A role for adipokines in cranial development?

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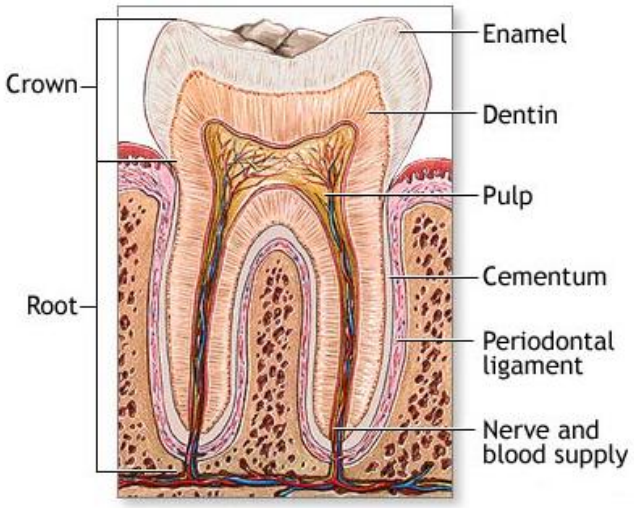
Abstract

During skeletal development, condensation of multipotential mesenchymal cells to differentiate towards the various cell types is an important process. Such processes include the formation of periodontal ligament cells (PDL cells), ameloblast and odontoblast cells. Even though the terminally differentiated cells differ in phenotype, they exhibit distinct similarities in the pattern of their secreted factors, which indicate a relationship between these cells. One of these common factors may be the adipokines; leptin, adiponectin and resistin, which all are involved in bone formation. Because both bone and dental tissue are mineralizing and have cells of mesenchymal origin, we are interested in examine if these adipokines also take part in dental development; are expressed in human pulp cells, PDL cells and/or odontoblasts and if so, try to identify their role.

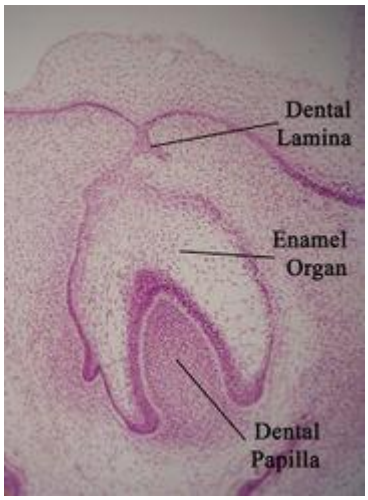
Pulp cells and PDL cells were incubated with dexametasone (Dex) and Enamel Matrix Derivative (EMD), respectively, and mRNA and cell culture medium were harvested after 1, 3, 7, and 14 days. The cell medium from pulp cells and PDL cells were analyzed using Luminex. The findings concluded that all the adipokines are expressed in PDL and pulp cells, and the protein expression was enhanced by either EMD or Dex, compared to the untreated control. Differentiation of pulp cells into odontoblasts, by Dex, enhanced the expression of adiponectin and leptin, whereas resistin was unaffected. EMD, known to stimulate periodontal repair, enhanced the secretion of resistin from both pulp and PDL cells.

Introduction

A tooth consists of two main parts: the crown, the visible part of the tooth, and the root, the anchor of the tooth that extends into the jawbone [Illustration 1.]. The outer surface of the crown is the *enamel*, which is produced by ameloblasts. The outer surface of the root is a layer of tough, yellowish, bone-like tissue, called *cementum*. Located under the enamel and cementum, is the *dentin*, which is a more porous tissue, that make up most of the tooth's mass. There are three types of dentin; primary, secondary and tertiary (reactionary) dentin. The *pulp* is the soft center of the tooth, which contains blood- and nerve supply. The dentin is produced by primary or secondary odontoblasts. The primary odontoblasts line the periphery of the pulp and are highly differentiated cells. They produce dentin both during tooth development and after completion of root formation. Primary and secondary dentin are terms sometimes used to designate dentin formed by primary odontoblasts before and after root development, respectively (Olgart and Bergenholtz, 2009). The primary odontoblast may also produce new dentin, sometimes termed tertiary or reactionary dentin, as a response to mild stimuli, e.g. slowly progressing caries (Bjørndal et al., 1999). Following injury or irritation, e.g. rapidly progressing caries, primary odontoblasts may die (Olgart and Bergenholtz, 2009). The mesenchymal cells in the pulp then have the ability to differentiate into odontoblast-like cells (secondary odontoblasts) and produce tertiary dentin (Fitzgerald et al., 1990). Secondary odontoblasts produce dentin at a rate that is dependent on the extent and duration of the injury (Olgart and Bergenholtz, 2009). Dentin formed by secondary odontoblasts becomes more irregular and amorphous and contains less dentinal tubules (Brännström et al., 1982).



1. Normal healthy tooth. Modified from http://www.nlm.nih.gov/medlineplus/ency/imagepages/1121.htm



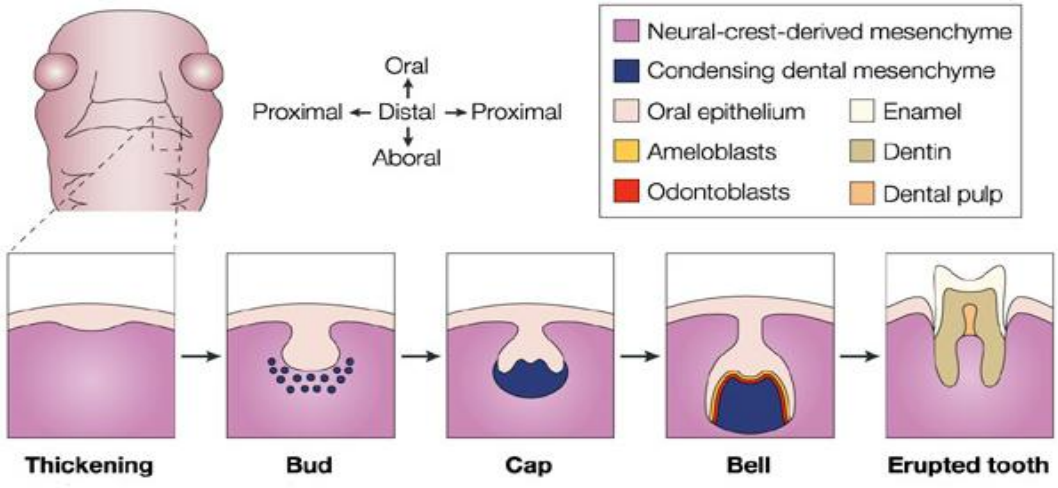
2. Histological view of a tooth bud. From http://en.wikipedia.org/wiki/File:Toothhistology11-17-05.jpg

During the odontogenesis, the enamel develops from the enamel organ and the dentin and pulp evolves from the dental papilla [Illustration 2.]. Nerves and blood vessels invade the dental papilla, and this is the reason for the pulps blood supply and innervation. *Periodontal ligament*, also named supporting ligament or PDL, is specialized connective tissue fibers that attach the tooth to the alveolar bone. These fibers help the tooth withstand the naturally substantial compressive forces which occur during for example chewing. PDL is derived from the dental follicle surrounding the tooth bud.

Dental development in humans

Tooth development can be divided into three overlapping phases: initiation, morphogenesis and histogenesis. The first sign of development is seen as a thickening of the oral epithelium. This epithelium invaginates into the underlying dental mesenchyme (ectomesenchymal tissue in origin, from the neural crest) to form a primary epithelial band. By the 7th week, the primary epithelial band divides into two processes: a buccally located vestibular lamina and a lingually situated dental lamina. The vestibular lamina contributes to the development of the vestibule of the mouth, delineating the lips and cheeks from the tooth-bearing regions. The dental lamina contributes to the development of the teeth (Berkowitz et al. 2009).

Tooth development can be divided into 3 stages; bud, cap and bell stage [Illustration 3.].



3. Overview of odontogenesis. Modified from http://www.nature.com/nrg/journal/v5/n7/fig_tab/nrg1380_F2.html

Bud stage

The enamel organ in the bud stage [Illustration 4.] appears as a simple, spherical to ovoid, epithelial condensation that is poorly morphodifferentiated and histodifferentiated. It is surrounded by mesenchyme. The cells of the tooth bud have a higher RNA content than those of the overlying oral epithelium, lower glycogen content and an increased oxidative enzyme activity. The epithelial component is separated from the adjacent dental mesenchyme by a basement membrane (Berkowitz et al. 2009).

Cap stage

By the 11th week morphogenesis has progressed, the deeper surface of the enamel organ invaginating to form a cap-shaped structure [Illustration 4.], but the enamel organ is still appearing relatively poorly histodifferentiated (Berkowitz et al. 2009).

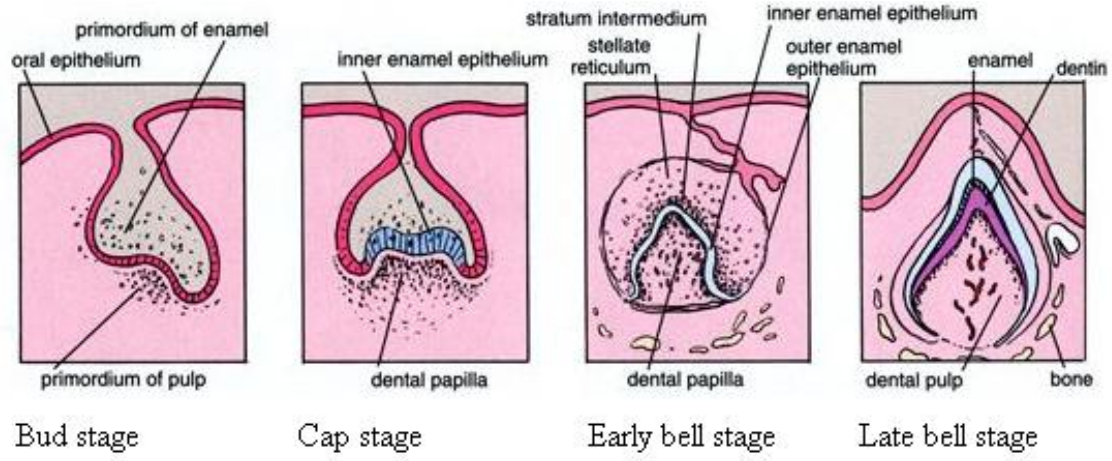
Early bell stage

During the early bell stage [Illustration 4.] the dental lamina loses connection with the oral epithelium. There is now a high degree of histodifferentiation with four distinct layers in the enamel organ, the outer enamel epithelium, stellate reticulum, stratum intermedium and internal enamel epithelium. A distinction develops between the more rounded cells in the central portion of the enamel organ and the peripheral cells which are becoming arranged to form the outer and internal enamel epithelium. The outer enamel epithelium cells are cubic in form and the internal cells are columnar and contain a lot of glycogen. The central cells of the enlarging enamel organ are separated and the intercellular spaces contains significant quantities of glycosaminoglycans. This creates an osmotic gradient and more water will be transported into the enamel organ, but because the cells are attached by desmosomes, the cells get a star-shaped appearance. Therefore these cells are termed the stellate reticulum. Between the inner enamel epithelium and stellate reticulum there are cells wit high alkaline phosphatase activity, termed stratum intermedium (Berkowitz et al. 2009).

Late bell stage

The late bell stage [Illustration 4.] of tooth development is associated with the formation of the dental hard tissues, commencing at about the 18th week. The dentinogenesis always precedes amelogenesis. The developing ameloblasts, the internal enamel epithelium,

influences the adjacent dental mesenchymal cells of the dental papilla to differentiate into odontoblasts. The odontoblasts then produce collagen and substances that mineralizes and becomes the predentine and dentine. The presence of dentine then induces the internal enamel epithelium to differentiate towards ameloblasts that secrete enamel (Berkowitz et al. 2009).



4. Bud stage, cap stage, early and late bell stage. Modified from http://faculty.ksu.edu.sa/khounganian/Pictures%20Library/TOOTH%20DEVELOPMENT.jpg

The enamel knot is a mass of cells in the center of the internal enamel epithelium. Recent studies of the enamel knot suggest it may represent an important signalling centre during tooth development (Berkowitz et al. 2009).

Enamel Matrix Derivate (EMD)

EMD includes a group of amelogenins derived from Hertwig's root sheet of porcine origin that have been used successfully in clinical applications to aid periodontal repair processes following inflammatory periodontal disease associated with attachment loss (Hammarstrøm et al, 1997; Sculean et al., 2001; Venezia et al., 2004). PDL cells exhibit several osteoblastic traits (Chou et al., 2002), and it has been shown that EMD had the ability to regulate cells of the osteoblastic lineage (Jiang et al., 2001). EMD also promotes periodontal ligament cell differentiation and osteoprotegrin, alkaline phosphatase and osteocalcin production, potentially resulting in a microenvironment supporting periodontal repair (Lassdorfer et al., 2007). The positive effect of EMD is through biomimetic stimulation of local growth factor

secretion and cytokine expression in periodontal tissues, inducing a regenerative process that mimics odontogenesis (Lyngstadaas et al., 2009) stimulating multiple mesenchymal cell types including fibroblasts (Lyngstadaas et al., 2001), osteoblasts (He et al., 2004), cementoblasts (Alhezaimi et al., 2009), and pluripotential mesenchymal stem cells into the osteoblast and/or chondroblast lineage (Ohyama et al., 2002) and many modes of EMD applications in tissue engineering might be speculated (Lyngstadaas et al., 2009; Satija et al., 2007).

Dexamethasone (Dex)

Dex is a synthetic glucocorticoid employed to induce osteogenic differentiation in vitro and found to regulate the commitment of progenitors derived from dental pulp to form odontoblast-like cells (Alliot-Licht et al., 2005). Pericytes are perivascular cells derived from mesenchymal stem cells or neurocrest cells (Amos et al., 2008), and these multi-linage cells have the ability to give rise to osteoblasts, chondrocytes, and adipocytes (Alliot-Licht et al., 2005). It is hypothesized that pericytes present in dental pulp tissue may be able to differentiate into odontoblast-like cells involved in reparative dentinogenesis (Fitzgerald et al. 1990; Shi and Gronthos 2003). Formation of tertiary dentin is always reactionary to different pathologies and is initiated by so called "transitional odontoblasts" (odontoblast-like cells) and partially fibroblasts (Mamaladze et al., 2010). Either the release of cytokines due to inflammatory events activates resident stem (progenitor) cells, or inflammatory cells or pulp fibroblasts undergo a phenotypic conversion into osteoblast/odontoblast-like progenitors implicated in reparative dentin formation (Goldberg et al., 2008). Series of publications have shown that dental pulp tissue contains post-natal stem cells co-expressing STRO-1 (a cell surface antigen used to identify osteogenic precursors in bone marrow stromal cells) and CD146 (a pericyte marker) suggesting indirectly that odontoblast-like progenitors derive from perivascular niche within dental pulp (Shi and Gronthos 2003). Administration of Dex increased the expression of STRO-1+ cells, demonstrating that Dex stimulates the differentiation of human dental pulp cells into odontoblast-like cells (Alliot-Licht et al., 2005).

Adipokines

Cells of mesenchymal origin share many characteristics in gene expression during differentiation and even though their phenotypes may differ markedly, mature adipocytes, chondrocytes and osteoblasts express and secret several common factors that underline the close relationship between these cells. One of these common factors are the adipokines; leptin, adiponectin and resistin, which all three has been found in bone (Reseland et al., 2001; Berner et al., 2004; Thommesen et al., 2006). Because mesenchymal precursor cells, which among others include odontoblasts and osteoblasts, have common characteristics in gene expression, it is reason to believe that dental tissue also express adipokines.

Leptin acts on the central nervous system, in particular the hypothalamus, suppressing food intake and stimulating energy expenditure as well in growth of different tissues (Meier et al., 2004). The leptin receptor (OB-R) exhibits homology with the interleukin-6 (IL-6) and belongs to the cytokine class 1 receptor family (Tartaglia et al., 1995). Leptin is involved in at least two different bone-controlling mechanisms, a direct stimulatory effect on bone growth, and/or an indirect suppressive effect on bone trough the hypothalamus (Reseland et al., 2002). Leptin is expressed in and secreted from primary cultures of human osteoblasts (Reseland et al., 2001). It has also been demonstrated that leptin have a direct effect on proliferation, differentiation, bone mineralization, and induces prolonged life span of human primary osteoblast by inhibiting apoptosis (Gordeladze et al., 2002).

Adiponectin exhibits various biological functions like increasing insulin sensitivity, protecting from hypertension, and suppression of atherosclerosis, liver fibrosis and tumor growth (Tilg et al., 2006). Transcription, translation and secretion of adiponectin in vitro have been demonstrated by primary human osteoblasts. Transcription of both adiponectin receptors (AdipoR1 and AdipoR2) in human osteoblasts have been observed, which indicates paracrine or endocrine effects of adiponectin on bone-forming cells (Berner et al., 2004). Adiponectin receptors (AR1 and AR2) are found in dental pulp cells, and administration of adiponectin enhances the expression of dentin sialophosphoprotein (DSPP) indicating that adiponectin indirectly might promote mineralization in pulp cells (Yasuda et al., 2008). High expressions of adiponectin in Meckels cartilage has been observed, as well as in cartilage of the developing os occipital and jaw of the developing vertebra (neck) in 18 days intra-uterine rat embryo. Adiponectin have also been identified in mandibular and jaw bone (Reseland,

Unpublished data). Adiponectin is structurally similar to tumor necrosis factor – alpha (TNF-a), receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegrin (OPG), which are all involved in osteoclastogenesis (Hu et al., 1996). Adiponectin regulates the TNF-α-induced nuclear transcription factor-κB activation, the signaling pathway of RANKL. OPG, RANK, and RANKL do also play a role in early tooth development, and there is a known relationship between RANKL in dentary bone mesenchymal cells and RANK and OPG in tooth germ (Aïoub et al., 2007). Immunostaining of adiponectin in the lower jaw of 18 days old rat embryos shows the protein present in front of the tooth, similar to the previous known expression of RANKL. In vitro and in vivo data indicate that adiponectin promotes resorption, and might either "make way" for the tooth and/or stimulate growth (Ohazama et al., 2004).

Resistin reduces insulin sensitivity in adipocytes, skeletal muscles and hepatocytes (Yagmur et al., 2006). Resistin is expressed in murine preosteoclasts and preosteoblasts, in primary human bone marrow stem cells and in mature human osteoblasts. Recombinant resistin increased the number of differentiated osteoclasts and stimulated NFkB promoter activity, even in absence of RANKL, indicating a role in osteoclastogenesis, and also stimulates osteoblast proliferation and cytokine release. Resistin may affect bone metabolism and remodeling via several mechanisms, where the two main actions seem to be the enhancement of osteoclasts differentiation and the recruitment of osteoblasts (Thommesen et al., 2006). More recently, important roles for resistin in the human immune response and inflammation, including induction of pro-inflammatory cytokines have been reported (Son et al., 2010).

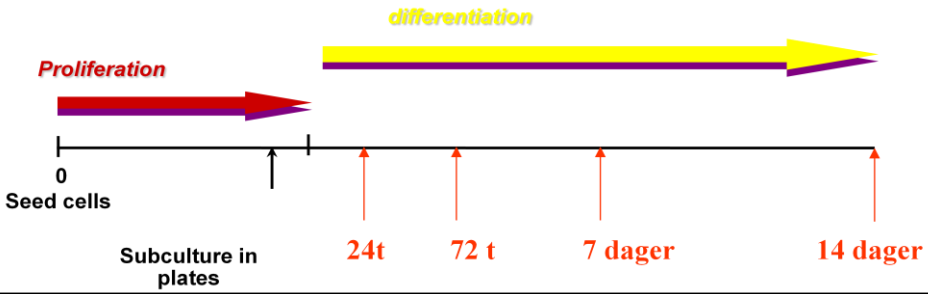
The expression of these adipokines has, to the authors' knowledge, not been identified in tooth–related tissues like pulp, ameloblasts, PDL cells or odontoblasts previously.

Materials and methods

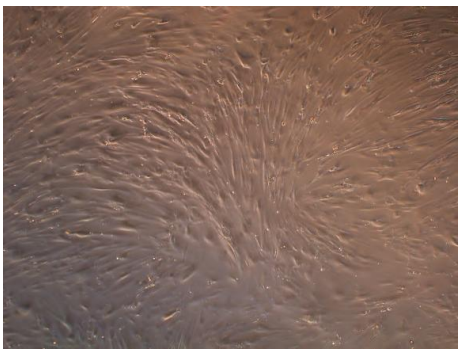
Cells and experimental design

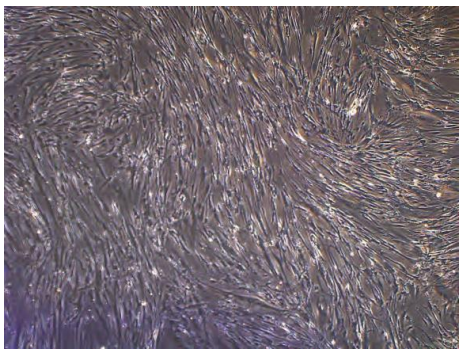
Human primary periodontal ligament cells (PDL) were cultured in DMEM (PAA laboratories GmbH, Pasching, Austria) containing 10% FCS, 50 U/ml penicillin and 50 µg/ml streptomycin. Cells were cultured in 6 well trays and treated with and without EMD (50 µg/ml) for 1, 3, 7, and 14 days. The medium was harvested and mRNA isolated at each time point [Illustration 5.]. Individual cellular experiments were repeated more than 3 times.

Human primary dental pulp cells were cultured in Modified Eagle's Medium (PAA laboratories GmbH, Pasching, Austria) containing 10% FCS, 50 U/ml penicillin and 50 µg/ml streptomycin. Cells were cultured in 6 well trays and treated with and without Dex (10^{-8}M) for 1, 3, 7, and 14 days [Illustration 6A and B]. The medium was harvested and mRNA isolated at each time point [Illustration 5.]. We also treated the pulp cells with EMD (50 µg/ml) for 1, 3, and 7 days. Individual cellular experiments were repeated more than 3 times. The human pulp cells might differentiate into odontoblasts after approximately 14 days, but in this study this was not verified by Realtime-PCR.



5. Expression of adipokines in periodontal ligament cells and human dental pulp cells. Overview of experimental design.





6A. Untreated pulp cells day 14. 6B. Pulp cells treated with Dex, day 14.

Isolating mRNA

Cells were lysed in lysis/binding buffer (100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, pH 8.0, 0.5 mM dithiothreitol [DTT], and 1% sodium dodecyl sulfate [SDS]) and mRNA was isolated using magnetic beads [oligo $(dT)_{25}$] as described by the manufacturer (Dynal AS, Oslo, Norway). Beads containing mRNA were resuspended in 10 mM Tris-HCl, pH 8.0, and stored at -70°C until use.

Luminex

Multianalyte profiling was performed using the membrane Luminex-100 system and the XY Platform (Luminex Corporation, Austin, TX). Calibration microspheres for classification and reporter readings as well as sheath fluid were also purchased from Luminex Corporation. Acquired fluorescence data were analyzed by the STarStation software (Version 2.0; Applied Cytometry Systems, Sheffield, UK). Prior to analysis, the samples were concentrated 10 times using MicrosepTM Centrifugal tubes with 3 KDa cut-off from Pall Life Science (Ann Armor, MI, USA). The concentrations of the adipokines (leptin, adiponectin and resistin) in the cell culture medium were determined using the Human Adipocyte Milliplex Kit (Millipore, Billerica, MA, USA). All analyses were performed according to the manufacturers' protocols.

Statistical analysis

Statistical comparison between groups and treatments was performed using the parametric one-way ANOVA test and post hoc Holm-Sidak tests (SigmaStat software; Systat Software Inc.; San Jose, CA). A probability of less than or equal to 0.05 was considered significant. Data were presented as a percentage of untreated cells (= 100 %) at each time point.

Results

Resistin

Resistin was found in human pulp cells (3.7 ± 5.6 pg/ml). Administration of EMD gave significant change of resistin secretion the first and third day in pulp cells (P = 0.005), respectively. Whereas adding Dex to the pulp cells induced no changes in the secretion of resistin. This tells us that resistin is expressed in human pulp cells, and that it is regulated by EMD. The expression is not altered by Dex-induced differentiation of the cells (Fig. 1.).

Resistin was also expressed in PDL cells (17.6 ± 15.1 pg/ml). We found EMD to induce a significant increase in resistin production at day three (P = 0.003). This tells us that resistin is expressed in PDL and regulated by EMD (Fig. 2.).

Adiponectin

Adiponectin was expressed in human pulp cells (98.8 ± 69.5 pg/ml), and incubation with Dex enhanced the adiponectin secretion (P = 0.025) compared to untreated pulp cells at day one. EMD however, had no significant effect on adiponectin in human pulp cells (Fig. 3.).

Adiponectin was also expressed in PDL cells (33.1 ± 27.2 pg/ml), however we found no significant changes in the expression upon EMD treatment (Fig. 4.).

Leptin

Leptin was expressed in human pulp cells (50.2 ± 7.3 pg/ml). Dex induced a significant increase in leptin secretion in human pulp cells after fourteen days incubation (P = 0.023), whereas EMD had no effect on leptin secretion from human pulp cells for the seven days of incubation (Fig. 5.).

Leptin is expressed in PDL cells (48.0 ± 0 pg/ml). Adding EMD had no effect on leptin secretion from PDL cells for the seven days of incubation (Fig. 6.).

Discussion and conclusions

We have verified that human pulp cells and PDL cells express the adipokines leptin, adiponectin or resistin in vitro. The analyzes show that adipokines are secreted from and regulated, either by EMD or Dex, in these cells.

We observe a significant increase in the resistin secretion after incubation with EMD in human pulp cells and PDL cells. EMD therefore has a regulatory effect on the resistin expression in pulp cells and PDL cells. This effect was not reproducible by any of the purified fraction of EMD, indicating that the whole enamel matrix derivative is more active than isolated components (Obregon-Whittle et al., unpublished data). Our results showed an acute significant EMD-induced increase in resistin secretion from dental pulp cells at day one and three. At day three our analyzes showed a significant EMD-induced increase in the resistin secretion in PDL cells.

Resistin was expressed in osteoblasts and osteoclasts, stimulates osteoblast proliferation and cytokine release as well as osteoclast differentiation and increased NFκB activity, indicating a role in bone metabolism and remodeling (Thommesen et al., 2006). Unpublished data showed an EMD-induced increase in resistin from mesenchymal stem cells and normal human osteoblasts. This might be one of the downstream factors or mechanisms inducing enhanced bone remodeling or executing the observed effects of EMD (Do, 2011). Odontoblasts and osteoblasts might have the same expression of adipokines. Knowing that resistin might have a role in bone metabolism and remodeling brings the idea that when adding EMD to odontogenic tissue we might also have an effect on dentin growth and repair.

Dex significantly enhanced the adiponectin and leptin secretion in human pulp cells compared to the untreated controls. Our results showed an acute significant Dex-induced increase in adiponectin secretion from dental pulp cells at day one. After day one the expression decreases, and remains stable during the differentiation of the cells. This tells us that adiponectin might induce a signal in the pulp cells or stimulate to differentiation. Leptin, on the other hand, shows opposite traits. Upon stimulation of pulp cell differentiation with Dex, leptin secretion continued increase the fourteen days of incubation studied. At day fourteen we observed a significant Dex-induced increase in the leptin secretion compared to untreated cells. This tells us that leptin expression might be similar to the expression in osteoblasts (Reseland et al., 2001), indicating a role in odontoblastic development, and

maturation or dentin mineralization. Mutations in leptin or leptin receptor have been found to induce a more yellowish color with a grainy texture on the surface of skull bones compared to wild-type mice, and frontal, parietal and occipital bones were found to be translucent. Between 50 and 90% of the teeth were found to be considerably wear compared to wild-type, indicating signs of hypomineralization, and there were increased fragility in both upper and lower jaw bones (Atar et al. 2008). This indicates that leptin signalling either directly or indirectly stimulate cranial and tooth development.

Work to be done

We have quantified the adipokine concentrations in cell culture medium, however the expression of mRNA needs to be verified. Samples are prepared for real-time RT-PCR analysis, so that the presens of the adipokine gene sequences in mRNA from human primary pulp cells and PDL cells can be verified.

The expression and secretion of adipokines from ameloblast needs to be examinated. The ameloblast-like cell line is murine, and analysis and quantitation requires a murine kit.

Identification of the proteins at various stages of craniofacial development using in situ hybridization might give some answers to the expression profile. Nevertheless, more effort is needed in order to elucidate and understand the role of adipokines in craniofacial development. What is the effect of the individual recombinant adipokines on proliferation and differentiation of pulp and odontoblasts, what is the effect on enamel protein expression in ameloblasts etc?

Acknowledgement

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Figure legends

Figure 1

The effect of Dex (10^{-8}M) and EMD (50 µg/ml) on resistin secretion from human pulp cells. The average on at least three individual cellular experiments with Dex and EMD, and data presented in percent of untreated cells at each time point.

Figure 2

The effect of EMD (50 µg/ml) on resistin secretion from human PDL cells. The average on at least three individual cellular experiments with EMD, and data presented in percent of untreated cells at each time point.

Figure 3

The effect of Dex (10^{-8}M) and EMD (50 µg/ml) on adiponectin secretion from human pulp cells. The average on at least three individual cellular experiments with Dex and EMD, and data presented in percent of untreated cells at each time point.

Figure 4

The effect of EMD (50 µg/ml) on adiponectin secretion from human PDL cells. The average on at least three individual cellular experiments with EMD, and data presented in percent of untreated cells at each time point.

Figure 5

The effect of Dex (10^{-8}M) and EMD (50 µg/ml) on leptin secretion from human pulp cells. The average on at least three individual cellular experiments with Dex and EMD, and data presented in percent of untreated cells at each time point.

Figure 6

The effect of EMD (50 µg/ml) on leptin secretion from human PDL cells. The average on at least three individual cellular experiments with EMD, and data presented in percent of untreated cells at each time point.

Fig 1

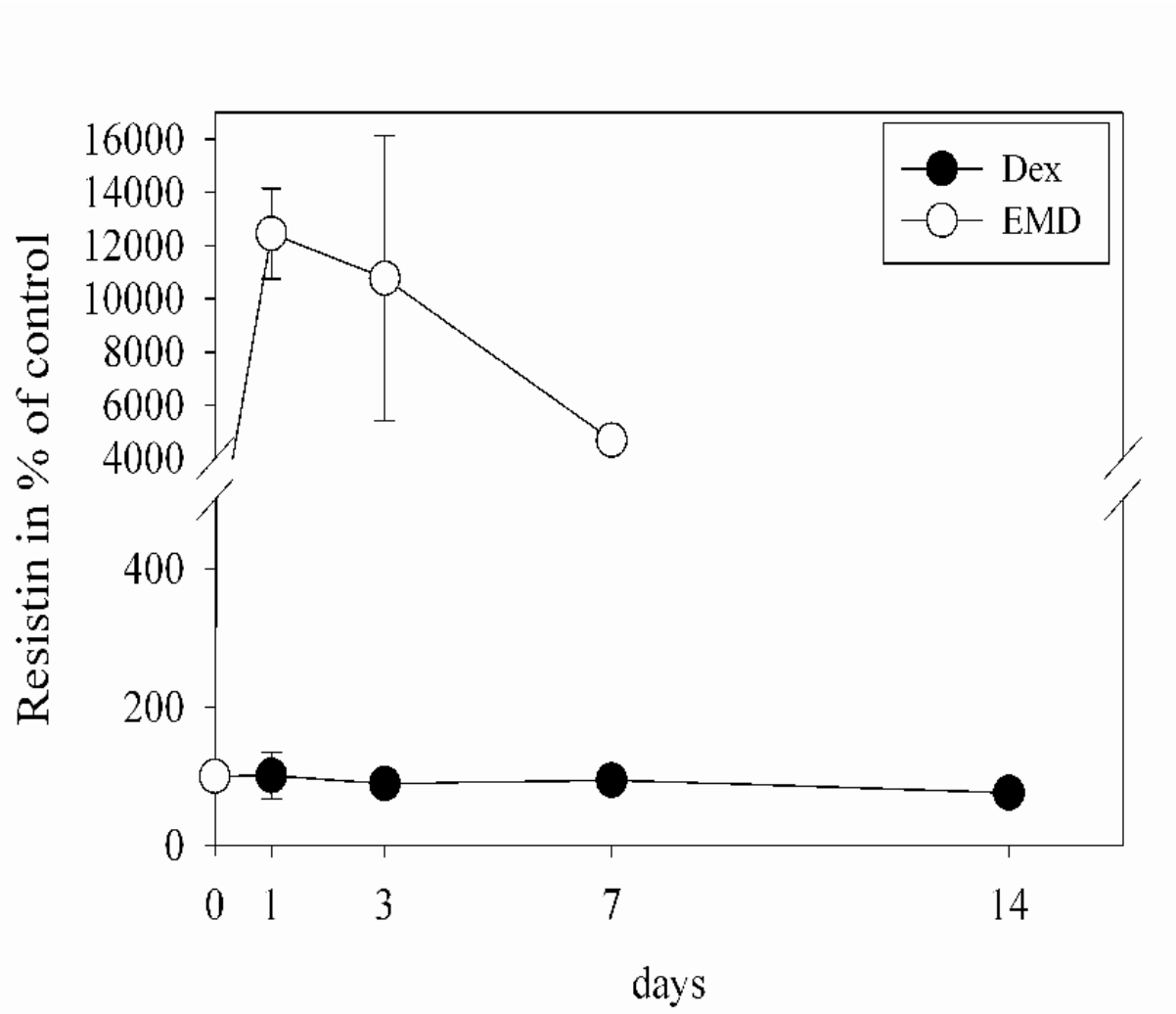
Resistin in % of control
16000
14000
12000
10000
8000
6000
4000
400
200
0
0
1
3
7
14
days
Dex
EMD

Fig 2

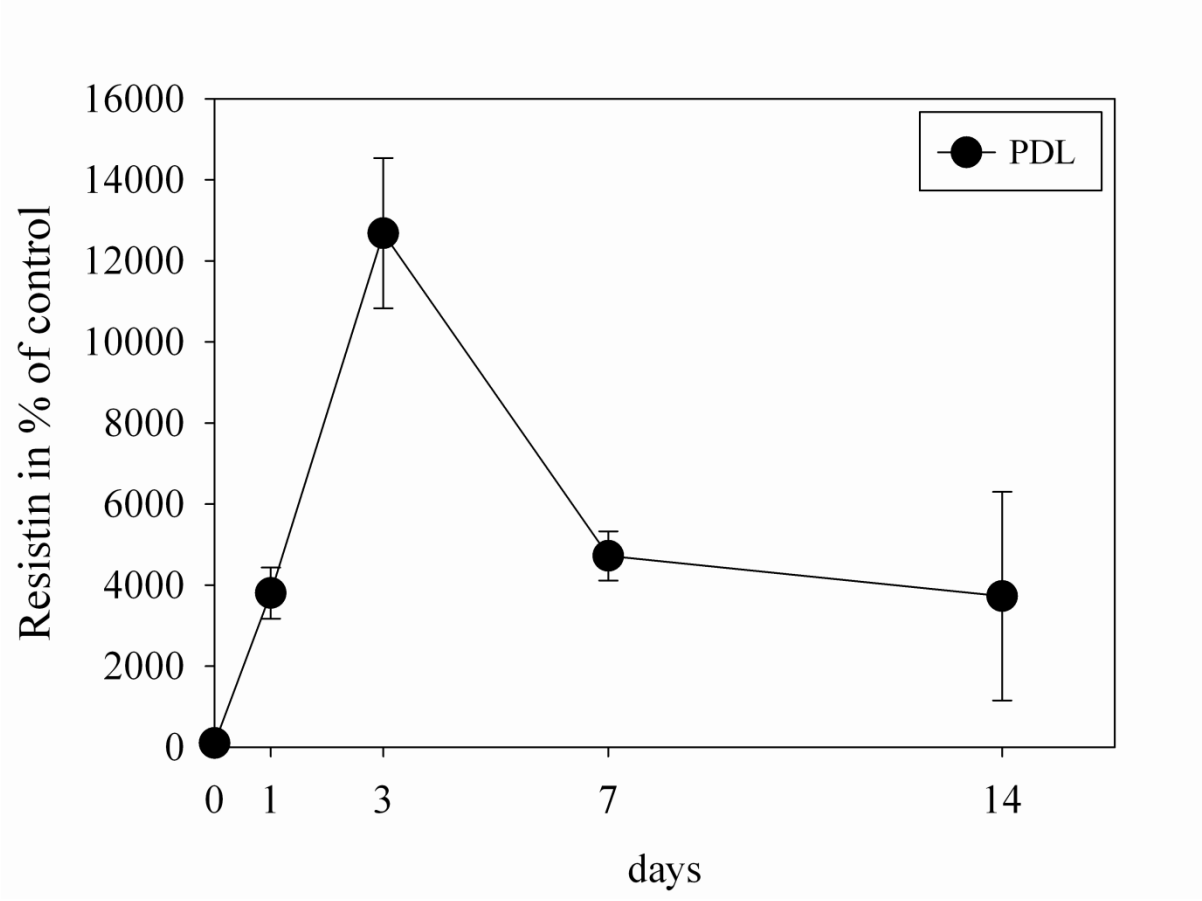
16000
14000
12000
10000
8000
6000
4000
2000
0
Resistin in % of control
PDL
0
1
3
7
14
days

Fig 3

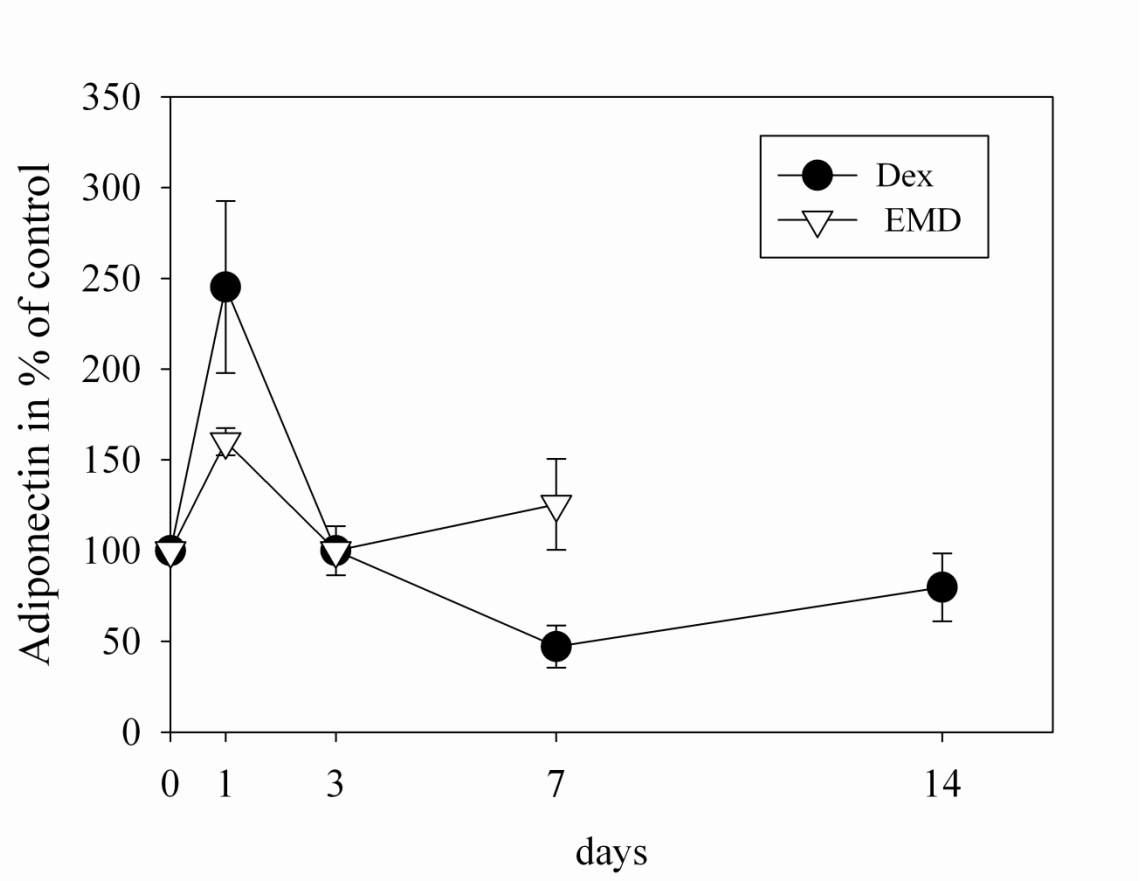
Adiponectin in % of control
350
300
250
200
150
100
50
0
0
1
3
7
14
days
Dex
EMD

Fig 4

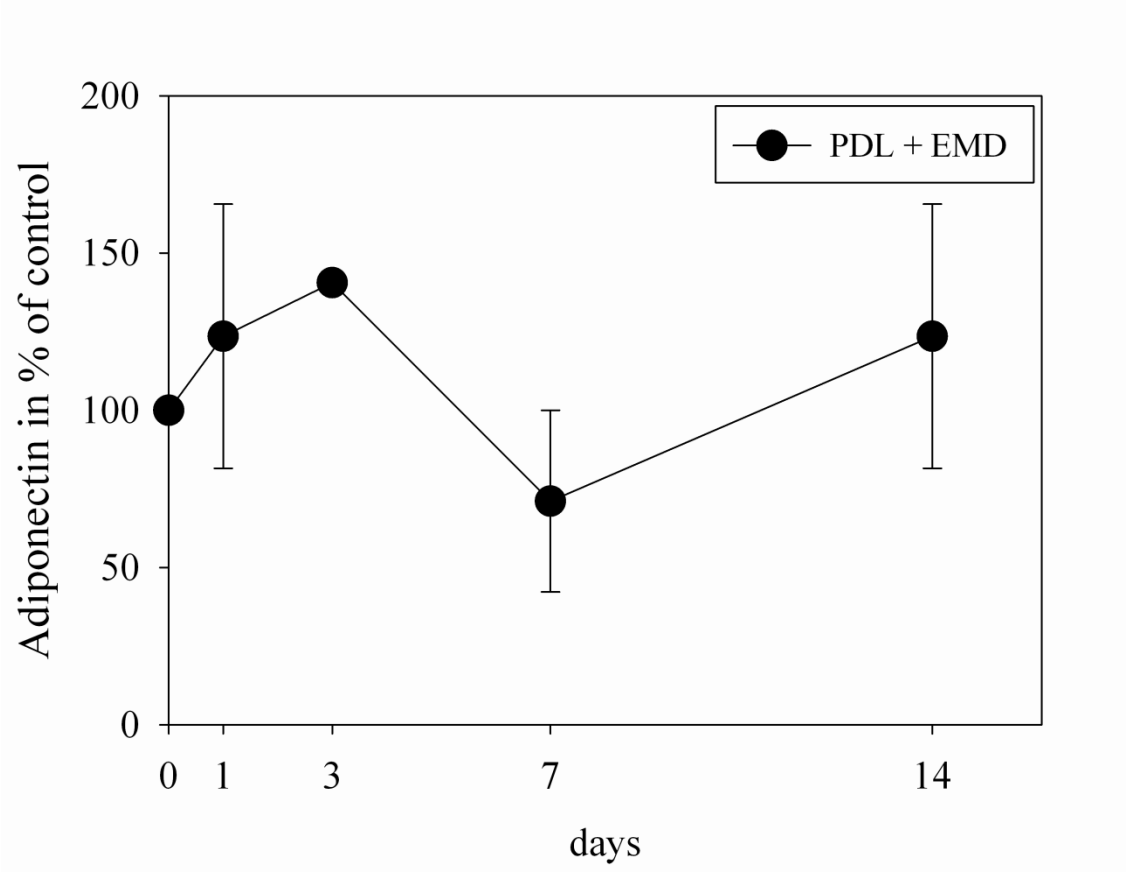
200
150
100
50
0
Adiponectin in % of control
PDL + EMD
0
1
3
7
14
days

Fig 5

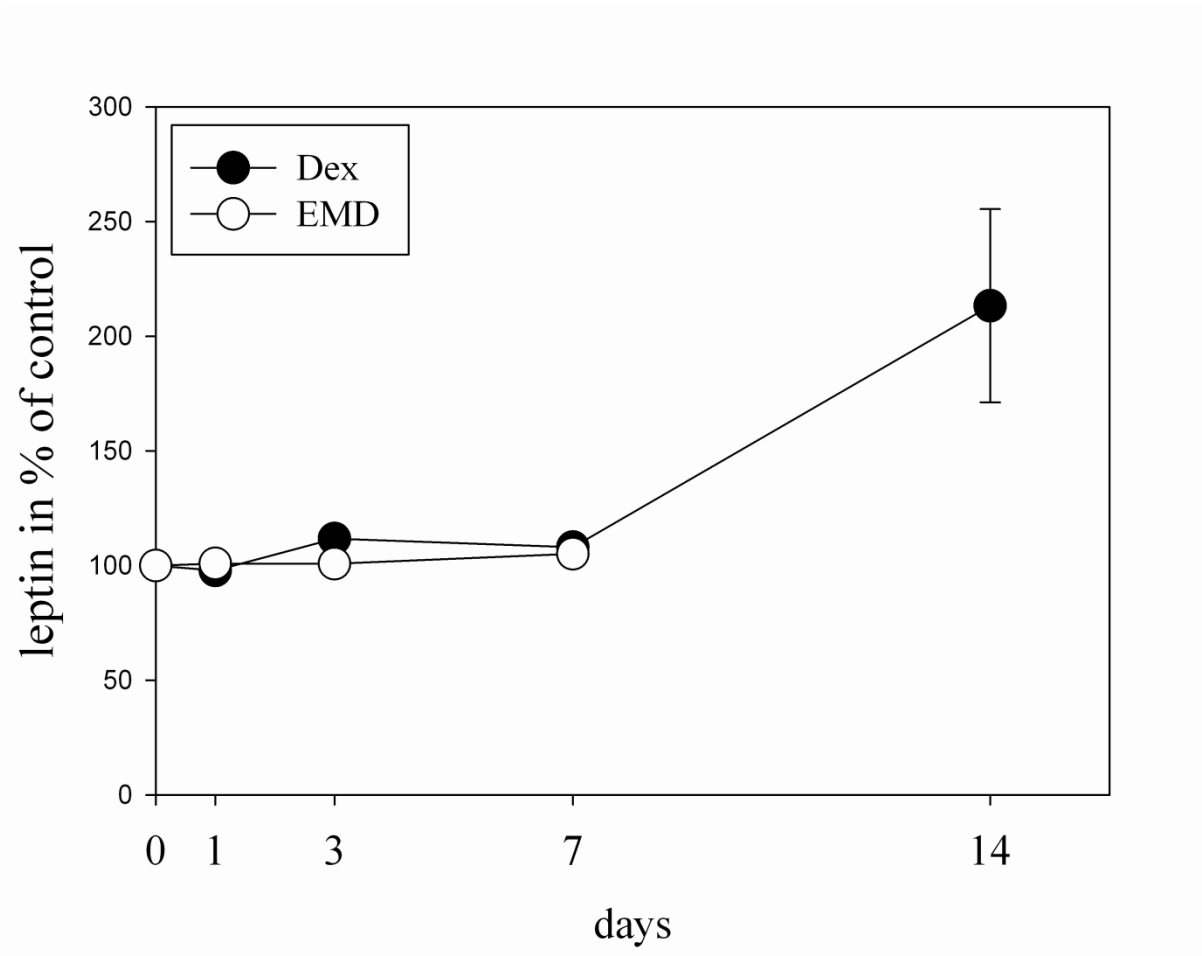
300
250
200
150
100
50
0
leptin in % of control
Dex
EMD
0
1
3
7
14
days

Fig 6