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Involvement of IGF-2, IGF-1R, IGF-2R and PTEN in development of human tooth germ – an immunohistochemical study

Darko Kero,[a] Livia Cigic,[a] Ivana Medvedec Mikic,[a] Tea Galic,[a] Mladen Cubela,[b] Katarina Vukojevic,[c] and Mirna Saraga-Babic[c]

[a]Study Program of Dental Medicine, School of Medicine, University of Split, Split, Croatia
[b]Dental Clinic Cubela, KMV Humskog bb, Mostar, Bosnia and Herzegovina
[c]Department of Anatomy, Histology and Embriology, School of Medicine, University of Split, Split, Croatia

ABSTRACT. Insulin-Like Growth Factor 2 (IGF-2) is a peptide hormone essential for prenatal growth and development. IGF-2 exerts its mitogenic effects via Insulin-Like Growth Factor 1 Receptor (IGF-1R), and is eliminated by binding to Insulin-Like Growth Receptor 2 (IGF-2R). IGF-2 is also negatively regulated by Phosphatase and Tensin Homolog (PTEN), a phosphatase mutated in various tumors. Not much is known about the interplay between these factors during human odontogenesis. In this study, expression patterns of IGF-2, IGF-1R, IGF-2R and PTEN were analyzed by double immunofluorescence in incisor human tooth germs during the foetal period of development between the 7th and 20th gestational week. Throughout the investigated period, IGF-2 was mostly expressed in enamel organ, whereas mild to moderate expression of PTEN could be seen in dental papilla and parts of enamel organ. Expression of IGF-1R was ubiquitous and displayed strong intensity throughout the entire enamel organ. In contrast, expression of IGF-2R had rather erratic pattern in enamel organ and dental papilla alike. Expression patterns of IGF-2, IGF-1R, IGF-2R and PTEN in highly proliferative cervical loops, as well as in differentiating pre-ameloblasts and pre-odontoblasts of cusp tip region during the early and late bell stages when enamel organ acquires definitive shape, indicate importance of these factors in crown morphogenesis of human incisor. Taken together, our data suggest the involvement of IGF-2, IGF-1R, IGF-2R and PTEN in temporo-spatial patterning of basic cellular processes (proliferation, differentiation) during normal tooth development. They are also relevant for improving knowledge of molecular basis of human odontogenesis.

KEYWORDS. differentiation, human tooth development, IGF-axis, IGF-2, IGF-1R, IGF-2R, proliferation, PTEN

Correspondence to Darko Kero, Email: dkero@mefst.hr, Study Program of Dental Medicine, School of Medicine, University of Split, Soltanska 2, 21000 Split, Croatia.
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INTRODUCTION

In the course of odontogenesis, human tooth germs undergo successive developmental stages of dental lamina, bud, cap and bell. Along with morphologically distinctive outlook, each of these stages is characterisedby a specific spatial pattern of basic cellular and tissue processes (proliferation, differentiation, apoptosis, epithelial-to-mesenchymal or mesenchymal-to-epithelial induction and transformation).[1–3] Various factors have been implicated in molecular regulation of such patterning, in particular, those encoded by homeobox genes which seem to be of paramount importance for initiation of tooth development and reciprocation of odontogenic potential between epithelial and mesenchymal parts of tooth germ during the entire course of odontogenesis.[4,5] Additionally, a number of growth factors have been shown to play diverse roles in directing both proliferation and differentiation of specific tooth germ cell populations within the context of each stage of development.[6,7] Unfortunately, not much is known about molecular basis of human odontogenesis, since most of the available data come from studies on tooth germs in animal models.[4,7,8] To partly address the issue, we set out to analyze expression patterns of Insulin-Like Growth Factor 2 (IGF-2), Insulin-Like Growth Factor 1 Receptor (IGF-1R), Insulin-Like Growth Factor 2 Receptor (IGF-2R), and Phosphatase and Tensin Homolog (PTEN) in human tooth germs during foetal period of development.

IGF-2 is a mitogenic peptide hormone with predominant role in prenatal growth and development. In humans, gain or loss of function of gene encoding IGF-2 may be involved in etiology of development disorders described in Beckwith-Wiedemann and Silver-Russell syndromes. The former is classically identified at birth by macrosomia, macroglossia, and omphalocele, while presence of other features such as asymmetry of the limbs, organomegaly, and neonatal hypoglycemia vary between individual cases.[9] In contrast, Silver-Russell syndrome is characterized by intrauterine and/or postnatal growth retardation, typically triangular shaped face and body asymmetry in a subset of affected individuals.[10] In mice, paternal inheritance of *Igf-2* null allele (due to genomic imprinting) results in foeto-placental growth retardation.[11] On a cellular level, the effects of IGF-2 are similar to that of Insulin-Like Growth Factor 1 (IGF-1) by promoting proliferation, differentiation, survival and migration of cells.[12,13] Functional versatility of IGF-2 must primarily be viewed within the context of IGF-axis, whose members such as Insulin-Like Growth Factor Binding Proteins (IGFBPs) and receptors IGF-1R and IGF-2R are involved in complex regulation of activity of both IGFs.[14] Namely, by binding IGF-1 and −2 in serum, IGFBPs facilitate endocrine delivery of these factors to target tissues in addition to IGFBP-free autocrine/paracrine delivery of IGF-1 and −2 from specific tissues.[14] Furthermore, both IGFs exert mitogenic and metabolic effects almost exclusively via IGF-1R (which binds IGF-1 with greater affinity than IGF-2), whereas IGF-2R has no signaling capacity, but rather serves to scuttle IGF-2 away from the cell surface targeting it for lysosomal degradation.[15,16]

Numerous studies indicate the importance of tumor suppressor PTEN in control of cell proliferation and apoptosis. Germline mutations of PTEN in human syndromes such as Cowden disease and Proteus syndrome (characterised by multiple hamartomas and other tumors), as well as its somatic mutations in a number of primary tumors, show that PTEN loss-of-function is responsible for rampant proliferation and extended cell survival in neoplastic tissues.[17–20] Counteracting IGF-1 and −2, PTEN's phosphatase activity was proven crucial for down-regulation of signaling pathways associated with IGFs main receptor IGF-1R, especially PI3K/Akt pathway, which promotes cell survival and progression of cell cycle.[21,22] Therefore, as a negative regulator of PI3K-Akt pathway PTEN is able to modulate IGFs action, which was demonstrated by the complex interplay between IGF-2 and PTEN in normal adult tissues and tumors.[11,23]

So far, the involvement of IGF-axis in embryonic and foetal development of human and animals has been relatively well investigated.[12,24–26] In developing human tooth germs, IGF-axis has been implicated in amelogenesis and root formation, but there are only few accounts on particular

members' roles (most notably IGF-1 and IGF-1R) during the earlier stages of tooth development.[27–31] On the other hand, involvement of PTEN in developing odontogenic tissues still remains to be investigated, even though the total mapping of PTEN expression patterns has been performed for developing human and mouse embryos.[32,33] Significant inter-species differences of PTEN expression patterns reported in those studies, support the occurrence of diverse phenotypes yielded by germline loss-of-function mutations of PTEN in humans and knockout mice, as much as they imply distinctive roles PTEN might play during normal development in various species.

Given the overall importance of IGF-axis in embryonic and foetal development and its intricate regulatory association with PTEN, the aim of this study was to analyze expression patterns of IGF-2, IGF-1R, IGF-2R and PTEN in developing human tooth germs. This should improve our insight into the molecular background of temporal and spatial patterning of basic cellular processes during human odontogenesis.

RESULTS

By the end of the 5th week of development, thickening of embryonic oral epithelium (dental lamina) heralds development of human tooth germs. This is followed by consecutive developmental stages (bud, cap and bell) in preparation for synthesis, secretion and maturation of enamel and dentin in tooth crown. In this study, the development of human incisor tooth germs was investigated in period between the 7th week and the 20th week of development.

Co-localization of IGF-2 and PTEN by double immunofluorescence

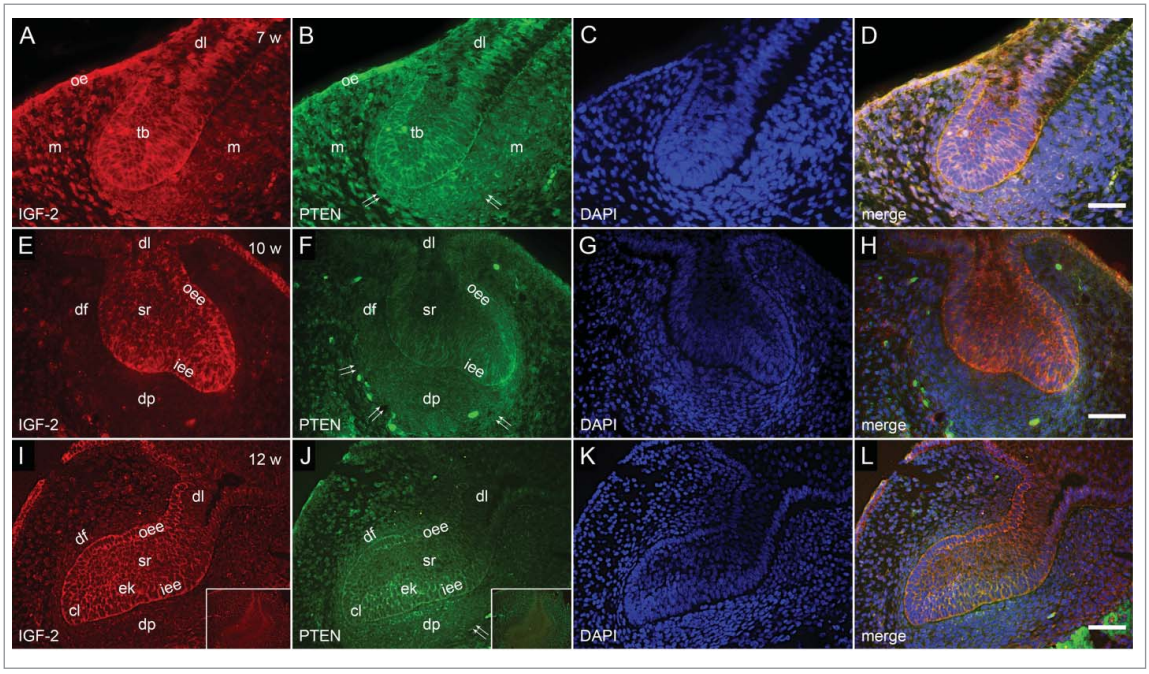
Co-localization of IGF-2 and PTEN by double immunofluorescence was performed on incisor tooth germs aged 7, 10, 12, 14 and 20 weeks of development (Fig. 1, Fig. 2, Fig. 3, Table 1).

In the 7th week of development, incisor tooth germ is in the bud stage of development. Moderate to strong expression of IGF-2 was observed in the whole tooth bud, especially in the cells at the bud's margin (prospective inner and outer enamel epithelia) and tip (prospective primary enamel knot) (Fig. 1a, d). The expression of IGF-2 in particular portions of dental lamina was either moderate or none, whereas oral epithelium expressed IGF-2 strongly. No expression of IGF-2 could be observed in mesenchymal tissue surrounding the bud (prospective dental papilla and dental follicle). The expression of PTEN in tooth bud, oral epithelium and dental lamina was similar to that described for IGF-2, with exception of moderate expression of PTEN throughout the dental lamina. Furthermore, narrow belt of mesenchymal tissue surrounding the tip of the bud, expressed PTEN moderately (Fig. 1b, d).

In the 10th week of development, incisor tooth germ is in the cap stage of development. The epithelial parts are now comprised of several distinctive tissues including inner and outer enamel epithelia enclosing the inner portion of prospective stellate reticulum. At this stage, dental papilla and dental follicle are discernible in mesenchymal parts of the tooth germ. Moderate expression of IGF-2 was observed in both enamel epithelia, however, at their confluence (prospective cervical loops) it was of strong intensity (Fig. 1e, h). IGF-2 was only mildly expressed in prospective stellate reticulum, whereas no expression of IGF-2 could be detected in dental papilla and dental follicle. PTEN displayed intensity of expression similar to that of IGF-2 with regard to outer enamel epithelium, prospective cervical loops and stellate reticulum and was mildly expressed in the inner enamel epithelium. However, throughout dental papilla and dental follicle PTEN was expressed moderately (Fig. 1f, h).

In the 12th week of development, incisor tooth germ is in the late cap stage of development. The expression pattern of IGF-2 remained the same as described in previous section with exception of slight increase of intensity in stellate reticulum, and decreased intensity in cervical loops (Fig. 1i, l). PTEN was still mildly expressed in the inner enamel epithelium, and moderately in enamel knot. Furthermore, area of dental papilla underlying the enamel knot expressed PTEN a bit more

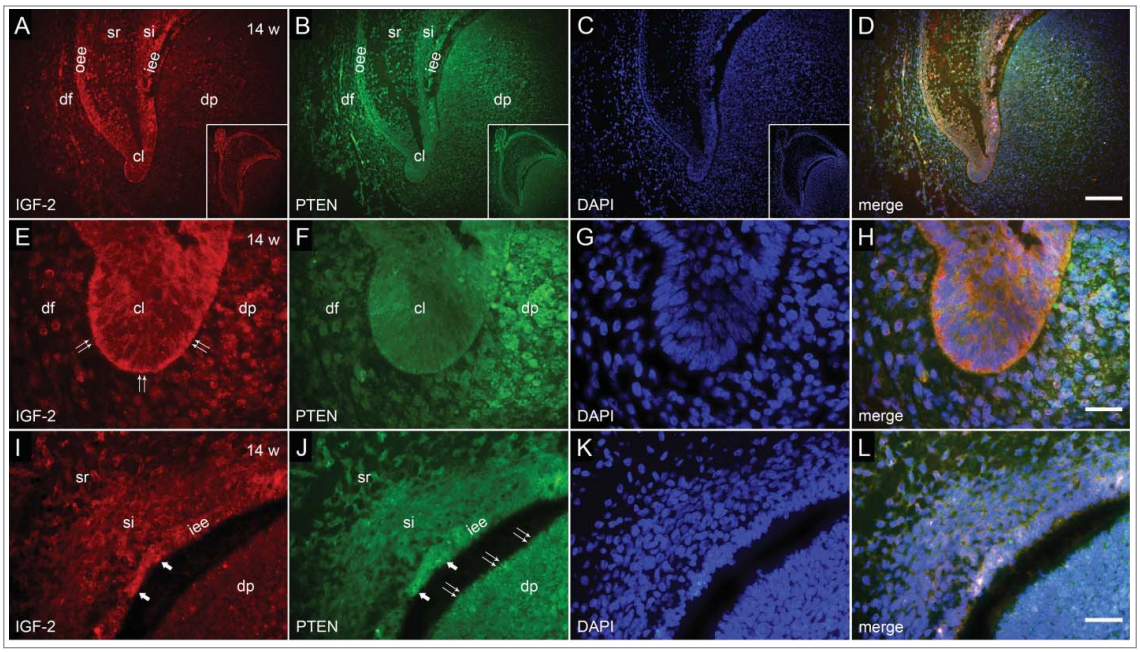
FIGURE 1. Co-localization of IGF-2 and PTEN by double immunofluorescence in human incisor tooth germs between 7th and 12th week of development. (a-d) Human incisor tooth germ in the 7th week of development (bud stage); (a) Moderate to strong expression of IGF-2 in the tooth bud. Jaw mesenchyme is negative to IGF-2; (b) Moderate to strong expression of PTEN in the tooth bud. Jaw mesenchyme is moderately positive to PTEN. Forefront of mesenchymal expression (arrows); (c) DAPI staining of nuclei; (d) Merging of a+b+c *(Magnification: ×40, scale bar: 25 μm)*. (e-h) Human incisor tooth germ in the 10th week of development – (cap stage); (e) Moderate expression of IGF-2 in outer and inner enamel epithelia, and stellate reticulum. Strong expression of IGF-2 in prospective cervical loop. Dental papilla and dental follicle are negative to IGF-2; (f) Moderate expression of PTEN in outer enamel epithelium, stellate reticulum and dental papilla. Strong expression of PTEN in prospective cervical loop. Forefront of expression in dental papilla (arrows); (g) DAPI staining of nuclei; (h) Merging of e+f+g (*Magnification: ×20, scale bar: 40 μm*). (i-l) Human incisor tooth germ in the 12th week of development (late cap stage); (i) Slight increase of expression of IGF-2 in stellate reticulum (*Inset*: negative control staining to IGF-2); (j) Inner enamel epithelium and primary enamel knot express PTEN moderately. Slight increase of expression of PTEN in restricted area of dental papilla underlying the enamel knot (*Inset*: negative control staining to PTEN); (k) DAPI staining of nuclei; (l) Merging of i+j+k (*Magnification: ×20, scale bar: 40 μm*)Designations: oral epithelium (oe), dental lamina (dl), tooth bud (tb), jaw mesenchyme (m), outer enamel epithelium (oee), inner enamel epithelium (iee), primary enamel knot (ek), stellate reticulum (sr), stratum intermedium (si), cervical loop (cl), enamel knot (ek), preameloblasts (pa), dental papilla (dp), dental follicle (df), pre-odontoblasts (po), forefront of marker's expression in tissue (double arrows); individual cells expressing marker (thick arrows).



intensely than the rest of dental papilla cells (Fig. 1j, l).

In the 14th week of development, incisor tooth germ is in the early bell stage of development. Located between the inner enamel epithelium and stellate reticulum, newly differentiated cell layer of stratum intermedium can now be seen. Due to completion of its developmental role, enamel knot disintegrates by this stage. IGF-2 was moderately expressed by the inner enamel epithelium at the site of future cusp tip (location of prospective pre-ameloblasts), whereas its expression in stratum intermedium at the same site was mild (Fig. 2a, i). Also, moderate expression of IGF-2

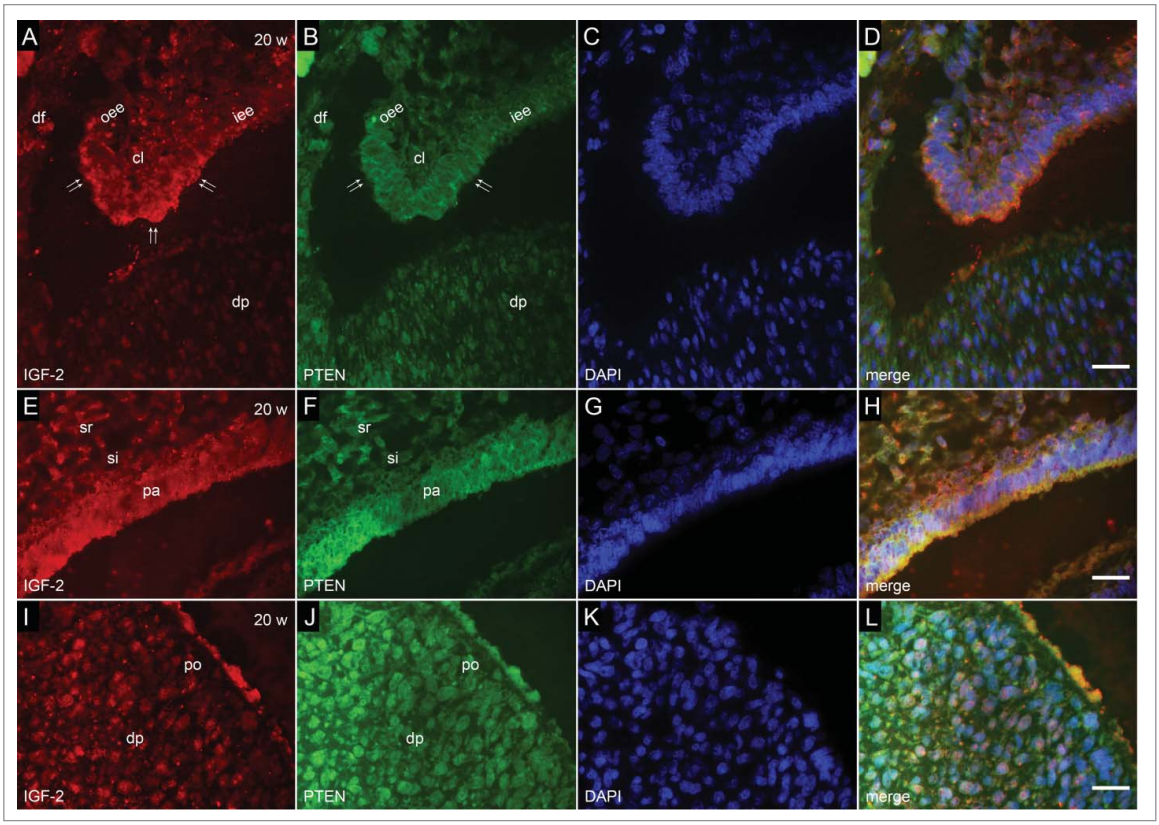
FIGURE 2. Co-localization of IGF-2 and PTEN by double immunofluorescence in human incisor tooth germ in the 14th week of development (early bell stage). (a-d) Human incisor tooth germ in the 14th week of development (early bell stage) (a) Expression of IGF-2 is mostly restricted to enamel organ (*Inset*: human incisor tooth germ in early bell stage, IGF-2 staining); (b) Expression of PTEN in enamel organ, dental papilla and dental follicle (*Inset*: Human incisor tooth germ in early bell stage, PTEN staining); (c) DAPI staining of nuclei (*Inset*: Human incisor tooth germ in early bell stage, DAPI staining of nuclei); (d) Merging of a+b+c (*Magnification: ×20, scale bar: 40 μm*). (e-h) Detail of cervical loop (e) Moderate expression of IGF-2 in the cervical loop and adjacent parts of enamel epithelia. Mild expression of IGF-2 in adjacent area of dental papilla; (f) Mild expression of PTEN in the cervical loop. Moderate expression of PTEN in adjacent area of dental papilla; (g) DAPI staining of nuclei; (h) Merging of e+f+g (*Magnification: ×100, scale bar: 10 μm*). (i-l) Detail of the site of future cusp tip (i) Moderate expression of IGF-2 in the inner enamel epithelium (prospective pre-ameloblasts) and mild expression in stratum intermedium; (j) Moderate expression of PTEN in the inner enamel epithelium. Moderate to strong expression of PTEN in prospective pre-odontoblasts of dental papilla; (k) DAPI staining of nuclei; (l) Merging of i+j+k (*Magnification: ×100; scale bar: 10 μm*).



could be seen in cervical loops and parts of outer enamel epithelium, while stellate reticulum barely expressed IGF-2 (Fig. 2a, d, e). Although the expression of PTEN displayed mostly similar pattern in enamel organ, only mild expression of PTEN was observed in cervical loops, whereas parts of dental papilla closely adjacent to inner enamel epithelium and cervical loops, expressed PTEN moderately (Fig. 2b, d, f, h). Furthermore, moderate to strong expression of PTEN was observed in cells of dental papilla at the site of future cusp tip (prospective pre-odontoblasts) (Fig. 2j, l).

In the 20th week of development, incisor tooth germ is in the late bell stage of development. The enamel organ significantly increases in size. Cells of the inner enamel epithelium at the site of future cusp tip differentiate into pre-ameloblasts, whereas the opposing cells of dental papilla begin to differentiate in pre-odontoblasts. While stratum intermedium is now compressed to only few layers of spindle-shaped cells, cervical loops continue to ingress into the underlying mesenchyme. IGF-2 and PTEN displayed the same expression pattern in proliferating cervical loops with a bit less intense expression of PTEN

FIGURE 3. Co-localization of IGF-2 and PTEN by double immunofluorescence in human incisor tooth germ in the 20th week of development (late bell stage). (a-d) Detail of cervical loop (a) Moderate expression of IGF-2 in the cervical loop and adjacent parts of enamel epithelia; (b) Mild expression of PTEN in the cervical loop; (c) DAPI staining of nuclei; (d) Merging of a+b+c (*Magnification: ×100; scale bar: 10 μm*). (e-h) Detail of enamel organ at the site of future cusp tip (e) Moderate to strong expression of IGF-2 in pre-ameloblasts. Moderate expression of IGF-2 in stratum intermedium; (f) Moderate to strong expression of PTEN in pre-ameloblasts. Mild expression of PTEN in stratum intermedium; (g) DAPI staining of nuclei; (h) Merging of e+f+g (*Magnification: ×100; scale bar: 10 μm*). (i-l) Detail of dental papilla at the site of future cusp (i) Mild expression of IGF-2 in pre-odontoblasts; (j) Moderate expression of PTEN in pre-odontoblasts; (k) DAPI staining of nuclei; (l) Merging of i+j+k (*Magnification: ×100; scale bar: 10 μm*).



in surrounding and underlying mesenchyme (Fig. 3a, b, d) Both IGF-2 and PTEN were strongly expressed by future cusp tip pre-ameloblasts, whereas moderate expression of PTEN was predominant in pre-odontoblasts of dental papilla (Fig. 3i, j, l).

IGF-1R immunofluorescent staining

Immunofluorescent staining to IGF-1R was performed on human incisor tooth germs aged 7, 10, 12, 14 and 20 weeks of development (Fig. 4, Fig. 5, Table 2).

In the 7th week of development (bud stage), IGF-1R was moderately expressed throughout the incisor tooth bud, mildly in dental lamina, whereas its expression in oral epithelium gradually increased from moderate to strong and very strong as the epithelium stretches away from the tooth bud (Fig. 4a, c). In jaw mesenchyme surrounding the bud, IGF-1R was expressed mildly.

TABLE 1. Expression of IGF-2 and PTEN in epithelial and mesenchymal parts of human incisor tooth germ between the 7th and 20th week of development.

Tooth germ parts		Epithelial								Mesenchymal		
Age (weeks)	Factor	tb	dl	oee	iee	cl	sr	si	pa	dp	df	po
7	IGF-2	++	+	/	/	/	/	/	/	–	/	/
	PTEN	++	++	/	/	/	/	/	/	+	/	/
10	IGF-2	/	+	++	++	+++	+	/	/	–	–	/
	PTEN	/	–	++	+	+++	+	/	/	++	++	/
12	IGF-2	/	+	++	++	++	++	/	/	–	–	/
	PTEN	/	–	++	+	++	++	/	/	++	++	/
14	IGF-2	/	++	++	++	++	+	+	/	+	–	/
	PTEN	/	–	++	++	+	+	+	/	++	++	/
20	IGF-2	/	+	++	++	++	+	++	+++	+	–	+
	PTEN	/	–	+	++	+	–	+	+++	++		++

Legends: tb – tooth bud; dl – dental lamina; oee – outer enamel epithelium; iee – inner enamel epithelium; cl – cervical loop; sr – stellate reticulum; si – stratum intermedium; pa – pre-ameloblasts; dp – dental papilla; df – dental follicle; po – pre-odontoblasts

Reactivity: – (absent); + (mild); ++ (moderate); +++ (strong); /(absence of tissue)

In the 10th week of development (cap stage), IGF-1R displayed moderate expression in inner and outer enamel epithelia, being mildly expressed in stellate reticulum and dental lamina (Fig. 4d, f). Slight increase in the intensity of expression could be observed in area of dental papilla closely adjacent to the inner enamel epithelium.

In the 12th week of development (late cap stage), IGF-1R increased the expression in both enamel epithelia, whereas moderate to strong expression of IGF-1R was present in enamel knot area and prospective cervical loops (Fig. 4g, i). The intensity of expression remained the same in dental lamina, stellate reticulum and dental papilla as observed in the 10th week of development.

In the 14th week of development (early bell stage), IGF-1R was strongly expressed by cervical loops, adjacent portions of inner and outer enamel epithelia, as well as in the inner enamel epithelium at the site of future cusp tip (prospective pre-ameloblasts) (Fig. 5a, c, d, f). IGF-1R was moderately expressed in stratum intermedium, while cells of dental papilla in the region of future cusp tip (prospective pre-odontoblasts), expressed IGF-1R mildly.

In the 20th week of development (late bell stage), similar pattern of expression of IGF-1R could be observed in inner enamel epithelium and stratum intermedium as described for the early bell stage (Fig. 5g, i). Mild expression of IGF-1R was seen in stellate reticulum, whereas it was strongly expressed in cervical loops (Fig. j, l). It should be noted that the overall expression of IGF-1R was increased throughout the dental papilla in comparison to previous developmental stage, being of moderate to strong intensity in pre-odontoblasts, and thus reflecting the intensity of expression of IGF-1R in pre-ameloblasts.

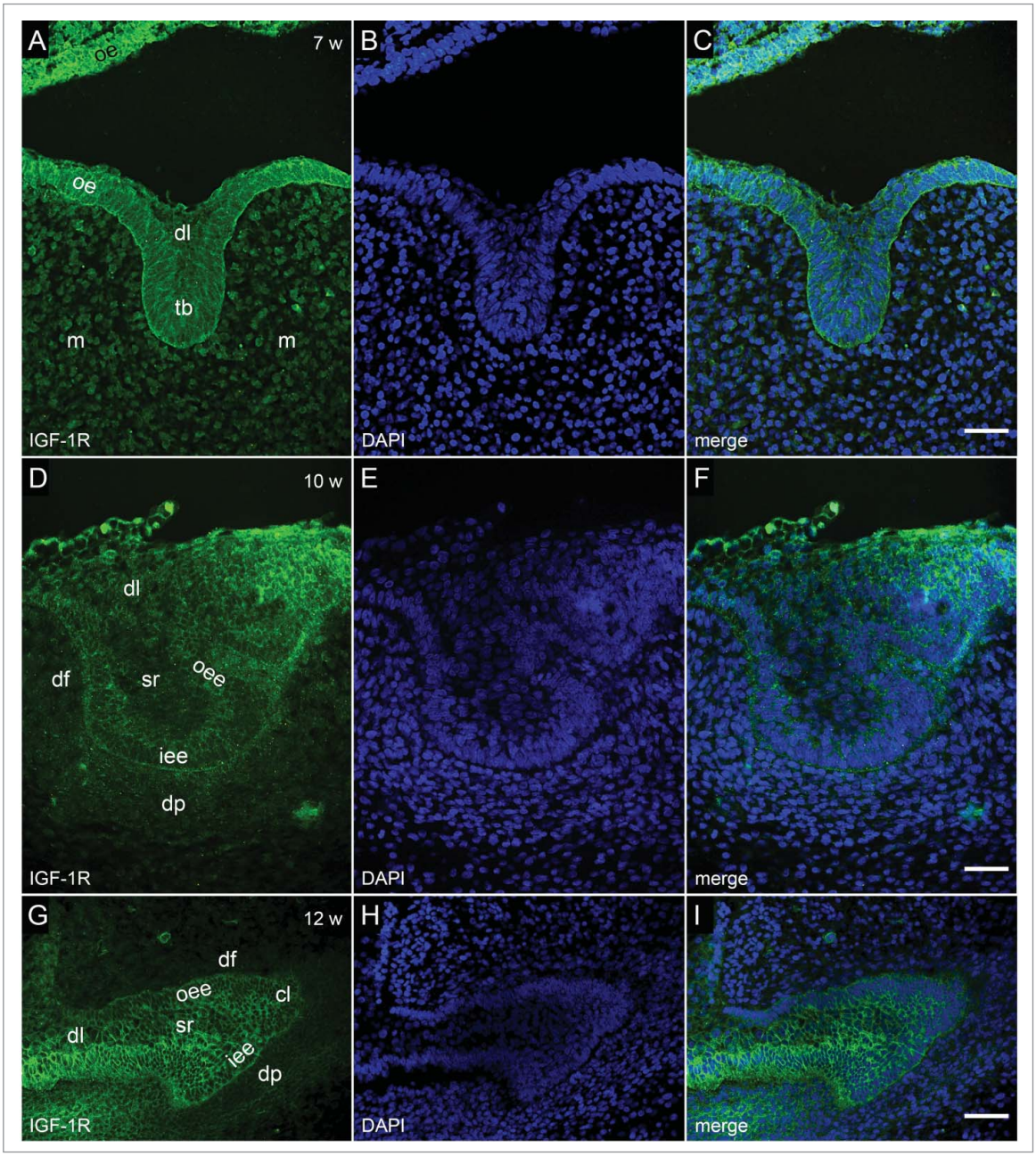
IGF-2R immunofluorescent staining

Immunofluorescent staining to IGF-2R was performed on human incisor tooth germs aged 7, 10, 12, 14 and 20 weeks of development (Fig. 6, Table 2).

In the 7th week of development (bud stage), IGF-2R was moderately expressed by individual cells sparsely distributed throughout the incisor tooth bud and surrounding jaw mesenchyme. Expression of IGF-2R was mild in dental lamina, whereas its expression in a strip of adjacent oral epithelium was of moderate intensity (Fig. 6a, c).

In the 10th week of development (cap stage), IGF-2R was expressed either mildly or

FIGURE 4. Immunofluorescent staining to IGF-1R in human incisor tooth germ between 7th and 12th week of development. (a-c) Human incisor tooth germ in the 7th week of development (bud stage) (a) Moderate expression of IGF-1R in tooth bud. Strong expression of IGF-1R in oral epithelium. Jaw mesenchyme is mildly positive to IGF-1R; (b) DAPI staining of nuclei; (c) Merging of a+b (*Magnification* ×*40; scale bar: 25* μ*m*). (d-f) Human incisor tooth germ in the 10th week of development (cap stage) (d) Moderate expression of IGF-1R in inner and outer enamel epithelia. Dental lamina, stellate reticulum and dental papilla are mildly positive to IGF-1R; (e) DAPI staining of nuclei; (f) Merging of d+e (*Magnification:* ×*40; scale bar: 25* μ*m*). (g-i) Human incisor tooth germ in the 12th week of development (late cap stage) (g) Strong expression of IGF-1R in the inner and outer enamel epithelia and prospective cervical loops. Stellate reticulum is moderately positive to IGF-1R, whereas dental expression of IGF-1R in dental lamina is mild; (h) DAPI staining of nuclei; (i) Merging of g+h (*Magnification:* ×*40; scale bar: 25* μ*m*).



moderately by individual cells located in inner and outer enamel epithelia, dental papilla and dental follicle (Fig. 6d, f). Similar scattered expression pattern of IGF-2R was observed in epithelial and mesenchymal parts of human incisor tooth germ in the 12th week of development (data not shown).

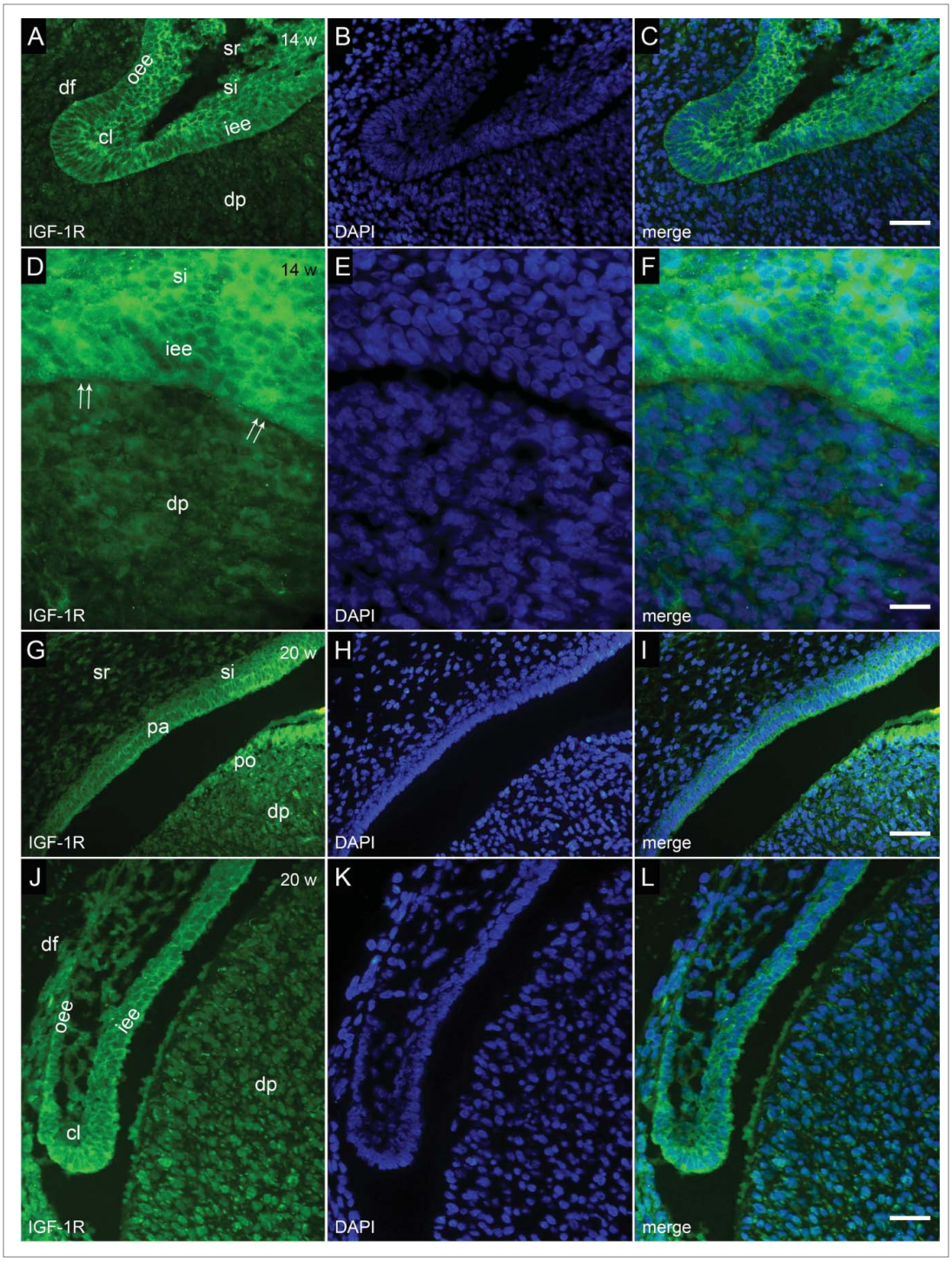
In the 14th and 20th week of development (early and late bell stage) IGF-2R displayed more cohesive expression patterns in cervical loops (Fig. 6g, i; Fig. 6m, o). At the site of future cusp tip, it was expressed moderately by individual cells of inner enamel epithelium and underlying dental papilla (Fig. 6j, l), as well as by some pre-ameloblasts (Fig. 6p, r). Pre-odontoblasts of dental papilla were mostly negative to IGF-2R.

DISCUSSION

Opposing functional roles of IGF-2 and PTEN with regard to promotion or suppression of cell proliferation and survival, led us to hypothesis that these factors might have mutually exclusive expression patterns in developing human tooth germs. Thus, IGF-2 was not expected to be co-expressed with PTEN in highly proliferative odontogenic tissues (i.e. tooth bud, cervical loops), or regions of odontogenic tissues which display transient proliferative activity (both in enamel organ and mesenchymal parts of the tooth germ). Present findings do not support such hypothesis with exception of those on expression patterns of IGF-2 and PTEN in dental papilla. Throughout the investigated period, dental papilla was positive to PTEN, while almost no expression of IGF-2 could be observed. This might reflect the notion that proliferative spurt in dental papilla significantly lags behind the proliferative spurt occurring in enamel organ.[1,34] However, continuous co-expression of these factors in enamel organ seems to be a common feature of developing human tooth germ. In development and malignant alteration, IGF-2 and PTEN have been reported to co-express as they act in tandem through a negative feedback loop, where differences in intensity of either of these 2 factors' expression affect the overall proliferative activity in particular tissue.[11] Similar effects can be seen in enamel organ's primary enamel knot, pre-ameloblasts at the site of future cusp tip and cervical loops, as all of these tissues co-express IGF-2 and PTEN while displaying either none, low or high proliferative activity, respectively.

While expressing IGF-2 with the same intensity as the inner enamel epithelium, cells of the primary enamel knot increased expression of PTEN during the late cap stage. These are among the first cells in

FIGURE 5. (For figure, see next page.) Immunofluorescent staining to IGF-1R in human incisor tooth germ in the 14th and 20th week of development (early and late bell stage). (a-c) Human incisor tooth germ in the 14th week of development (early bell stage) (detail of cervical loop) (a) Strong expression of IGF-1R in the cervical loop, adjacent portions of the inner and outer enamel epithelia and stratum intermedium; (b) DAPI staining of nuclei; (c) Merging of a+b (*Magnification: ×40; scale bar: 25 μm*). (d-f) Human incisor tooth germ in the 14th week of development (early bell stage) (detail at the site of future cusp tip) (d) Strong expression of IGF-1R in the cells of inner enamel epithelium (prospective pre-ameloblasts) and stratum intermedium. Underlying cells of dental papilla (prospective odontoblasts) are mildly positive to IGF-1R; (e) DAPI staining of nuclei; (f) Merging of d+e (*Magnification: ×100; scale bar: 10 μm*). (g-i) Human incisor tooth germ in the 20th week of development (late bell stage (detail at the site of future cusp tip) (g) Strong expression of IGF-1R in pre-ameloblasts and pre-odontoblasts. Moderate expression of IGF-1R in stratum intermedium and dental papilla. Stellate reticulum is mildly positive to IGF-1R; (h) DAPI staining of nuclei; (i) Merging of g+h (*Magnification: ×20; scale bar: 40 μm*). (j-l) Human incisor tooth germ in the 20th week of development (late bell stage) (detail of cervical loop) (j) Strong expression of IGF-1R in the cervical loop, adjacent portions of the inner and outer enamel epithelia and stratum intermedium. Dental papilla is moderately positive to IGF-1R; (k) DAPI staining of nuclei; (l) Merging of j+k (*Magnification: ×40; scale bar: 25 μm*).



enamel organ to arrest the cell cycle as they anchor at the site of inner enamel epithelium folding (future cusp tip). During the late cap stage, removal of the primary enamel knot is already underway in order to be completed by the beginning of the early bell stage. It is still unclear whether this occurs exclusively due to apoptosis, cell migration or

TABLE 2. Expression of IGF-1R and IGF-2R in epithelial and mesenchymal parts of human incisor tooth germ between the 7th and 20th week of development.

Tooth germ parts		Epithelial								Mesenchymal		
Age (weeks)	Factor	tb	dl	oee	iee	cl	sr	si	pa	dp	df	po
7	IGF-1R	++	+	/	/	/	/	/	/	+	/	/
	IGF-2R	++*	+	/	/	/	/	/	/	++*	/	/
10	IGF-1R	/	+	++	++	++	+	/	/	+	−	/
	IGF-2R	/	+	+*	++*	++*	−	/	/	++*	+	/
12	IGF-1R	/	+	+++	+++	+++	+	/	/	+	−	/
	IGF-2R	/	+	+*	++*	+*	+	/	/	++*	+	/
14	IGF-1R	/	+	+++	+++	+++	+	++	/	+	−	/
	IGF-2R	/	++*	+*	++*	++*	+	+*	/	++*	+	/
20	IGF-1R	/	+	++	+++	+++	+	++	+++	++	−	+++
	IGF-2R	/	++*	+*	++*	+++*	+	++*	++*	++*	+	+*

Legends: tb – tooth bud; dl – dental lamina; oee – outer enamel epithelium; iee – inner enamel epithelium; cl – cervical loop; sr – stellate reticulum; si – stratum intermedium; pa – pre-ameloblasts; dp – dental papilla; df – dental follicle; po – pre-odontoblasts

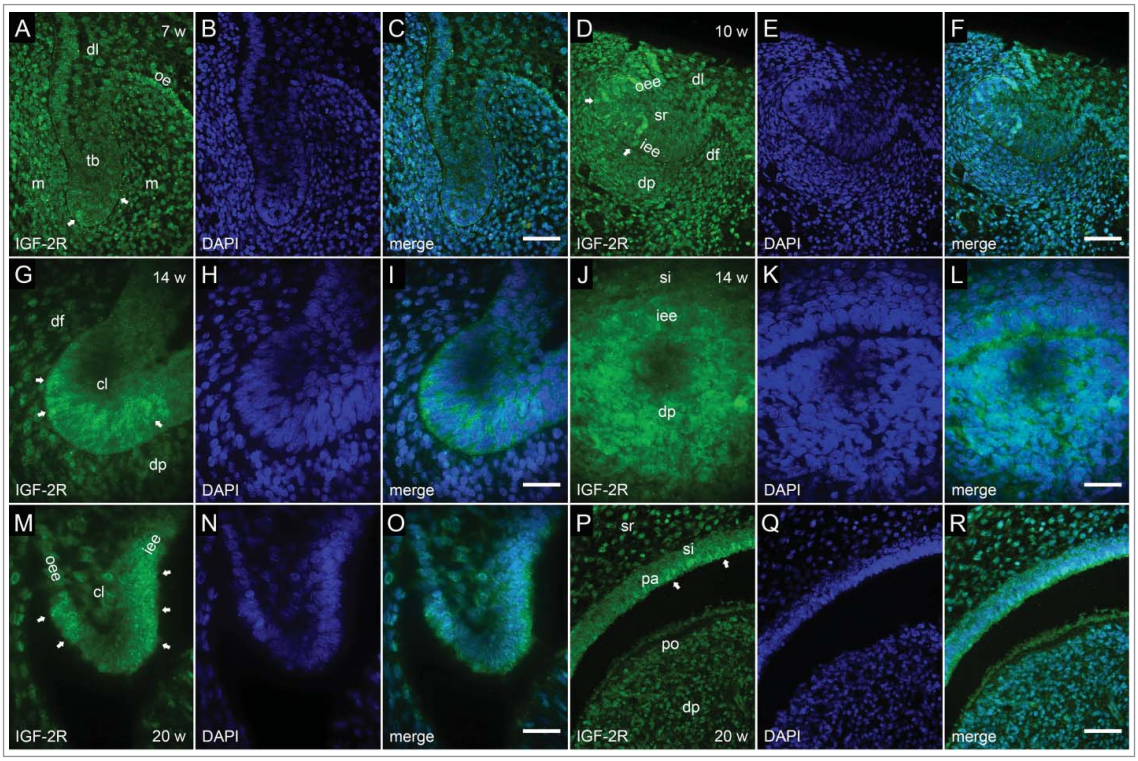
Reactivity: – (absent); + (mild); ++ (moderate); +++ (strong); /(absence of tissue); *(expression in individual cells)

combination of the 2 processes.[35–37] There are indications that apoptosis might be a prime mechanism of the primary enamel knot removal. Firstly, in addition to anti-proliferative effects, PTEN is also known to attenuate cellular migration.[17,38, 39] Thus, the increase of expression of PTEN in the primary enamel knot prior to its removal might also be suggestive of reduced migratory potential of the primary enamel knot cells. Secondly, even though the primary enamel knot expressed IGF-2, it was negative to IGF-1 as previously described.[28] Since IGF-1 and −2 promote cell survival, this might point to an increasing susceptibility of the primary enamel knot cells to apoptosis.[25,40,41] Thirdly, in contrast to developing premolars and molars, incisor tooth germs have only a single site of inner enamel epithelium folding provided by the primary enamel knot. Absent need of secondary enamel knots (which serve as sites of additional epithelial folding and are believed to be partly populated by the migrating cells of the primary enamel knot), it is plausible that the primary enamel knot of incisor tooth germ could be terminated exclusively by apoptosis.[35]

Highly proliferative confluence regions of inner and outer enamel epithelia (prospective cervical loops) and cervical loops co-expressed IGF-2 and PTEN in somewhat different manner than the primary enamel knot. Namely, cervical loops displayed slight increase of expression of IGF-2 with comparable decrease of expression of PTEN. These alterations in expression of IGF-2 and PTEN might be required for directed ingrowth of cervical loops into the underlying mesenchymal tissue, which ultimately causes elongation of enamel organ during the course of early and late bell stage. Similar example of negative feedback loop between IGF-2 and PTEN has been proposed to be crucial for regulation of terminal length of developing mammary gland ducts.[23] On the other hand, differentiating pre-ameloblasts of the future cusp tip started to express IGF-2 and PTEN with equally strong intensity by the late bell stage, whereas the population of PTEN-positive cells in dental papilla directly opposed to pre-ameloblasts, began to express IGF-2. Such co-expression pattern of IGF-2 and PTEN is indicative to delayed onset of differentiation of pre-odontoblasts.

The expression of IGF-1R in human incisor tooth germ was ubiquitous throughout the investigated period, meaning that both epithelial and mesenchymal tissues of the human incisor tooth germ possess the ability to harness the effects of IGF-1 and −2.[42] Although IGFs are exclusively expressed in enamel organ, the intensity of expression of IGF-1R

FIGURE 6. Immunofluorescent staining to IGF-2R in human incisor tooth germ between the 7th and 20th week of development (bud to late bell stage). (a-c) Human incisor tooth germ in the 7th week of development (bud stage) (a) Moderate expression of IGF-2R in sparsely distributed cells of the tooth bud and jaw mesenchyme; (b) DAPI staining of nuclei; (c) Merging of a+b (*Magnification: ×40; scale bar: 25 μm*). (d-f) Human incisor tooth germ in the 10th week of development (cap stage) (d) Moderate expression of IGF-2R in sparsely distributed cells of enamel organ and dental papilla; (e) DAPI staining of nuclei; (f) Merging of d+e (*Magnification: ×40; scale bar: 25 μm*). (g-i) Human incisor tooth germ in the 14th week of development (early bell stage) (detail of cervical loop) (g) Moderate expression of IGF-2R in the cervical loop; (h) DAPI staining of nuclei; (i) Merging of g+h (*Magnification: ×100; scale bar: 10 μm*). (j-l) Human incisor tooth germ in the 14th week of development (early bell stage) (detail at the site of future cusp tip) (j) Mild to moderate expression of IGF-2R by individual cells of the inner enamel epithelium and underlying dental papilla; (k) DAPI staining of nuclei; (l) Merging of j+k (*Magnification: ×100; scale bar: 10 μm*). (m-o) Human incisor tooth germ in the 20th week of development (late bell stage) (detail of cervical loop) (m) Moderate to strong expression of IGF-2R in cervical loop. Individual cells at the very tip of the cervical loop are negative to IGF.2R; (n) DAPI staining of nuclei; (o) Merging of m+n (*Magnification: 100×; scale bar: 10 μm*). (p-r) Human canine tooth germ in the 20th week of development (late bell stage) (detail at the site of future cusp tip) (p) Moderate expression of IGF-2R in small clusters of pre-ameloblasts. Pre-odontoblasts are mostly negative to IGF-2R; (q) DAPI staining of nuclei; (r) Merging of p+q (*Magnification: 100×; scale bar: 10 μm*).



can still be positively correlated with that, since IGF-1R was strongly expressed in enamel organ in contrast to only mild expression in dental papilla and dental follicle. It should also be noted that during the bud stage, non-odontogenic oral epithelium expressed IGF-1R more strongly than the tooth bud itself. The scope of this study enables us only to speculate whether downregulation of IGF-1R normally takes place at the onset of tooth development in particular regions of embryonic oral epithelium with odontogenic

potential. Similarly to IGF-1R, IGF-2R was expressed in all tissues of human tooth germ. However, the distribution and intensity of its expression in enamel organ were rather erratic throughout the investigated period and could not be closely correlated with that of IGF-2. These findings indicate that although most tissues in human tooth germ do possess the ability to suppress IGF-2 via IGF-2R, negative regulation of IGF-2 is simultaneously executed by alternative mechanisms.[14,25]

In conclusion, described features of expression of IGF-2 and PTEN imply versatile roles and synchronous involvement of these factors in stage-specific spatial patterning of basic cellular processes (proliferation, differentiation, cell survival) during human tooth development. Expression pattern of IGF-1R seemed to be more context-dependent than that of IGF-2R, and was, in contrast, well correlated with the presence of IGF-1 and −2 in tissues of developing human incisor tooth germs. Unfortunately, data presented here allow us only to speculate about the acting modes of delivery of IGFs (autocrine/paracrine) in odontogenic tissues. Therefore, further studies on distribution of IGFs mRNA transcripts and IGFBPs in human tooth germs are still needed in order to fully disclose the nature of involvement of IGF-axis during human odontogenesis.

MATERIALS AND METHODS

Tissue procurement and processing

For this study, 10 human fetuses aged between 7 and 20 weeks of development were obtained after spontaneous abortions and tubal pregnancies from the Department of Pathology, University Hospital in Split, Croatia. The samples were stored in the form of histological sections and kept at −24°C as a part of archive human collection of the human material belonging to the Department of Anatomy, Histology and Embryology, School of Medicine, University of Split. Approval for tissue processing was given by the Ethical and Drug Committee of University Hospital in Split (Class: 033–081/11–03/0005, No: 2181-198-03-04/10-11-0024, 2011) in accordance with Helsinki Declaration.[43] For assessment of gestational age of the human fetuses, external measurements were used.[44] Analysis was performed only on foetal tissues from head areas and/or parts of jaws containing tooth germs. Following tissue fixation with 4% paraformaldehyde in phosphate-buffered saline (PBS) and dehydration in graded ethanol dilutions, foetal tissues were paraffin-embedded and cut in frontal or transversal planes (serial 7 μm sections). Tissue sections were then mounted on glass slides for microscopic examination using Olympus BX51 light microscope (Olympus, Tokyo, Japan). Once the adequate tissue preservation was confirmed by the examination of control sections stained with haematoxylin and eosin, sections were selected for single and double immunofluorescent staining.

Double immunofluorescent staining

Tissue sections were deparaffinised in xylene and descending ethanols, rehydrated in distilled water and left incubating for 30 min in 0.1% H_2O_2 for suppression of endogenous peroxidase activity. After that, sections were washed in PBS and put in sodium citrate buffer for 15 min heating at 95°C in microwave oven. Prior the 24 hrs incubation in dark chamber with primary antibodies, sections were left to cool down to room temperature. Primary antibodies used for double immunofluorescent staining were as follows: goat monoclonal anti-human IGF-2 antibody (1:500; ab123812 Abcam, UK), and mouse monoclonal anti-human PTEN antibody (1:200; MAB3847, R&D Systems, MN, USA). Rabbit polyclonal anti-human IGF-1R antibody (1:50; ab39398 , Abcam, UK) and mouse monoclonal anti-human IGF-2R antibody (1:100; ab2733, Abcam, UK) were used for single immunofluorescent staining. Once the incubation with primary antibodies was done, sections were primed for 1 hr incubation in dark chamber with secondary antibodies as follows: donkey anti-goat Alexa Fluor 594 (1:300; ab150136, Abcam, UK), donkey anti-mouse Alexa Fluor

488 (1:300; ab150105, Abcam, UK), donkey anti-mouse Alexa Fluor 488 (1:300; ab150105, Abcam, UK), and donkey anti-rabbit Streptavidin Alexa Fluor 488 (1:300; Invitrogen Molecular Probes Inc., Eugene, OR, USA). When the incubation in secondary antibody/antibodies was over, sections had to be washed in PBS and counterstained with 4′6-diamidino-2-phenylindole (DAPI) for 2 min to stain nuclei. Sections were once again rinsed in PBS, air-dried, mounted and cover-slipped (Immuno-Mount; Shandon, Pittsburgh, PA). Antibody specificity control was also performed by omission of primary antibodies from the staining procedure.

Immunofluorescence images were made by SPOT Insight camera (Diagnostic Instruments, USA), mounted on Olympus BX61 microscope (Olympus, Tokyo, Japan). Acquisition and merging of images were performed by CellA® and SpotAdvanced® software followed by the image-plate assembly in Adobe Photoshop® CS6. Expression patterns of all antibodies used in the present study were analyzed semi-quantitatively (Table 1, Table 2).

ABBREVIATIONS

IGF-2	Insulin-Like Growth Factor 2
IGF-1R	Insulin-Like Growth Factor 1 Receptor
IGF-2R	Insulin-Like Growth Factor 2 Receptor
PTEN	Phosphatase and Tensin Homolog

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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AUTHOR CONTRIBUTIONS

Darko Kero designed the study, performed selection and double immunofluorescent staining of tissue sections (IGF-2/PTEN), acquired, interpreted and processed data, assessed the literature review reports and wrote the manuscript. Livia Cigic performed double immunofluorescent staining (IGF-2/PTEN), acquired and interpreted data, reviewed part of the literature (IGF-2, PTEN/functional studies) and wrote Materials and Methods and Results sections of the manuscript. Ivana Medevedec Mikic performed single immunofluorescent staining (IGF-1R, IGF-2R), acquired and interpreted data, reviewed part of the literature (IGF-1R, IGF-2R/functional studies) and wrote Results section of the manuscript. Tea Galic performed single immunofluorescent staining (IGF-1R), reviewed part of the literature (IGF-2 and PTEN in organogenesis and odontogenesis), and was in charge for proofreading of the manuscript. Mladen Cubela performed single immunofluorescent staining (IGF-2R), reviewed part of the literature (IGF-1R and IGF-2R in organogenesis and odontogenesis), and was in charge for prrofreading of the manuscript. Katarina Vukojevic and Mirna Saraga Babic co-designed the study, provided inputs on staining procedures and data interpretation, revised and edited the manuscript.

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