Original

Expression of the IGF-1, IGFBP-3 and IGF-1 receptors in dental pulp stem cells and impacted third molars

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Abstract: IGF-1 regulates the metabolism of hard dental tissue through binding to the IGF-1 receptor on target cells. Furthermore, IGF-binding-protein-3 promotes the accessibility of IGF-1. The aim of this study was to investigate the expression of IGF-1, IGFBP-3 and IGF-1R in STRO-1-positive dental pulp stem cells (DPSCs) and fully impacted wisdom teeth in relation to tooth development. Third molars were surgically removed from 60 patients and classified into two groups: teeth showing ongoing development (group 1) and teeth that had completed root shaping (group 2). The transcript and protein levels of IGF-1, IGFBP-3 and IGF-1R were investigated using RT-PCR and immunohistochemistry. The expression of the same proteins was also analyzed in DPSCs. The teeth from group 1 showed significantly stronger expression of IGF-1 and IGF-1R. The major sources of all of the proteins investigated immunohistochemically in sections of wisdom teeth were odontoblasts, cementoblasts and cell colonies in the pulpal mesenchyme. These colonies were identified as stem cells in view of their positivity for STRO-1, and the cells were subsequently sorted by flow cytometry. These DPSCs

demonstrated high levels of pluripotency markers and IGF-1 and IGF-1R. We conclude that members of the IGF-1 family are involved in the late stage of tooth development and the process of pulpal differentiation. (J Oral Sci 55, 319-327, 2013)

Keywords: dental pulp stem cell; dentinogenesis; IGF-1; IGF-1 receptor; odontogenesis.

Introduction

Dental tissue engineering and the regeneration of functional tooth-tissue structures involve multidisciplinary approaches based on the interaction of three basic key elements: stem cells, morphogens and scaffolds. In this connection, locally niched stem cells, and their differentiation and pluripotency, are of considerable importance, together with the morphogens, growth factors and cytokines that are intrinsically involved in the process of odontogenesis (1,2). One such factor playing a role in dental development is insulin-like growth factor 1 (IGF-1), a single-chain polypeptide showing approximately 50% structural homology with insulin. IGF-1 binds to the insulin receptor but with lower affinity than insulin, and therefore has lower metabolic capabilities (3,4). Moreover, IGF-1 and two other proteins, IGF-1-binding-protein-3 (IGFBP-3) and the IGF-1 receptor (IGF-1R), serve as regulators of cellular proliferation and the response to growth hormone (GH). It has been suggested that IGF-1 is involved in most endocrine, paracrine and autocrine

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stimuli affecting organogenesis, and the proliferation of skeletal tissue in particular (5). Ninety-nine percent of serum-free IGF-1 is bound to IGFBPs and released by proteolytic cathepsins and matrix metalloproteases (6,7). IGFBP-3 may inhibit cell proliferation through competitive binding to IGF-1, and also affect cell proliferation irrespective of the presence of IGF-1 (8). Furthermore, among all the IGFBPs, IGFBP-3 has the highest concentration in postnatal serum, responding to IGF-1 treatment with increased expression. IGFBP-3 may also have a direct role in bone formation by acting on the growth plate (9). Especially in bones, IGF-1 is regulated by GH, parathyroid hormone and steroids, whereas in gonads its release is induced by steroids (10). More than ten years ago it was demonstrated for the first time that IGF-1 and GH may have a major influence on the mitotic activity of cells participating in odontogenesis (11). Studies of cultured mouse molars revealed that insulin, IGF-1 and IGF-2 were able to increase the synthesis of enamel matrix proteins, indicating their involvement in amelogenesis (12,13). Furthermore, the dentinogenic ability of IGF-1 has been demonstrated in direct pulp-capping experiments, IGF-1 mediating the formation of dentinal hard tissue (14). Treatment of human pulp fibroblast cell cultures with IGF-1 has been shown to increase cell proliferation, alkaline phosphatase activity, and DNA synthesis (15). IGF-1 has also been shown to induce the differentiation of mesenchymal stem cells in human bone marrow (16). The presence of multipotent stem cells in dental pulp is necessary for odontoblast differentiation and is involved in numerous pulpal healing processes. These mesenchymal cells, named dental pulp stem cells (DPSC), can be distinguished using STRO-1 as a marker (17-19). Dental stem cells have been reported to contain high amounts of IGF-1, in contrast to fibroblasts or human bone marrow-derived mesenchymal stem cells (20,21). These findings clearly demonstrate the importance of growth factors in cellular differentiation and dental development.

All of these previously published investigations were mainly animal studies aimed at demonstrating the association between the IGF-1 family and odontogenesis. However, recent studies focusing only on the effects of IGF-1 have yielded contradictory results. Caviedes-Bucheli et al. reported that IGF-1 was down-regulated during the late stage of human tooth development, and that IGF-1 was overexpressed in third molars that had completed their development (22). On the other hand, another study demonstrated up-regulation of IGF-1R in human wisdom teeth with incomplete root development (23). Due to these inconsistencies in the expression

patterns of IGF-1 and IGF-1R, our present study focused on the expression of IGF-1, IGFBP-3 and IGF-1R at different stages of root development in human third molars, and the interaction of these proteins with pluripotent pulpal progenitors. Our working hypothesis was that IGF-1, IGFBP-3 and IGF-1R were associated with human tooth root development and the pulpal mesenchyme. As a null hypothesis we considered that the IGF-1 family played no role in the late stages of tooth development.

Materials and Methods

Patients

Totally impacted third molars ($n = 60$) were surgically removed from patients between the ages of 15 and 59 years. All the patients were free of acute root or periodontal infections that could have affected the third molars. The teeth were fully impacted and did not communicate with the oral cavity; all had been extracted for orthodontic, prophylactic or prosthetic reasons. This study was approved by the ethics committee of the Martin Luther University, Faculty of Medicine (13.01.2010). All patients provided written informed consent. Four wisdom teeth were used to isolate DPSCs, 12 were dissected for immunohistochemical staining, and 44 were used for preparation of RNA.

FACS analysis and culture of human dental pulp stem cells (DPSCs)

A single-cell suspension was prepared from the removed dental pulp in accordance with recently published protocols, and cultured in α -MEM medium (Invitrogen, Karlsruhe, Germany) containing 200 μ M L-ascorbic acid 2-phosphate (Sigma-Aldrich GmbH, Steinheim, Germany), 2 mM L-glutamine (Merck KGaA, Darmstadt, Germany), and 10% FCS (Biowest, Nuaille, France) at 37°C under 5% CO₂ (24,25). The third passage of the primary-cultured pulpal cells was incubated in PBS with a monoclonal antibody against the mesenchymal stem cell marker STRO-1 (10 μ g/mL; R&D Systems, Minneapolis, MN, USA) for 30 min at 0°C. After a wash in PBS, the cells were incubated for an additional 30 min at 0°C with phycoerythrin (PE)-conjugated goat anti-mouse antibody (1:50 Sigma-Aldrich GmbH). A FACS Vantage (BD Biosciences, Heidelberg, Germany) was used for fluorescence-activated cell sorting. STRO-1-positive and -negative cells were then cultured in α -MEM medium (Invitrogen) at 37°C under 5% CO₂, and finally mRNA and proteins were extracted.

Adipogenic and osteogenic differentiation

For osteogenic differentiation, the cells were treated

with α -MEM, 200 μ M ascorbic acid 2-phosphate, 1 μ M dexamethasone, 10 mM glycerol 3-phosphate (all from Sigma-Aldrich), and 10% FCS. After morphological changes became apparent, the cells (2-3 weeks) were fixed with 2% formaldehyde (Sigma-Aldrich) and washed with PBS. Calcium deposits were stained with Alizarin red S for 20 min and then washed with distilled water.

For adipogenic differentiation, the cells were stimulated with adipogenic induction medium (α -MEM, 50 μ M dexamethasone, 10 μ g/mL bovine insulin, 100 μ M indomethacin, 500 μ M 3-isobutyl-1-methylxanthine (Sigma-Aldrich), and 10% FCS). The cells were then treated with this medium for 2 days, followed by adipogenic differentiation medium (α -MEM, 50 μ M dexamethasone, 10 μ g/mL bovine insulin, 5 μ M rosiglitazone [Alexis Corporation, Lucerne, Switzerland], and 10% FCS) for 3 days. This cycle was repeated until differentiation was observed. Then the adipogenic DPSCs were fixed with 2% formaldehyde (Sigma-Aldrich) and washed with 50% ethanol after 2-3 weeks. Oil Red O staining (Sigma-Aldrich) was performed for 20 min, and then the cells were washed with 50% ethanol (26).

RT-PCR

The patients were divided into two groups according to tooth developmental stage. The first group included patients with ongoing tooth development and the second group included patients in whom the root shaping process had been completed. After surgical extraction, all teeth were immediately frozen in liquid nitrogen. Prior to RNA extraction the teeth were crushed and homogenized for 20 s (Mikro-Dismembrator, B. Braun Biotech Int., Melsungen, Germany). The RNA was then extracted using Trizol reagent in accordance with the manufacturer's instructions (Invitrogen, Karlsruhe, Germany).

Total RNA was extracted from complete teeth with surrounding tissues including the dental bud or periodontal ligament, or from non-confluent STRO-1-positive and -negative DPSCs using Trizol reagent in accordance with the manufacturer's instructions (Invitrogen). The cDNA was synthesized using a reverse transcriptase kit (Superscript II, Gibco BRL, Invitrogen, Karlsruhe, Germany). RT-PCR was performed with specific primer pairs for IGF1 (sense 5'-ATCCTTCTCTCTCATTCTTC-3'; antisense 5'-GATACACAGACACAGATAAAG-3') (Sequence ID: NM_001111283.1), IGFBP3 (sense 5'-TCAGAGCACAGATACCCAG-3'; antisense 5'-ACAGCCGCCTAAGTCAC-3') (Sequence ID: NM_001013398.1), IGF1R (sense 5'-TCCACATCCTGCTCATCTCC-3'; antisense 5'-AGAAGTCACGGTC-

CACACAG-3') (Sequence ID: NM_000875.3), Oct-4 (sense 5'-GACCATCTGCCGCTTGAGGCTCTG-3'; antisense 5'-GCGCCGGTTACAGACACACTCGG-3') (Sequence ID: NM_002701.4), Nanog (sense 5'-CAATGTGTGACGCAG-3'; antisense 5'-ATTCAGC-CAGTGTCCAG-3') (Sequence ID: NM_024865.2), Sox-2 (sense 5'-TGTCTACTCGCAGCAG-3'; antisense 5'-GCGTGAGTGTGGATGG-3') (Sequence ID: NM_003106.3) and 18S (sense 5'-GTTGGTGGAGC-GATTGTCTGG-3'; antisense 5'-AGGGCAGGGACT-TAATCAACGC-3') (Sequence ID: NR_003286.2) as normalizing markers.

The PCR products were resolved on 1% agarose gel containing 0.05% ethidium bromide and scanned (Kodak Digital Science Image station 440CF; Eastman Kodak, Rochester, NY, USA). The colon carcinoma cell line SW 480 was used as a positive control for all amplicons (27,28). The intensity of the PCR amplicons was calculated semi-quantitatively in comparison with a positive control (C+) defined as 100%. Kodak Digital Science ID V3.0.2 software was used for all calculations.

Immunohistochemistry

The surgically extracted third molars were immediately frozen. Freshly cut cryo-embedded serial 8- μ m sections of 12 teeth were subjected to immunohistochemistry to investigate the cellular localization of the studied proteins. The teeth were decalcified with 300 mM EDTA for 2-5 weeks, and then embedded in OCT-Matrix (Cell Path Ltd, Newton Powys, UK).

Briefly, consecutive cryostat sections were cut using a special steel knife on a Micron HM560 Cryostat (both from MICROM GmbH, Walldorf, Germany), mounted on Superfrost slides (Erie Scientific Company, Portsmouth, NH, USA), and fixed in a mixture of 97% ice-cold methanol and 3% H₂O₂ for 20 min. The slides were incubated for 1 h at 37°C with monoclonal antibodies (mAb) against IGF-1 (H-70), IGFBP-3 (H-98), and IGF-1R α (H-78, all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:100. Staining with the monoclonal antibody against STRO-1 (1:50; R&D Systems, Minneapolis, MN, USA) was performed for 10 h at 6°C. After washing with PBS, the sections were incubated for exactly 30 min with a 1:1,000 dilution of biotinylated goat anti-mouse secondary antibody (DAKO GmbH, Hamburg, Germany). Detection of immunoreactive proteins was performed by the avidin-biotin complex method with 15% 3,3'-diaminobenzidine (DAKO) solution as the chromogen. Afterwards the samples were counterstained with hematoxylin followed by intensive washing with distilled water.

Fig. 1 Representative immunoreactivity of IGF-1 in the apical region and dental pulp of wisdom teeth. **a** Note strong IGF-1 staining in the dental papilla mesenchyme (green arrow), odontoblasts (black arrow) and cementoblasts (blue arrow). **b** Immunoreactivity for IGF-1 in the odontoblasts is evident. **c** IGF-1 staining of aggregated cells in the dental pulp. **d** In stained sections, only the odontoblastic layer (arrow) and single pulp cell clusters like those shown in (c) are positive. **e** Strong IGFBP-3 staining of the odontoblast zone (black arrow), pulpal mesenchyme (green arrow) and the dental follicle (blue arrow). **f** Intense IGFBP-3 staining of mesenchymal cells of the dental papilla (green arrows), the young odontoblast layer (black arrow) and cementoblasts (blue arrow). **g** Cell colonies connected to blood vessels distant from pericytes show strong IGFBP-3 immunoreactivity. **h** In fully developed areas, odontoblasts (arrows) show positive staining. **i** Diffuse IGF-1R α staining of the cytoplasm of young odontoblasts (black arrow) and focal low expression in the mesenchyme of the dental follicle (blue arrow). **j** IGF-1R α is especially evident in the cytoplasm of young odontoblasts (black arrow), pulp mesenchyme (green arrows) and cementoblasts (blue arrow). **k** IGF-1R α -immunopositive cell colonies distant from pericytes connected to blood vessels. **l** The fully developed areas show strong staining only in mature odontoblasts (arrows) and intrapulpal cell colonies (j).

STRO-1-positive and -negative cells were cultured on slides for 24 h and then fixed with 97% ice-cold methanol and 3% H₂O₂ for 20 min. This was followed by incubation with the monoclonal antibodies against IGF-1, IGFBP-3 and IGF-1R α (1:100, all from Santa Cruz Biotechnology) in accordance with the protocol described above.

Western blotting

The cells were washed twice with PBS prior to protein extraction. Proteins were extracted using a lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 2% pharmalyte (all Amersham Bioscience, Freiburg, Germany), and 2% DTT (Invitrogen). About 10 μ g of total protein lysate from untreated DPSCs and STRO-1-negative cells was separated on 10% polyacrylamide SDS-PAGE gels and blotted on a PVDF membrane (Amersham Biosciences). Blocking was performed for 1 h at 20°C in 5% non-fat milk powder/1 $\times$ TBST (Tris-buffered saline/0.05% Tween 20/pH 7.5). After three washes with

1 $\times$ TBST, the membranes were incubated overnight at 4°C with specific antibodies against IGF-1, IGFBP-3, and IGF-1R α (1:1,000; all Santa Cruz) and β -actin (1:10,000; Sigma). Secondary goat-anti-rabbit IgG-HRP (1:2,000, sc-2004, Santa Cruz Biotechnology) antibodies were applied for 1 h at 20°C. Immunoreactive bands were visualized using an ECL Detection Kit (Amersham Biosciences) and analysed with Kodak Image System 440cf (Eastman Kodak). Detection of β -actin was used for normalization, and SW 480 (C+) served as a positive control. The relative contents of the investigated proteins in STRO-1-positive and negative cells were calculated semi-quantitatively.

Statistical analysis

Statistical analysis was carried out with SPSS 12.0 software. All experimental parameters were assessed for statistical significance using ANOVA and Student's *t*-test. Levels of significance were defined as $P < 0.05^*$

and $P < 0.005^{**}$.

Results

Immunohistochemistry

The immunohistochemical investigations were performed using specific antibodies against IGF-1, IGFBP-3 and IGF-1R, and demonstrated restricted and focal cell-specific staining. The major sources of all proteins were not the pulpal tissues in general, but the layer of odontoblasts, nerve fibers and blood vessels within the pulp, and

Fig. 2 Immunohistochemical staining of the dental pulp for STRO-1. STRO-1-positive cell colonies are located in the dental pulp adjacent to blood vessels.

the cementoblasts on the root surface. The majority of the sections investigated showed strong cytoplasmic staining for IGF-1 within odontoblasts and cementoblasts (Fig. 1a, b). This protein was expressed particularly strongly in the developing and differentiating apical mesenchyme. In this connection, young preodontoblasts and dental follicles were positively stained for IGF-1 (Fig. 1a, b). Single cell colonies were also identified in the pulp of both immature and developed teeth (Fig. 1c). Furthermore, odontoblasts were positive for IGF-1 in developed teeth (Fig. 1d). IGFBP-3 was observed in the cytoplasm of odontoblasts and cementoblasts (Fig. 1e, f), as well as single colonies in the pulpal mesenchyme and connective tissues (Fig. 1g). IGFBP-3-staining was found in completely developed odontoblasts (Fig. 1h). Strong IGF-1R α -staining was observed in the mesenchyme of apical pulp sections from developing teeth. These positive staining mainly included cells connected with blood vessels in the pulpal tissue (Fig. 1i, j). Furthermore, cell clusters were stained for IGF-1R α (Fig. 1k). In developed areas, the odontoblastic layer was positive for IGF-1R α (Fig. 1l).

Interestingly, these intrapulpal cell colonies (Fig. 1c, g, k) were also detectable with the mesenchymal stem cell marker STRO-1 (Fig. 2).

Fig. 3 **a** Semi-quantitative evaluation of the results of RT-PCR for **b** IGF-1, **c** IGF-1R and **d** IGFBP-3. **a** Representative examples of IGF-1, IGFBP-3 and IGF-1R expression evaluated as total mRNA expression in both groups. 18S was used for normalization. **b** IGF-1 expression in the two groups. Note that group 1 showed significantly higher expression of IGF-1 than group 2. **c** Expression of IGF-1R was significantly lower in group 2 than in group 1. **d** IGFBP-3 expression was lower in group 2 than in group 1, but not to a significant degree (* $P < 0.05$; ** $P < 0.005$).

Fig. 4 **a** FACS analysis. In primary pulp cultures, 1.5% of the cells were STRO-1-positive. **b** Expression of IGF-1, IGFBP-3 and IGF-1R in DPSCs. STRO-1-positive DPSCs showed much stronger expression of Oct-4, Nanog and Sox-2 than STRO-1-negative cells. **c** Semi-quantitative RT-PCR ($*P < 0.05$; $**P < 0.005$). **d, e** Alizarin red S staining after treatment with osteogenic medium. **d** STRO-1-positive DPSCs show stainable calcium deposits. **e** No changes are evident in STRO-1-negative cells. **f, g** Oil Red O staining after treatment with adipogenic medium. Intracellular lipids are stainable in STRO-1-positive **f** but not in negative **g** cells.

RT-PCR of human wisdom teeth

The RT-PCR analysis revealed strong expression of IGF-1 mRNA in wisdom teeth obtained from patients in group 1. In contrast, teeth from patients in group 2 showed significantly weaker expression of IGF-1 mRNA ($P = 0.011$) (Fig. 3a, b), which coincided with significantly decreased expression of IGF-1R ($P = 0.013$) (Fig. 3a, c) and reduced levels of IGFBP-3 (Fig. 3a, d) within this group. There were no evident differences in the relationship between the expression of any of the investigated proteins and gender (data not shown).

Fig. 5 Expression of IGF-1, IGFBP-3 and IGF-1R in DPSCs. **a** STRO-1-positive DPSCs showed much stronger mRNA-expression of IGF-1, IGFBP-3 and IGF-1R in comparison to STRO-1-negative cells. 18S amplicons were used for normalization. **b** Semi-quantitative evaluation of the results of RT-PCR. Immunohistochemical staining of **c-e** STRO-1-positive and **f-h** negative cells for **c, f** IGF-1, **d, g** IGFBP-3 and **e, h** IGF-1R α . STRO-1-positive cells showed stronger immunoreactivity for **c** IGF-1 and **e** IGF-1R α than STRO-1-negative (**f, h**). However, no differences in IGFBP-3-expression were observed between STRO-1 positives (**d**) and -negatives (**g**). Note the colony-forming units among the STRO-1-positive cells (**c-e**). **i** Immunoblotting of STRO-1-positive and -negative cells using antibodies against IGF-1, IGFBP-3 and IGF-1R α , normalized relative to β -actin. **j** Graphical analysis of the Western blots demonstrated significantly higher expression of IGF-1 and IGFBP-3 in STRO-1-positive cells ($*P < 0.05$; $**P < 0.005$).

Isolation and differentiation of DPSCs, and their expression of IGF family members

In order to separate STRO-1-positive dental pulp stem cells from pulpal fibroblasts, flow cytometry was performed. Our aim was to investigate the expression of IGF-1, IGFBP-3 and IGF-1R in these mesenchymal progenitors in comparison with STRO-1-negative cells.

STRO-1-positive sorted cells accounted for 1.5% of total cells (Fig. 4a).

To confirm the isolation of stem cells, RT-PCR for pluripotency markers was performed. The STRO-1-positive stem cells showed significantly increased expression of Oct-4 ($P = 0.0004$), Sox-2 ($P = 0.021$) and Nanog ($P = 0.012$) in comparison with STRO-1-negative cells (Fig. 4b, c). Furthermore, the DPSCs were divisible into osteogenic and adipogenic lineages (Fig. 4d, f), unlike STRO-1-negative cells (Fig. 4e, g), on the basis of Alizarin red S and Oil Red O staining.

In order to validate the results of *in situ* IGF-1, IGFBP-3 and IGF-1R expression in third molars, our *in vitro* investigations focused on STRO-1-positive DPSCs. Undifferentiated DPSCs demonstrated significantly stronger expression of IGF-1 ($P = 0.001$) and IGF-1R ($P = 0.033$) mRNA than the corresponding STRO-1-negative controls (Fig. 5a, b). Expression of IGF-1 (Fig. 5c, f), IGFBP-3 (Fig. 5d, g) and IGF-1R α (Fig. 5e, h) protein was also investigated by immunohistochemical staining of both STRO-1-positive and -negative cells. STRO-1-positive cells showed stronger immunoreactivity for IGF-1 (Fig. 5c) and IGF-1R α (Fig. 5e) than STRO-1-negative cells (Fig. 5f, h). There were no immunohistochemically detectable differences in IGFBP-3 expression between STRO-1-positive (Fig. 5d) and STRO-1-negative (Fig. 5g) cells. Furthermore, the STRO-1-positive cells demonstrated colony-forming units, which are a typical characteristic of mesenchymal stem cells (Fig. 5 c-e). These results were confirmed by immunoblotting (Fig. 5i, j); STRO-1-positive cells over-expressed IGF-1 ($P = 0.023$) and IGF-1R α ($P = 0.025$) relative to STRO-1-negative cells. No significant differences ($P = 0.87$) in IGFBP-3 expression (Fig. 5i, j) were evident, reflecting the results of immunohistochemistry (Fig. 5 d, g).

Discussion

The present study was able to confirm our hypothesis that IGF-1, IGFBP-3 and IGF-1R play a role in the late stages of human tooth development, and thus the null-hypothesis was rejected. The strongest expression of IGF-1, IGFBP-3 and IGF-1R was evident in teeth with ongoing root development, especially in the incomplete apical base, where high proliferation and differentiation rates are normally observed (29). Furthermore, in accordance with previous studies of the potential roles of the IGF family in odontogenesis, we found that the expression of IGF-1, IGFBP-3 and IGF-1R declined when tooth development had been completed. On the other hand, immature teeth showed increased levels of expression

of all three members of the IGF-1 family investigated (23,30,31). Moreover, we were able to show that members of this family of growth factors were mainly expressed in the area of apical growth and differentiation, along with mesenchymal progenitor cells. On the other hand, in complete developed structures, only odontoblasts and cementoblasts were immunopositive, while pulpal tissues were mostly negative. These findings suggest that the IGF-1 family has a significant role in the differentiation of mesenchymal cells into pulpal cells, including fibroblasts, cementoblasts and odontoblasts.

The different findings reported by Caviedes-Bucheli et al., indicating decreased IGF-1 expression in the dental pulp of human wisdom teeth with immature roots, might have been attributable to technical factors (22). In the present study we homogenized the teeth completely (including the dental follicle, apical papilla and dental pulp) for preparation of mRNA, whereas Caviedes-Bucheli et al. investigated only the dental pulp (22). As we consider that the root shaping process also involves surrounding tissues such as the dental follicle, periodontium and apical papilla, we included them in our preparations. On the other, the major conclusion reached by Caviedes-Bucheli et al. was that fully developed teeth retained lifelong expression of IGF-1 and its receptor, and that this growth factor continued to be involved in pulpal regeneration. As we demonstrated detectable levels of IGF-1 and IGF-1R in fully developed teeth, mainly in the odontoblastic layer, we concur with Caviedes-Bucheli et al. on this issue (22).

We also detected IGF proteins in cell colonies in the dental pulp, and found that these cell clusters were also positive for the mesenchymal stem cell marker STRO-1, suggesting the presence of dental pulpal stem cells (DPSCs). Moreover, immunohistochemical staining showed that these cells were partly associated with blood vessels, which are reportedly the preferred location of DPSCs (18). Our *in vitro* data demonstrated that DPSCs, identified using STRO-1 as a stem cell marker, had an increased level of pluripotency defined by the expression of Oct-4, Nanog and Sox-2 (31). Furthermore, the isolated DPSCs were able to differentiate into osteoblasts and adipocytes, as reported previously (17,19,24,32,33). The isolated cells showed all of the characteristics of mesenchymal progenitors. In addition, we demonstrated increased expression of IGF-1, IGFBP-3 and IGF-1R, correlating with the high levels of IGF family members in teeth with ongoing root development. It is noteworthy that STRO-1-negative cells were either negative or only slightly positive for the investigated pluripotency markers and IGF family members. This suggested that

IGF-1 family members are expressed mainly in the pulpal mesenchyme component possessing pluripotent potential, whereas differentiated pulpal cells have weaker expression of IGF-1 and IGF-1R. These data support our hypothesis that members of the IGF family are involved in the differentiation of mesenchymal progenitors into pulpal cells (16,34).

In summary, we have demonstrated the involvement of IGF-1, IGFBP-3 and IGF-1R in the late stages of human tooth development and the influence of these proteins on the pulpal mesenchyme. The expression patterns of the investigated morphogen IGF-1 might provide a guide for developing future strategies for the extra-corporal biosynthesis of dentin and engineering of hard tissues, which would have considerable implications for regenerative dentistry.

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