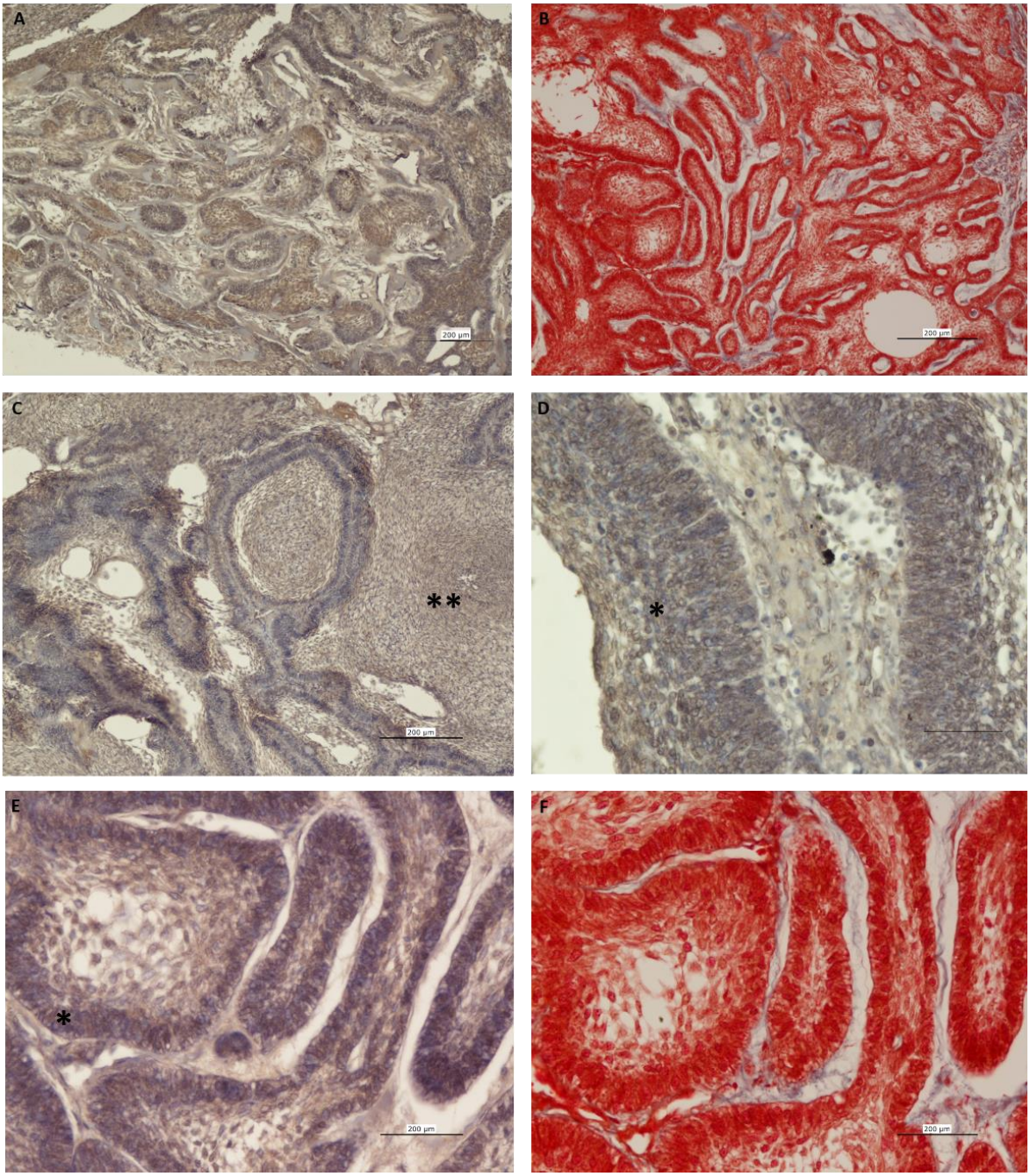

Figure 12: FAPα is expressed in the plexiform subtype of human ameloblastoma (A, C) Paraffin sections of an ameloblastoma stained with DAB staining for FAPα and **(A, C, D, E)** showing positivity both for epithelium clusters (*) and matrix (**). Masson's Trichrome Staining showing collagen concentration **(B, F)**. Scale bars represent 200 µm.

Discussion

-FAPα expression in the dental follicle during root formation

In this study we hypothesized that FAPα plays a role in dental root development. For that, in the first part of our project, we aimed to study FAPα expression in the dental follicle and the corresponding stem cells involved in dental root development. It is well accepted that dental follicle differentiates into fibroblasts that compose the periodontal ligament in order to bind the tooth to the bone (47) and other studies have proposed it as the source of cementoblasts and osteoblasts (48). In fact, the dental follicle is composed of a heterogeneous cell population, probably related to these different fates during development. To provide an approach on this, we aimed to characterize this tissue using FAPα expression. The majority of DFSCs were positive for FAPα and vimentin distributed throughout the extracellular matrix, which matches the stem cell expression profile of DFSCs in culture. In addition, we found epithelial clusters (remnants of HERS) as confirmed by positivity for the epithelial marker E-cadherin. Within these remnants, epithelial cells were observed with either positive or negative expression of FAPα, but negativity for vimentin. FAPα is also considered as a marker for EMT during tumour development and coincides with vimentin upregulation (49). It is possible that epithelial cells are in transition to become mesenchymal cells but haven't achieved the fully differentiated phenotype with expression of vimentin. Therefore, it is not surprising to find a small percentage of FAPα+vimentin- in the dental follicle. However, at the edge of the epithelial remnants, FAPα+vimentin+ cells were observed indicating a completed EMT process. We hypothesize that the collagenase activity of FAPα could induce a disintegration of the epithelial remnants leading to migration of EMT induced epithelial cell in the ECM. A similar observation was made in the family of MMPs which are expressed in epithelial cells during EMT and promote cell invasion (50). Moreover, TGF- β1 has been proved to regulate EMT of HERS (27), as well as induce FAPα expression in BM-MSCS (13), what could be the link of our observations.

Interestingly, we found the presence of FAPα not only on the plasma membrane of these cells, but also on the nucleus by 3-D volume rendering. This would be normally unexpected due to the collagenase function of FAPα, however, in one of the others big groups of enzymes that degrade collagen i.e. MMPs, an expression on the nucleus of some cells during physiological and pathological situations was also found. The role of intracellular MMPs is poorly understood, but some authors demonstrated a relation between the nuclear expression of these enzymes and accumulation and fragmentation of oxidized DNA in neurons (51) or apoptosis in neural stem cells (52). However, this hypothetical translocation mechanism from the plasma membrane to the nucleus should be studied further.

-New histological insights in the apical papilla and identification of FAPα expression

In the second part of our project, we have studied the expression of FAPα in the apical papilla and SCAPs in culture. Detailed information on the structural organization of this tissue is lacking. In 2008, a first histological description was reported by Sonoyama et al., revealing a cell rich zone attaching the apical papilla to the dental pulp. However, no structural identification was provided (32). Therefore, before discussing FAPα expression, we have performed a thorough histological analysis of the apical papilla.

Macroscopically, the apical papilla is composed of multiple subunits depending on the number of developing roots. Within each subunit, we have identified two novel histological regions: the cortex fibrosa and the medulla.

At the periphery of each subunit, the cortex fibrosa is composed of a simple cuboidal epithelium with a high cellular density layer adjacent to it. Cells in the cortex fibrosa are embedded in a high density apparently cross-linked collagen type I and both components are aligned in the same direction. We hypothesize that the cortex fibrosa could provide mechanical support to the underlying tissue namely the medulla, which accounts for the largest fraction of the apical papilla. The medulla consists of spindle-shaped cells indicating the presence of SCAPs, which are routinely used in cell differentiation studies (53). Interestingly, the collagen distribution is clearly different from the cortex fibrosa, presenting less organized and diffuse distribution.

Backwards to our protein of interest, the cortex fibrosa in young patients demonstrates a mixed population of either FAPα+vimentin+ or FAPα-vimentin+ cells. The expression profile of the latter phenotype resembles undifferentiated fibroblasts which could contribute to the highly cross-linked and aligned collagen fibrils (54). On the other hand, the medulla of consists of a high concentration of FAPα+vimentin+ cells resembling the expression profile of SCAPs in culture. It is possible that this higher positivity for FAPα in the medulla is related to the collagenase function that SCAPs are using to migrate to the periphery of the tissue. In this case, it may be postulated that the common factor between the different expression patterns of FAPα in embryogenic development, tumour microenvironment and MSCs is the remodeling of ECM through its collagenase activity.

Furthermore, unpublished data of our group has shown a high frequency of vesicles in the medulla. These results made us propose vesicle secretion as an alternative mechanism for collagen degradation in the apical papilla, in which FAPα is not only expressed on the plasma membrane/nucleus of SCAPs, but also in the secreted vesicles (55).

-Apical papilla remodelling during root maturation

After defining the basic histological organization of the apical papilla, we moved our focus to the progressive remodelling of this tissue during root maturation. Macroscopically, our data demonstrated that the apical papilla decreases in size during root formation from patients of 14 years old compared to patients from 20 years old. There are two clinical studies that clearly show an involvement of this tissue on the correct formation of the root. In one of them, apical papilla is removed at early stage of development and root formation is halted, whereas in other, dental pulp was removed and apical papilla retained leading to a normal root-tip formation (33). Subsequently, we have posed in the following questions: which is the fate of the SCAPs that confine it? And at last, what is the role of FAPα during remodelling?

First, a quantification of the cell size showed a significant reduction in cell area in the medulla of older patients suggesting a decrease in number in SCAPs. Double staining of FAPα and vimentin revealed FAPα+vimentin+ cell populations in the medulla, what could be due to enhanced migration towards the cortex fibrosa.

Cell migration plays a fundamental role during development/tissue repair and it goes accompanied by chemotaxis, a process triggered by the binding of chemokines to receptors. The chemokine SDF-1 (also known as CXCL12) and its receptor CXC chemokine receptor type 4 (CXCR4) are known to be involved in developmental processes such as vascularization or migration (56). Several studies show how SDF-1 can recruit MSCs to different tissues contributing to wound repair (57). Specifically, in the dental tissue, it was shown how SDF-1 could induce hDPSCs migration indicating a novel approach for pulp repair (58). Not only in DPSCs, but also in SCAPs, the expression of CXCR4 was shown in the paravascular region of the apical papilla and migration was enhanced using SDF-1 as inductor (59). However, the molecular mechanisms and signals that link SDF-1 with migration remain unknown.

To test whether SCAPs that we previously reported positive for FAPα responded to SDF-1 as a migration inductor, a transwell assay was performed and the results agree with this late study and migration was enhanced with higher concentrations of SDF-1. In that case, the following question rises up: what is the reason why SDF-1 is released and by which type of cells?

SDF-1 is mainly produced by osteoblasts, fibroblasts and endothelial cells in the bone marrow (60). Given this, it could be possible that the fibroblast layer found underneath the epithelium in the cortex fibrosa release SDF-1 to induce SCAPs migration. To test that, SDF-1 immunostaining was performed, and it becomes apparent that this suggestion could be reliable since the positivity for this chemokine was higher at the cortex fibrosa of the tissue where probably most of fibroblasts are located. Nevertheless, the molecular mechanisms behind the release of SDF-1 by fibroblasts should be tested further.

Moreover, a co-location of SDF-1 and FAPα was found. However, if FAPα is involved in migration, there must be some factor that triggers its expression as well. We showed how SCAPs when stimulated with TGF- β1, developed a migrating-phenotype, and a relation between TGF-β1 and FAPα was established in BM-MSCs where TGF-β1 increased FAPα expression and provoked enhanced migration (13). These findings are suggesting that the activation of FAPα in SCAPs could

be due to the action of TGF-β1. In addition, TGF-β1 showed positivity in the ECM, and it has been shown that TGF-β1 is stored in the matrix as a latent complex and can be posteriorly activated by cell contractile force (61). In apical papilla tissue sections, immunostaining of TGF-β1 showed positivity for regions in the ECM co-located with FAPα+ cells, which corroborates our suggestion. Since not only FAPα expression is induced by TGF-β1 (13), but also vimentin (62), it is possible that the lower expression of TGF-β1 in the medulla is also related to the vimentin- cell population in older patients.

On the other hand, TGF-β1 is a signalling molecule involved in plenty processes such us cell differentiation, proliferation and chemotaxis, in addition to development and homeostasis. It has been shown that TGF-β1 is present in BM microenvironment and controls the quiescence of stem cells (63). Among all the different pathways which TGF-β1 can activate, there is a division between two main routes. The first one is the "canonical pathway" in which smad proteins are phosphorylated and processes like apoptosis or EMT are activated. The second one is composed all the non-smad pathways, including the binding to GTP-binding proteins and RhoA or Ras, or the famous MAPK pathway with the activation of Erk (64). The molecular mechanisms behind the activation of FAPα through TGF-β1 remain largely unknown. However, either the canonical or the non-smad pathway are possibly candidates of FAPα activation, what constitute an interesting topic of study for future research.

Summarizing, TGF-β1 increases FAPα expression and it is related to differentiation and proliferation, and SDF-1 induces migration of SCAPs. However, is it possible to find an association between SDF-1 and TGF-β1? Whereas a cooperation was established in periodontal ligament cells where TGF- β1 appeared to increase SDF-1 expression through the smad-pathway (65), another study indicated that TGF-β1 downregulated SDF-1 expression influencing migration and adhesion in BM-MSCS (66). Future experiments should assess the link between SDF-1 and TGF-β1 in cell migration which remains unknown.

Since vascularization is a crucial process for development, the fate/location of these blood vessels and nerves is important to understand the tissue re-organization in time. Sonoyama et al. already stated that apical papilla contained less vascular components than dental pulp. Blood vessels develop during tooth development in the cap phase and are formed in the dental papilla that finally will become pulp (67). Quantification of number of blood vessels/area showed differences being higher in the cortex fibrosa than in the medulla, what could be related to the high concentration of SDF-1, which has been proved to control blood vessels formation (68). Nonetheless, the organization of the vascularity during root maturation and apical papilla remodelling requires further investigation.

-The role of FAPα in ameloblastoma

As stated previously, the interactions between the epithelium and the mesenchyme during tooth development are crucial. In fact, due to an impaired signalling of these interactions, the development of the tooth is disrupted, and ameloblastoma is formed. To consider the possibility that FAPα is also involved in disrupted tooth formation, we aimed to investigate its presence in two subtypes of ameloblastoma. According to the World Health Foundation (WHF), ameloblastoma is classified as a benign tumour with aggressive behaviour in which surgery is needed and the recurrence rate is high. Among different described subtypes, there are some common characteristics we corroborated in our study such as the basic structure of stellate fibroblasts and epithelium networks/islands. However, besides the cells which were more tightly packed in the follicular than in the plexiform, both invasive epitheliums demonstrated positive expression for FAPα. These findings suggest that FAPα is taking part again into the remodeling of the extracellular matrix to form these epithelium structures. Nonetheless, cells within the extracellular matrix also appear to be positive for FAPα, indicating the presence of myofibroblasts.

Taking a deeper look into the molecular mechanism, a transcriptome analysis was published describing some altered pathways in several subtypes of ameloblastoma. Results included variations in cell-cycle regulation, inflammation processes and a remarkable one, the MAPKinase pathway. The MAPK pathway is activated in ameloblastoma through BRAF mutation and Ras activation and this metabolic route is involved in cancer pathogenesis. Considering the previous evidences related to routes activated by TGF- β1 and FAPα, it's possible that this mutation is responsible of FAPα expression in ameloblastoma.

Furthermore, another study about differential gene expression on different types of ameloblastoma showed a lower expression of RhoGTPAses (69), one of the enzymes that FAPα appears to inactivate. RhoGTPases such as RhoA regulate migration of MSCs by the re-arrangement of cytoskeleton, and it was shown that depletion of FAPα increased RhoGTPase (13). RhoA has a heterogeneous role in MSCs. Whereas in some cells it promotes migration, in some other cells like fibroblasts, inhibition of RhoA promotes migration by reducing these features (70). In addition, in BM-MSCs migration was inhibited when RhoA was overexpressed due to FAPα depletion. Given this, this is perhaps the reason why FAPα inhibits this enzyme and due to a high expression of FAPα in ameloblastoma, the expression of RhoA decreases. However, information about the activation of FAPα is missing to fully understand this mechanism.

Interestingly, we found a remarkable phenotype inside of some of the follicles in ameloblastoma patients. Depending on the sample, giant cells were discovered in the interior of the follicles next to the epithelial layers. These are designed as "giant" due to the notable difference in size compared to the rest of the cells in the niche. In some cases, these giant cells were positive for FAPα, but their phenotype and function are currently unknown.

Apart from these giant cells, we suspect that tumour-infiltrating inflammatory cells are present in ameloblastoma, and more precisely, tumour-associated macrophages (TAMs). These are considered an important group of immunosuppressive cells residing within the tumour environment (71) and promote tumour growth and angiogenesis through both a paracrine and

autocrine effect (72). Interestingly, a relation between FAPα and TAMs has been established in ovarian cancer where FAPα non-enzymatic function promoted the generation of TAMs affecting the tumour progression (73). Regarding these evidences and together with the role of FAPα in the tumour previously designated, we suggest that FAPα enhances epithelial invasion in ameloblastoma throughout an enzymatic and non-enzymatic function.

Conclusion

FAPα is a protein of keen interest due to its heterogeneous expression. Dental follicle and apical papilla are dynamic tissues where FAPα plays a role in the remodelling of the extracellular matrix through an enzymatic function with the cleavage of collagen and a non-enzymatic function which remains largely unknown. The development of the apical papilla and the migration of SCAPs is controlled by TGF-β1 and SDF-1, growth factors that are also related to FAPα activation. Taken together, our findings about the role on FAPα in dental stem cells will provide more insight in the fundamental mechanism of end-stage root development and will offer new perspectives in ameloblastoma formation.

References

1. Dunn JS. Anatomy. A Regional Atlas of the Human Body. Journal of Anatomy. 1987;155:214-5.
2. Sharpe PT. Dental mesenchymal stem cells. Development. 2016;143(13):2273-80.
3. Goodman JD, Rozypal TL, Kelly T. Seprase, a membrane-bound protease, alleviates the serum growth requirement of human breast cancer cells. Clinical & Experimental Metastasis. 2003;20(5):459-70.
4. Kelly T, Huang Y, Simms AE, Mazur A. Fibroblast activation protein-alpha: a key modulator of the microenvironment in multiple pathologies. Int Rev Cell Mol Biol. 2012;297:83-116.
5. Pineiro-Sanchez ML, Goldstein LA, Dodt J, Howard L, Yeh Y, Tran H, et al. Identification of the 170-kDa melanoma membrane-bound gelatinase (seprase) as a serine integral membrane protease. J Biol Chem. 1997;272(12):7595-601.
6. Rettig WJ, Garin-Chesa P, Healey JH, Su SL, Ozer HL, Schwab M, et al. Regulation and heteromeric structure of the fibroblast activation protein in normal and transformed cells of mesenchymal and neuroectodermal origin. Cancer Res. 1993;53(14):3327-35.
7. Rettig WJ, Garin-Chesa P, Beresford HR, Oettgen HF, Melamed MR, Old LJ. Cell-surface glycoproteins of human sarcomas: differential expression in normal and malignant tissues and cultured cells. Proc Natl Acad Sci U S A. 1988;85(9):3110-4.
8. Milner JM, Kevorkian L, Young DA, Jones D, Wait R, Donell ST, et al. Fibroblast activation protein alpha is expressed by chondrocytes following a pro-inflammatory stimulus and is elevated in osteoarthritis. Arthritis Res Ther. 2006;8(1):R23.
9. Jacob M, Chang L, Pure E. Fibroblast activation protein in remodeling tissues. Curr Mol Med. 2012;12(10):1220-43.
10. Bosman FT, Stamenkovic I. Functional structure and composition of the extracellular matrix. J Pathol. 2003;200(4):423-8.
11. Aertgeerts K, Levin I, Shi L, Snell GP, Jennings A, Prasad GS, et al. Structural and kinetic analysis of the substrate specificity of human fibroblast activation protein alpha. J Biol Chem. 2005;280(20):19441-4.
12. Zhang M, Xu L, Wang X, Sun B, Ding J. Expression levels of seprase/FAPalpha and DPPIV/CD26 in epithelial ovarian carcinoma. Oncol Lett. 2015;10(1):34-42.
13. Chung KM, Hsu SC, Chu YR, Lin MY, Jiaang WT, Chen RH, et al. Fibroblast activation protein (FAP) is essential for the migration of bone marrow mesenchymal stem cells through RhoA activation. PLoS One. 2014;9(2):e88772.
14. Lee HO, Mullins SR, Franco-Barraza J, Valianou M, Cukierman E, Cheng JD. FAP-overexpressing fibroblasts produce an extracellular matrix that enhances invasive velocity and directionality of pancreatic cancer cells. BMC Cancer. 2011;11:245.
15. Niedermeyer J, Garin-Chesa P, Kriz M, Hilberg F, Mueller E, Bamberger U, et al. Expression of the fibroblast activation protein during mouse embryo development. Int J Dev Biol. 2001;45(2):445-7.
16. Tillmanns J, Hoffmann D, Habbaba Y, Schmitto JD, Sedding D, Fraccarollo D, et al. Fibroblast activation protein alpha expression identifies activated fibroblasts after myocardial infarction. J Mol Cell Cardiol. 2015;87:194-203.
17. Bae S, Park CW, Son HK, Ju HK, Paik D, Jeon CJ, et al. Fibroblast activation protein alpha identifies mesenchymal stromal cells from human bone marrow. Br J Haematol. 2008;142(5):827-30.
18. Yagi H, Soto-Gutierrez A, Kitagawa Y, Tilles AW, Tompkins RG, Yarmush ML. Bone marrow mesenchymal stromal cells attenuate organ injury induced by LPS and burn. Cell Transplant. 2010;19(6):823-30.
19. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. Nature. 2002;420(6916):629-35.
20. Goldberg M, Kulkarni AB, Young M, Boskey A. Dentin: structure, composition and mineralization. Front Biosci (Elite Ed). 2011;3:711-35.

21. Orban BBJ, Bhaskar SSN. Orban's Oral Histology and Embryology: C. V. Mosby; 1986.
22. Caton J, Tucker AS. Current knowledge of tooth development: patterning and mineralization of the murine dentition. J Anat. 2009;214(4):502-15.
23. Nam H, Kim JH, Kim JW, Seo BM, Park JC, Kim JW, et al. Establishment of Hertwig's epithelial root sheath/epithelial rests of Malassez cell line from human periodontium. Mol Cells. 2014;37(7):562-7.
24. Sonoyama W, Seo BM, Yamaza T, Shi S. Human Hertwig's epithelial root sheath cells play crucial roles in cementum formation. J Dent Res. 2007;86(7):594-9.
25. Zeisberg M, Neilson EG. Biomarkers for epithelial-mesenchymal transitions. J Clin Invest. 2009;119(6):1429-37.
26. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009;119(6):1420-8.
27. Chen J, Chen G, Yan Z, Guo Y, Yu M, Feng L, et al. TGF-beta1 and FGF2 stimulate the epithelial-mesenchymal transition of HERS cells through a MEK-dependent mechanism. J Cell Physiol. 2014;229(11):1647-59.
28. Reeve CM, Wentz FM. The prevalence, morphology, and distribution of epithelial rests in the human periodontal ligament. Oral Surgery, Oral Medicine, Oral Pathology. 1962;15(7):785-93.
29. Cerri PS, Katchburian E. Apoptosis in the epithelial cells of the rests of Malassez of the periodontium of rat molars. J Periodontal Res. 2005;40(5):365-72.
30. Lungova V, Radlanski RJ, Tucker AS, Renz H, Misek I, Matalova E. Tooth-bone morphogenesis during postnatal stages of mouse first molar development. J Anat. 2011;218(6):699-716.
31. Li J, Parada C, Chai Y. Cellular and molecular mechanisms of tooth root development. Development. 2017;144(3):374-84.
32. Sonoyama W, Liu Y, Yamaza T, Tuan RS, Wang S, Shi S, et al. Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. J Endod. 2008;34(2):166-71.
33. Huang GT, Sonoyama W, Liu Y, Liu H, Wang S, Shi S. The hidden treasure in apical papilla: the potential role in pulp/dentin regeneration and bioroot engineering. J Endod. 2008;34(6):645-51.
34. Wang J, Zhang F, Zhang J, Tang W, Tang A, Peng H, et al. Key issues and recent progress of high efficient organic light-emitting diodes. Journal of Photochemistry and Photobiology C: Photochemistry Reviews. 2013;17:69-104.
35. Hemmat S, Lieberman DM, Most SP. An introduction to stem cell biology. Facial Plast Surg. 2010;26(5):343-9.
36. Ullah I, Subbarao R B, Rho G J. Human mesenchymal stem cells - current trends and future prospective. Biosci Rep. 2015;35(2).
37. Arana-Chavez VE, Massa LF. Odontoblasts: the cells forming and maintaining dentine. Int J Biochem Cell Biol. 2004;36(8):1367-73.
38. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A. 2000;97(25):13625-30.
39. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, et al. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A. 2003;100(10):5807-12.
40. Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet. 2004;364(9429):149-55.
41. Morsczeck C, Gotz W, Schierholz J, Zeilhofer F, Kuhn U, Mohl C, et al. Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. Matrix Biol. 2005;24(2):155-65.
42. Reichart PA, Philipsen HP, Sciubba JJ. The new classification of Head and Neck Tumours (WHO)--any changes? Oral Oncol. 2006;42(8):757-8.
43. Bachmann AM, Linfesty RL. Ameloblastoma, solid/multicystic type. Head Neck Pathol. 2009;3(4):307-9.

44. Sehic A. Understanding ameloblastomas through tooth development2015.
45. Reichart PA, Philipsen HP, Sonner S. Ameloblastoma: biological profile of 3677 cases. Eur J Cancer B Oral Oncol. 1995;31B(2):86-99.
46. Fuchigami T, Koyama H, Kishida M, Nishizawa Y, Iijima M, Kibe T, et al. Fibroblasts promote the collective invasion of ameloblastoma tumor cells in a 3D coculture model. FEBS Open Bio. 2017;7(12):2000-7.
47. Cahill DR, Marks SC, Jr. Tooth eruption: evidence for the central role of the dental follicle. J Oral Pathol. 1980;9(4):189-200.
48. Bosshardt DD, Schroeder HE. Cementogenesis reviewed: a comparison between human premolars and rodent molars. Anat Rec. 1996;245(2):267-92.
49. Vuoriluoto K, Haugen H, Kiviluoto S, Mpindi JP, Nevo J, Gjerdrum C, et al. Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer. Oncogene. 2011;30(12):1436-48.
50. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nature reviews Molecular cell biology. 2014;15(3):178-96.
51. Yang Y, Candelario-Jalil E, Thompson JF, Cuadrado E, Estrada EY, Rosell A, et al. Increased intranuclear matrix metalloproteinase activity in neurons interferes with oxidative DNA repair in focal cerebral ischemia. J Neurochem. 2010;112(1):134-49.
52. Tonti GA, Mannello F, Cacci E, Biagioni S. Neural stem cells at the crossroads: MMPs may tell the way. Int J Dev Biol. 2009;53(1):1-17.
53. Wang L, Yan M, Wang Y, Lei G, Yu Y, Zhao C, et al. Proliferation and osteo/odontoblastic differentiation of stem cells from dental apical papilla in mineralization-inducing medium containing additional KH(2)PO(4). Cell Prolif. 2013;46(2):214-22.
54. Ghersi G, Dong H, Goldstein LA, Yeh Y, Hakkinen L, Larjava HS, et al. Regulation of fibroblast migration on collagenous matrix by a cell surface peptidase complex. J Biol Chem. 2002;277(32):29231-41.
55. Laghezza Masci V, Taddei AR, Gambellini G, Giorgi F, Fausto AM. Microvesicles shed from fibroblasts act as metalloproteinase carriers in a 3-D collagen matrix. J Circ Biomark. 2016;5:1849454416663660.
56. Murdoch C. CXCR4: chemokine receptor extraordinaire. Immunological reviews. 2000;177:175-84.
57. Hu C, Yong X, Li C, Lu M, Liu D, Chen L, et al. CXCL12/CXCR4 axis promotes mesenchymal stem cell mobilization to burn wounds and contributes to wound repair. The Journal of surgical research. 2013;183(1):427-34.
58. Li M, Sun X, Ma L, Jin L, Zhang W, Xiao M, et al. SDF-1/CXCR4 axis induces human dental pulp stem cell migration through FAK/PI3K/Akt and GSK3β/β-catenin pathways. Scientific Reports. 2017;7:40161.
59. Liu JY, Chen X, Yue L, Huang GT, Zou XY. CXC Chemokine Receptor 4 Is Expressed Paravascularly in Apical Papilla and Coordinates with Stromal Cell-derived Factor-1alpha during Transmigration of Stem Cells from Apical Papilla. J Endod. 2015;41(9):1430-6.
60. Yun HJ, Jo DY. Production of stromal cell-derived factor-1 (SDF-1)and expression of CXCR4 in human bone marrow endothelial cells. Journal of Korean medical science. 2003;18(5):679-85.
61. Hinz B. The extracellular matrix and transforming growth factor-beta1: Tale of a strained relationship. Matrix Biol. 2015;47:54-65.
62. Yoshida K, Saito T, Kamida A, Matsumoto K, Saeki K, Mochizuki M, et al. Transforming growth factor-beta transiently induces vimentin expression and invasive capacity in a canine mammary gland tumor cell line. Research in veterinary science. 2013;94(3):539-41.
63. Hinge A, Filippi MD. Deconstructing the Complexity of TGFbeta Signaling in Hematopoietic Stem Cells: Quiescence and Beyond. Curr Stem Cell Rep. 2016;2(4):388-97.
64. Kubiczkova L, Sedlarikova L, Hajek R, Sevcikova S. TGF-beta - an excellent servant but a bad master. J Transl Med. 2012;10:183.

65. Hasegawa T, Chosa N, Asakawa T, Yoshimura Y, Fujihara Y, Kitamura T, et al. Differential effects of TGF-beta1 and FGF-2 on SDF-1alpha expression in human periodontal ligament cells derived from deciduous teeth in vitro. Int J Mol Med. 2012;30(1):35-40.
66. Wright N, de Lera TL, Garcia-Moruja C, Lillo R, Garcia-Sanchez F, Caruz A, et al. Transforming growth factor-beta1 down-regulates expression of chemokine stromal cell-derived factor-1: functional consequences in cell migration and adhesion. Blood. 2003;102(6):1978-84.
67. H. Ross M, J. Reith E, Romrell L. Histology : A Text and Atlas1995.
68. Abduelmula A, Huang R, Pu Q, Tamamura H, Morosan-Puopolo G, Brand-Saberi B. SDF-1 controls the muscle and blood vessel formation of the somite. Int J Dev Biol. 2016;60(1-3):29-38.
69. Heikinheimo K, Jee KJ, Niini T, Aalto Y, Happonen RP, Leivo I, et al. Gene expression profiling of ameloblastoma and human tooth germ by means of a cDNA microarray. J Dent Res. 2002;81(8):525-30.
70. Cox EA, Sastry SK, Huttenlocher A. Integrin-mediated adhesion regulates cell polarity and membrane protrusion through the Rho family of GTPases. Mol Biol Cell. 2001;12(2):265-77.
71. Wing K, Sakaguchi S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nat Immunol. 2010;11(1):7-13.
72. Chen P, Huang Y, Bong R, Ding Y, Song N, Wang X, et al. Tumor-associated macrophages promote angiogenesis and melanoma growth via adrenomedullin in a paracrine and autocrine manner. Clin Cancer Res. 2011;17(23):7230-9.
73. Hou CM, Qu XM, Zhang J, Ding TT, Han W, Ji GC, et al. Fibroblast activation proteins-α suppress tumor immunity by regulating T cells and tumor-associated macrophages. Experimental and Molecular Pathology. 2018;104(1):29-37.

Auteursrechtelijke overeenkomst

Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:
The role of FAPalpha in dental stem cells and root development

Richting: **Master of Biomedical Sciences-Environmental Health Sciences**
Jaar: **2018**

in alle mogelijke mediaformaten, - bestaande en in de toekomst te ontwikkelen - , aan de Universiteit Hasselt.

Niet tegenstaand deze toekenning van het auteursrecht aan de Universiteit Hasselt behoud ik als auteur het recht om de eindverhandeling, - in zijn geheel of gedeeltelijk -, vrij te reproduceren, (her)publiceren of distribueren zonder de toelating te moeten verkrijgen van de Universiteit Hasselt.

Ik bevestig dat de eindverhandeling mijn origineel werk is, en dat ik het recht heb om de rechten te verlenen die in deze overeenkomst worden beschreven. Ik verklaar tevens dat de eindverhandeling, naar mijn weten, het auteursrecht van anderen niet overtreedt.

Ik verklaar tevens dat ik voor het materiaal in de eindverhandeling dat beschermd wordt door het auteursrecht, de nodige toelatingen heb verkregen zodat ik deze ook aan de Universiteit Hasselt kan overdragen en dat dit duidelijk in de tekst en inhoud van de eindverhandeling werd genotificeerd.

Universiteit Hasselt zal mij als auteur(s) van de eindverhandeling identificeren en zal geen wijzigingen aanbrengen aan de eindverhandeling, uitgezonderd deze toegelaten door deze overeenkomst.

Voor akkoord,

Amaya Garrido, Ana

Datum: **12/06/2018**