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Expression of cytokeratin 8, vimentin, syndecan-1 and Ki-67 during human tooth development

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Abstract Spatio-temporal immunolocalizations of cytokeratin 8 (CK8), vimentin, syndecan-1 and Ki-67 were analyzed in ten human incisors and canine tooth germs between the 7th and 20th developmental weeks. CK8 expression was mild to moderate in the epithelial tooth parts, while it shifted from absent or mild in its mesenchymal parts, but few cells, sparsely distributed throughout the tooth germ, strongly expressed CK8. As development progressed, CK8 expression increased to strong in preameloblasts, while expression of vimentin increased to moderate in the epithelial and mesenchymal tooth parts, particularly in the dental papilla and sac. Co-expression of CK8 and vimentin was observed in some parts of the tooth germ, and was increasing in the differentiating preameloblasts and preodontoblasts. Syndecan-1 showed characteristic shift of expression from epithelial to mesenchymal tooth parts, being particularly strong in dental papilla, sac and cervical loops, while co-expression of Ki-67/syndecan-1 was strong in the dental papilla. Our study demonstrated spatio-temporal expression and restricted co-expression of the investigated markers, indicating participation of CK8 and vimentin in cell proliferation and migration, and differentiation of preodontoblasts and preameloblasts. Our data also suggest involvement of syndecan-1 in morphogenesis of the developing tooth crown and cervical loops, and together with CK8 and vimentin in differentiation of preameloblasts and preodontoblasts.

Introduction

Human tooth development undergoes several distinctive phases through successive appearance of dental crest, bud, cap, and bell developmental stages. Morphogenesis of different teeth is a consequence of spatio-temporal appearance of cell proliferation, apoptosis and cell differentiation, as well as cell-to-cell and cell-matrix interactions (Lesot and Brook 2009; Townsend et al. 2009). Firstly observed developmental process during odontogenesis is cell proliferation, which is usually visualized by the Ki-67 nuclear antigen. Investigations on different stages of human tooth germs disclosed importance of restricted spatio-temporal cell proliferation for normal tooth growth and morphogenesis, while its selective decrease was associated with initiation of cell differentiation (Kalibovic Govorko et al. 2010; Kero et al. 2014; Guven et al. 2007). Reorganization of inner enamel epithelium characterized by modulations in cell–cell junctions and cytoskeleton changes was observed during post-mitotic ameloblasts elongation and polarization (Nishikawa 1992), while their terminal differentiation was controlled by cell-to-matrix interactions (Lesot and Brook 2009).

Intermediate filament proteins (IFs) are basic building units of cytoskeleton. Apart from their well-established roles in maintenance of cell shape (Doherty and McMahon 2008),

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cell adhesion and directional motility (Eriksson et al. 2009), these proteins participate in signal transduction (Eriksson et al. 2009), modulation of different cellular proteins properties (Liao et al. 1997) and protection from apoptosis (Gilbert et al. 2001). Among distinct classes of IFs, cytokeratins (CKs) are typically expressed in epithelial cells, whereas vimentin can be found in cells of mesenchymal origin. Unlike vimentin, CK filaments assemble to form pairs expressed characteristically in different types of adult epithelia. By now, co-expression of vimentin and CKs was found in certain structures of epithelially derived enamel organ of tooth germ (Heikinheimo et al. 1989; Kasper et al. 1989). Co-expression of vimentin and CKs is believed to be the hallmark of epithelial-to-mesenchymal, or mesenchymal-to-epithelial transformation of developing tissues, and in tooth germ it was shown to occur at the bell stage (Heikinheimo et al. 1989). Thus, vimentin and CKs were co-expressed in stellate reticulum of the enamel organ, while co-expression of various simple and stratified epithelia CKs was observed in the most parts of enamel organ (Couwenhoven and Schwartz 1988; Domingues et al. 2000; Heikinheimo et al. 1989; Kasper et al. 1989; Lesot et al. 1982). The initial increase of simple epithelia CKs expression was restricted to regions of embryonic oral epithelium (dental lamina), and was followed by the expression of stratified epithelia CKs further in development (Hosoya et al. 2010; Kasper et al. 1989). Previous studies of IFs expression patterns during tooth development, performed either on animal (Couwenhoven and Schwartz 1988; Hosoya et al. 2010; Lesot et al. 1982; Nakai et al. 1986; Ravindranath et al. 2001; Webb et al. 1995) and human tissues (Domingues et al. 2000; Heikinheimo et al. 1989; Kasper et al. 1989), disclosed their astounding complexity. Stage-specific co-expression of vimentin and CKs was observed in enamel organ and dental papilla (Heikinheimo et al. 1989; Lesot et al. 1982; Suzuki et al. 1999). However, the existing data on particular simple epithelia CKs [namely cytokeratin 8 (CK8)] expression patterns during human tooth development are still incomplete and sometimes contradictory. The reasons for such discrepancies in IF patterning might be either in the narrow focus of those studies omitting earlier stages of human tooth development prior to bell stage (Domingues et al. 2000; Heikinheimo et al. 1989), or in searching for pools of simple epithelia CKs instead of individual polypeptides (Heikinheimo et al. 1989; Kasper et al. 1989). However, proper IF patterning in odontogenesis holds great importance, because some studies on transgenic mice have shown that alterations of IF networks within the odontoblasts interfered with secretory activity of both odontoblasts and ameloblasts, causing abnormal tooth development (Berteretche et al. 1993).

Organization and composition of cytoskeleton may be profoundly affected by varying levels of syndecans (Saunders et al. 1989) switching it from epithelia-like to mesenchyme-like type of cytoskeleton. There is also evidence of co-localization of syndecans and some components of cytoskeleton such as actin filaments (Bernfield et al. 1992; Jalkanen et al. 1987). Generally, syndecans are single transmembrane proteins that have the affinity to bind to various extracellular matrix components, enzymes, peptide growth factors, transcription factors and cell adhesion molecules (Bernfield et al. 1992), which are responsible for a diverse set of syndecan functions. So far, the roles and expression patterns of syndecans during tooth development have been extensively studied in mice (Bai et al. 1994; David et al. 1993; Muto et al. 2007; Salmivirta et al. 1991; Vainio et al. 1989; Vainio and Thesleff 1992) showing that syndecan-1 has quite distinctive expression patterns from some other members of the syndecan family and is mostly expressed in epithelial tissues and only transiently in mesenchymal tooth germ parts. These studies imply complementary roles of syndecans during odontogenesis as has been shown for other proteoglycans of extracellular matrix (Hou et al. 2012). Syndecan-1 is predominantly expressed in proliferating parts of tooth germ in a stage-specific manner tightly regulated by epithelial-mesenchymal interactions (Vainio et al. 1991; Vainio and Thesleff 1992), and seems to take part in the plethora of processes involved in tooth development spanning from cell condensation and growth control (Vainio et al. 1991; Vainio and Thesleff 1992) in the earliest stages, through amelogenesis and dentinogenesis (Muto et al. 2007) in the advanced stages of tooth development. In Msx1 mutant, syndecan-1 was specifically reduced in dental mesenchyme, causing failure of tooth morphogenesis such as arrest of molar tooth development (Maas et al. 1996). In spite of abundant previous research, there are no studies on syndecan-1 expression patterns during human tooth development.

In the present study we analyze expression and co-expression of simple epithelia CK8 and vimentin, and syndecan-1 and Ki-67 during the successive stages of the early embryonic and fetal human tooth development. Our findings of their expression patterns are compared to those reported in previous studies, and discussed with respect to their contributing role in fundamental developmental processes such as proliferation, differentiation and epithelial-to-mesenchymal transition in odontogenic tissues.

Materials and methods

Tissue procurement and processing

A total of ten human fetuses collected after tubal pregnancies or spontaneous abortions from the Department of Pathology, University Hospital in Split, Croatia, were
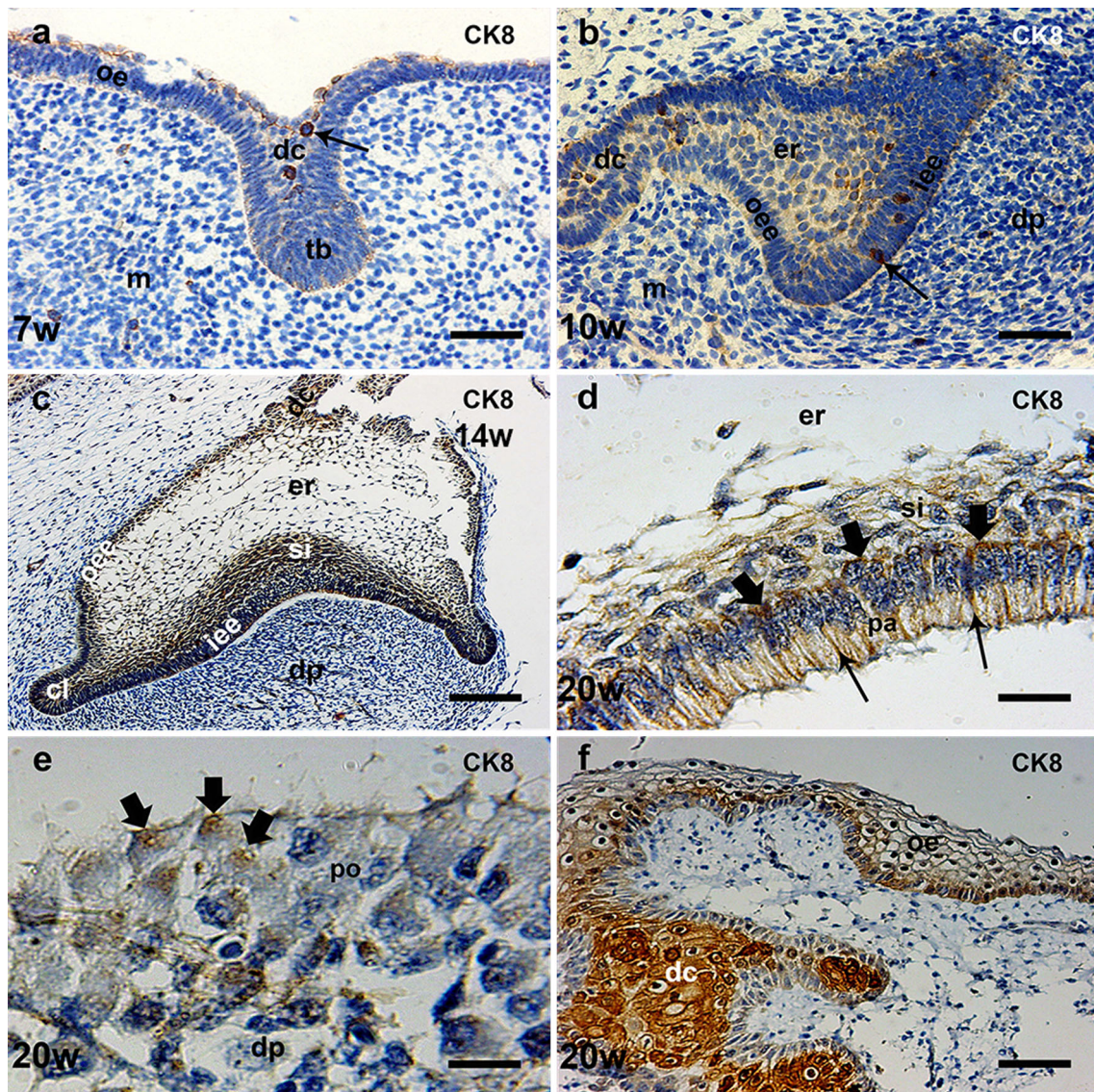
Fig. 1 CK8 immunohistochemical staining of developing human tooth germ. Human incisor (**a–c**) and canine (**d–f**) tooth germ between 7th and 20th developmental week: oral epithelium (*oe*), dental crest (*dc*), tooth bud (*tb*), outer enamel epithelium (*oee*), inner enamel epithelium (*iee*), cervical loop (*cl*), enamel/stellate reticulum (*er*), stratum intermedium (*si*), dental papilla (*dp*), dental sac (*ds*), jaw mesenchyme (*m*), preameloblasts (*pa*), preodontoblasts (*po*). *Brown* staining of CK8-positive cells. CK8 staining in individual cells (*arrows*). **a** Human incisor tooth germ in the 7th developmental week. Cell strongly positive to CK8 (*thin arrow*). Magnification: ×40, *scale bar* 25 µm. **b** Human incisor tooth germ in the 10th developmental week. Cell strongly positive to CK8 (*thin arrow*). Magnification: ×40, *scale bar* 25 µm. **c** Human incisor tooth germ in the 14th developmental week. Note unequally *brown* staining of CK8-positive cells. Magnification: ×20, *scale bar* 50 µm. **d** Human canine tooth germ in the 20th developmental week—cusp tip area of enamel organ. CK8 staining of basal (*thick arrows*) and lateral (*thin arrows*) cytoplasmic portions of preameloblasts. Magnification: ×100, *scale bar* 10 µm. **e** Human canine tooth germ in the 20th developmental week—cusp tip area of dental pulp: CK8-positive preodontoblasts (*thick arrows*). Magnification: ×100, *scale bar* 10 µm. **f** Human canine tooth germ in the 20th developmental week—dental crest and adjacent region of oral epithelium. Magnification: ×40, *scale bar* 25 µm. (Color figure online)

current analyses of fetal tissues. Following fixation with 4 % paraformaldehyde in phosphate-buffered saline (PBS) and dehydration in graded ethanol dilutions, tissues were paraffin-embedded. Serial 7 µm tissue sections cut in frontal or transverse plane were mounted on glass slides and examined using an Olympus BX51 light microscope (Olympus, Tokyo, Japan). Prior the application of

immunohistochemistry, every 10th section was stained by haematoxylin and eosin to confirm adequate tissue preservation.

Immunohistochemical staining

Sections were deparaffinized in xylene and rehydrated in water, followed by incubation for 30 min in 0.1 % H_2O_2 in order to suppress endogenous peroxidase activity. Sections for immunhistochemical staining with syndecan-1 and CK8 were washed in PBS and microwaved in sodium citrate buffer for 15 min at 95 °C. Sections were then left to cool down (room temperature) before incubation with rabbit monoclonal anti-human CK8 (1:500, ab59400; Abcam plc, Cambridge, UK) and mouse anti-human syndecan-1 (1:100, B-A38; Abcam plc, Cambridge, UK) for 1 h at room temperature. After primary antibodies incubation, sections were washed with PBS followed by 30 min incubation with biotinylated secondary antibody (mouse UniTect ABC Kit, Oncogene; Boston, MA, USA) at room temperature. Afterwards, sections were once more washed in PBS, stained with diaminobenzidine (DAB) and then rinsed in distilled water. To finalize the procedure, counterstaining with haematoxylin to stain nuclei, and separate dehydration of sections in ethanol and xylene were done. Cells positive to syndecan-1 or CK8 displayed brown-stained cytoplasms. Other tissues (located in the same sections) which stain specifically with the primary antibodies were considered as positive internal controls for syndecan-1 and CK8. Staining was cancelled in case of omission of primary antibodies.

Double immunofluorescence

Following deparaffinization of the sections and before their incubation with primary antibodies, the processing procedures were as described previously. These combinations of primary antibodies were selected for double immunofluorescent antibody staining: (1) mouse monoclonal anti-human Ki-67 (1:200, M7240; DAKO, Glostrup, Denmark) with mouse monoclonal anti-human syndecan-1 (1:100, B-A38; Abcam plc, Cambridge, UK); (2) rabbit monoclonal anti-human CK8 (1:500, ab59400; Abcam plc, Cambridge, UK) with goat polyclonal anti-human vimentin (1:800, AF2105; R&D Systems. Minneapolis, MN, USA). Secondary antibodies used for immunofluorescence: (1) goat anti-rabbit Texas Red (1:200, sc-2780, Santa Cruz Biotechnology, Inc.; Santa Cruz, CA, USA), (2) goat anti-mouse Rhodamine (1:50, AP124R; Jackson Immuno Research Lab; West Grove, PA, USA), (3) anti-rabbit FITC (AP132F; Chemicon,

Fig. 2 Co-localization of CK8 and vimentin by double immunofluorescence. (**a–d**) Human incisor tooth germ in the 8th developmental week: oral epithelium (*oe*), dental crest (*dc*), tooth bud (*tb*), jaw mesenchyme (*m*), cells strongly expressing specific marker (*arrows*). **a** Moderate expression of CK8 in epithelial parts of the tooth germ, and complete absence of it in the underlying mesenchyme. Few cells strongly positive to CK8 can be seen in the dental crest and tooth bud (*thin arrows*). CK8 immunofluorescent staining. Magnification: ×40, scale bar 25 μm. **b** Stronger vimentin expression in epithelial parts of tooth germ (dental crest and tooth bud) than in the underlying mesenchyme. Vimentin immunofluorescent staining. Magnification: ×40, scale bar 25 μm. **c** DAPI staining of nuclei in the tooth germ. DAPI immunofluorescent staining. Magnification: ×40, scale bar 25 μm. **d** Merging of A + B + C, shows reveals co-localization of CK8 and vimentin mostly in the epithelial parts of tooth germ (dental crest and tooth bud). Magnification: ×40, scale bar 25 μm. **e–h** Human incisor tooth germ in the 12th developmental week: dental crest (*dc*), outer enamel epithelium (*oee*), inner enamel epithelium (*iee*), enamel knot (*ek*), enamel reticulum (*er*), dental papilla (*dp*), dental sac (*ds*), cells strongly expressing specific marker (*arrows*). **e** Moderate expression of CK8 in epithelial parts of tooth germ with few highly positive cells in inner and outer enamel epithelia (*arrows*), enamel reticulum and dental crest. Dental papilla and sac mostly do not express CK8. CK8 immunofluorescent staining. Magnification: ×40, scale bar 25 μm. **f** Predominant expression of vimentin in dental papilla and sac as compared with mildly positive epithelial parts. Vimentin immunofluorescent staining. Magnification: ×40, scale bar 25 μm. **g** DAPI staining of nuclei in the tooth germ. DAPI immunofluorescent staining, Magnification: ×40, scale bar 25 μm. **h** Merging of E + F + G, reveals co-localization of CK8 and vimentin in epithelial parts of the tooth germ. Magnification: ×40, scale bar 25 μm. **i–l** Future cusp tip area of the human incisor enamel organ in the 14th developmental week: inner enamel epithelium (*iee*), stratum intermedium (*si*), enamel reticulum (*er*), dental papilla (*dp*), cells strongly expressing specific marker (*arrows*). **i** Moderate to strong expression of CK8 in stratum intermedium and inner enamel epithelium and its absence in dental papilla. Highly postive cells to CK8 in the stratum intermedium (*thin arrows*). CK8 immunofluorescent staining. Magnification: ×40, scale bar 25 μm. **j** Moderate expression of vimentin in stratum intermedium, inner enamel epithelium and dental papilla. Slightly increased expression of vimentin at the border between inner enamel epithelium and stratum intermedium (*thick arrows*). Vimentin immunofluorescent staining. Magnification: ×40, scale bar 25 μm. **k** DAPI staining of nuclei in the tooth germ. DAPI immunofluorescent staining. Magnification: ×40, scale bar 25 μm. **l** Merging of I + J + K, reveals co-localization of CK8 and vimentin in epithelial parts of future cusp tip area of the human incisor enamel organ. Magnification: ×40, scale bar 25 μm. **m–p** Future cusp tip area of the human canine enamel organ in the 20th developmental week: preameloblasts (*pa*), enamel reticulum (*er*), cells strongly expressing specific marker (*arrows*). **m** Strong expression of CK8 in the basal portions (*thick arrows*) of preameloblasts close to stratum intermedium. Moderate expression of CK8 delineating lateral portions of preameloblasts cell membranes. CK8 immunofluorescent staining. Magnification: ×40, scale bar 25 μm. **n** Expression of vimentin in the basal portions (*thick arrows*) of preameloblasts at their interface with stratum intermedium. Vimentin immunofluorescent staining. Magnification: ×40, scale bar 25 μm. **o** DAPI staining of nuclei in the future cusp tip area of canine enamel organ. DAPI immunofluorescent staining. Magnification: ×40, scale bar 25 μm. **p** Merging of M + N + O reveals colocalization of CK8 and vimentin in the basal portions of preameloblasts. Magnification: ×40, scale bar 25 μm
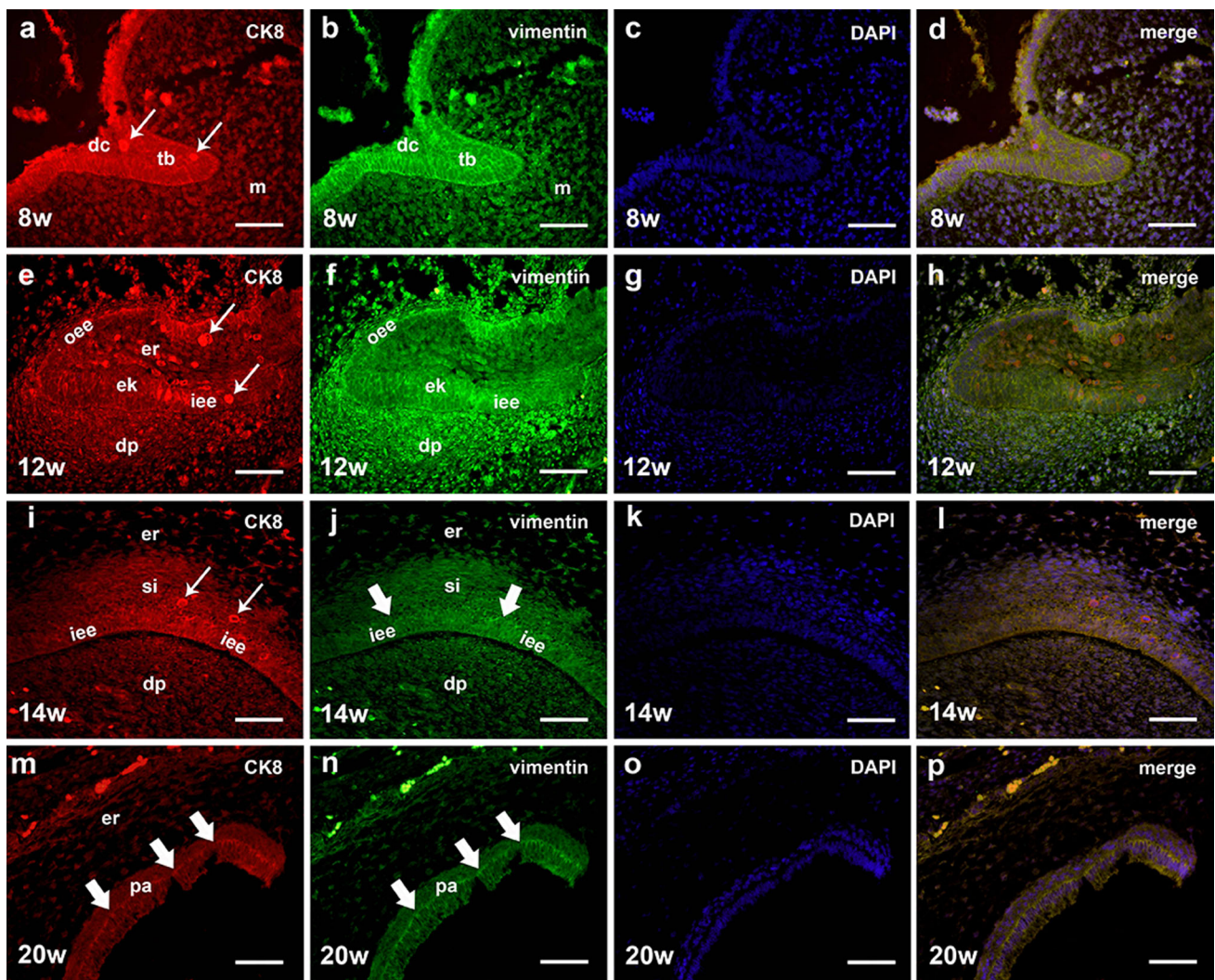
Results

After secondary antibody incubation, the sections were washed in PBS and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to stain nuclei. After a final rinsing in PBS, sections were mounted, air-dried and cover-slipped (Immuno-Mount; Shandon, Pittsburgh, PA). As a specificity control, the primary antibody was omitted from the staining procedure. Immunofluorescence images were taken by SPOT Insight camera (Diagnostic Instruments, USA), mounted on an Olympus BX61 microscope. The acquisition and processing of images using Olympus CellA® software was completed by their assembly in Adobe Photoshop®.

Between the 7th and 20th developmental week, the human tooth germ undergoes morphologically distinctive developmental stages starting with the bud stage, followed by the cap stage and subsequent formation of fully functional enamel organ in the bell stage.

CK8 immunohistochemical staining

In the 7th developmental week, the incisor tooth germ is in the bud stage of development. Tooth bud is formed by ingrowth of dental crest, i.e. dental lamina, which is part of oral epithelium, into the underlying mesenchymal tissue. Mildly positive CK8 staining can be seen in cells occupying the outer perimeter of the tooth bud at its interface with underlying mesenchymal tissue. However, some cells of the dental crest and those in the adjacent regions of oral epithelium strongly express CK8. In contrast to epithelial parts of the developing tooth germ, CK8 expression is completely absent in the underlying mesenchymal tissue of dental papilla, except for some rarely positive cells (Figs. 1a, 2a; Table 1).

In the 10th developmental week, the incisor tooth germ is in the cap stage of development. Due to enlargement of

epithelial parts of the tooth germ, inner and outer enamel epithelia are already discernible, enclosing the enamel (stellate) reticulum. In this stage of development, CK8 expression is still mild, whereas few cells, which can be seen within the epithelial parts of the tooth germ and particularly within the inner enamel epithelium, strongly express CK8. Condensing mesenchymal cells of dental papilla and dental sac mildly expresses CK8, except for some cells which display strong expression of CK8 (Fig. 1b; Table 1). Similar expression pattern of CK8 is observed in the 12th developmental week (Fig. 2e; Table 1).

In the 14th developmental week, the incisor tooth germ is in the early bell stage of development. The enamel organ is now enlarged and differentiated into histologically distinct tissues, adding stratum intermedium between the inner and outer enamel epithelia. Inner and outer enamel epithelium and dental crest maintain the same mild intensity of CK8 expression. Moderate to strong expression of CK8 is displayed throughout the entire stratum intermedium, and inner enamel epithelium. In addition, few highly CK8-positive cells can also be seen in those tissues. The underlying dental papilla mostly does not express CK8 or very mildly, except for several strongly positive cells scattered throughout the papilla (Figs. 1c, 2i; Table 1).

In the 20th developmental week, the canine tooth germ is in the late bell stage of development. Enamel organ further increases its size. At the site of future cusp tip, differentiating tall columnar cells of the inner enamel epithelium, i.e. preameloblasts, can be seen directly opposed by the amassing preodontoblasts of the dental papilla. Along with thinning of outer enamel epithelium, while stratum intermedium is reduced to 3–4 layers of flattened cells. The differentiating preameloblasts express CK8 throughout their cytoplasm, but the expression is the most intensive in the basal portions of the cells and in the area of cell contacts between the neighboring preameloblasts (Fig. 1d). Stratum intermedium mildly expresses CK8, whereas no observable changes in CK8 expression patterns can be seen in the outer enamel epithelium and dental crest. Enamel reticulum appears to be negative (Fig. 2m). In contrast to the mesenchymal parts of the tooth, where CK8 is strongly expressed only in some cells, area of the future preodontoblasts moderately expresses CK8 (Fig. 1e; Table 1). CK8 expression is also observed in the dental crest (Fig. 1f).

Co-localization of CK8 and vimentin by double immunofluorescence

In addition to CK8 immunohistochemical staining, its co-expression with vimentin was performed for the same developmental stages of tooth germ. Between the 7th (Fig. 2b) and the 10th–12th developmental week (Fig. 2f), continuous expression of vimentin in epithelial parts of tooth germ is mild in the dental crest, and increases to moderate in the inner and outer enamel epithelia, while it peaks by the 12th developmental week in future dental papilla and dental sac (Fig. 2f; Table 1). Merging of DAPI nuclear stain with CK8/vimentin markers, reveals their co-expression in primarily epithelial but rarely in mesenchymal parts of the tooth, with predominance of CK8 over vimentin expression

Table 1 Expression of cytokeratin 8 (CK8) and vimentin (VIM) in epithelial and mesenchymal parts of tooth germ between the 7th and 20th developmental week

Tooth germ parts:		Epithelial						Mesenchymal		
Age (weeks)	IF	dc	iee	oee	er	si	pa	dp	ds	po
7	CK8	+ +++*	/	/	/	/	/	− +++*	/	/
	VIM	+	/	/	/	/	/	+	+	/
10–12	CK8	+ +++*	+ +++*	+ +++*	+ +++*	/	/	+ +++*	+ +++*	/
	VIM	+	++	++	+	/	/	++	++	/
14	CK8	+ +++*	++	+	+	++ +++*	++	+ +++*	+ +++*	
	VIM	+	++	+	++	++	++	++	++	
20	CK8	+ +++*	+	++	−	+	+++	+ +++*	+ +++*	+ ++
	VIM	+	++	+	+	+	++	++	++	++

dc dental crest, *iee* inner enamel epithelium, *oee* outer enamel epithelium, *er* enamel reticulum, *si* stratum intermedium, *pa* preameloblasts, *dp* dental papilla, *ds* dental sac (dental follicle), *po* preodontoblasts

Reactivity: − (absent); + (mild); ++ (moderate); +++ (strong); +++* (strong in few cells); / (absence of structure)

only in some strongly positive cells. Mesenchymal parts of the tooth, e.g. dental papilla and dental sac, predominantly express vimentin (Fig. 2c, d, g, h; Table 1).

Between the 14th and 20th developmental week (early bell stage; Fig. 2i–l), dental papilla and dental sac express vimentin moderately in comparison to previously observed developmental stage. Inner enamel epithelium and differentiating ameloblasts, as well as stratum intermedium and enamel reticulum at the site of future cusp tip moderately express vimentin (Fig. 2j).

In the 20th week, vimentin expression gradually decreases to mild in the stratum intermedium and enamel reticulum (Fig. 2n; Table 1). The intensity of vimentin expression in these structures decreases as we move towards cervical loops. Inner enamel epithelium continues to express vimentin significantly, especially the basal portions of preameloblasts (Fig. 2n). Merging of DAPI nuclear stain with CK8/vimentin markers shows their co-expression in inner enamel epithelium including ameloblasts, stratum intermedium and enamel reticulum at the site of future cusp tip (Fig. 2k, l, o, p). In the same area, weaker co-expression of CK8 and vimentin is displayed by

the underlying preodontoblasts and some diffusely distributed cells in the dental papilla, which express vimentin moderately (not shown).

Syndecan-1 immunohistochemical staining

In the 7th–8th developmental week, in the incisor tooth germ syndecan-1 is strongly expressed by the dental crest, moderately in the outer enamel epithelium, and very mild or not at all in the slightly invaginated inner enamel epithelium. However, a few rows of cells in developing dental papilla and dental sac show moderate expression of syndecan-1, matching the early condensation of mesenchymal tissue (Fig. 3a; Table 2).

In the 9th developmental week, the incisor tooth germ is at the early cap stage. The expression pattern of syndecan-1 remains unchanged in both enamel epithelia and dental crest. Significant expansion of syndecan-1 expression is displayed by the mesenchymal tissue of dental papilla and dental sac, fully matching its condensation around the epithelial parts of tooth germ and sharply demarcating tooth germ from the jaw mesenchyme (Fig. 3b; Table 2).
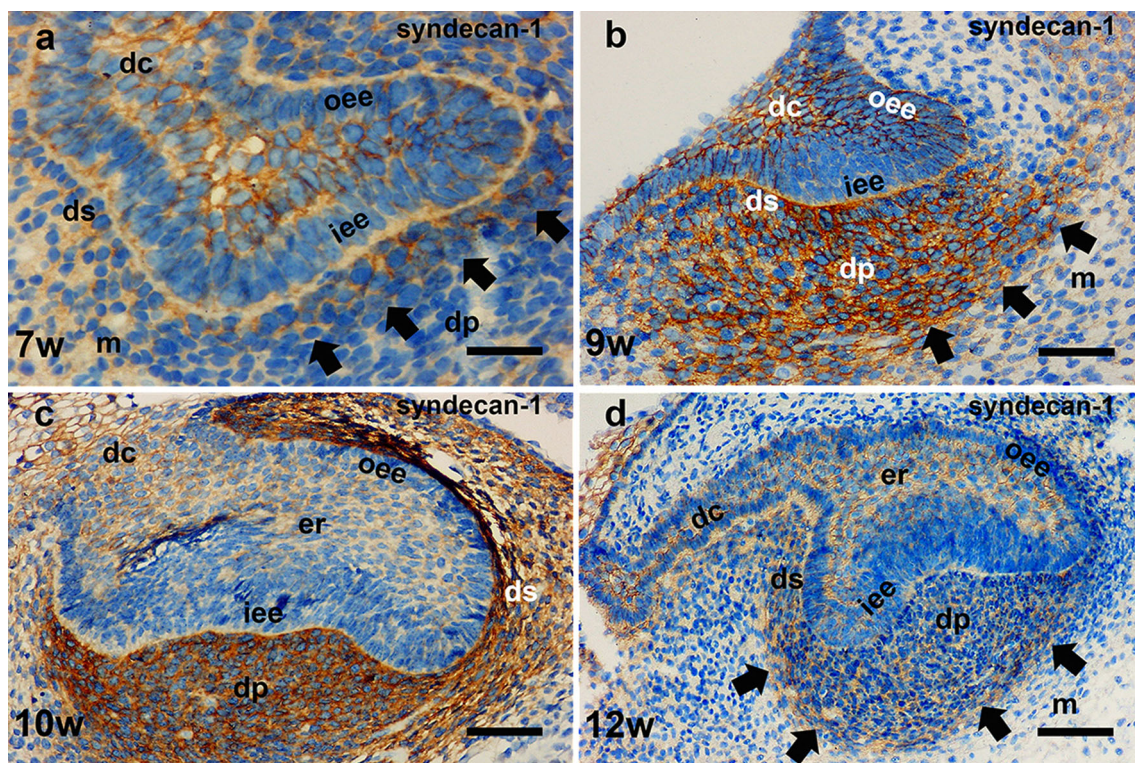
Fig. 3 Syndecan-1 immunhistochemical staining of developing human tooth germ. Human incisor (**a–d**) tooth germ between 8th and 12th developmental week: dental crest (*dc*), outer enamel epithelium (*oee*), inner enamel epithelium (*iee*), enamel/stellate reticulum, dental papilla (*dp*), dental sac (*ds*), jaw mesenchyme (*m*). *Brown* staining of syndecan-1 positive cells. Forefront of syndecan-1 expression (*arrows*). **a** Human incisor tooth germ in the 8th developmental week—late bud stage. Magnification: ×100, *scale bar* 10 μm. **b** Human incisor tooth germ in the 9th developmental week—early cap stage. Magnification: ×40, *scale bar* 25 μm. **c** Human incisor tooth germ in the 10th developmental week—cap stage. Magnification: ×40, *scale bar* 25 μm. **d** Human incisor tooth germ in the 12th developmental week—late cap stage. Magnification: ×20, *scale bar* 50 μm. (Color figure online)

Table 2 Expression of syndecan-1 in epithelial and mesenchymal parts of the tooth germ between the 7th and 20th developmental week

Syndecan-1	Tooth germ parts:						
Age (weeks)	Epithelial					Mesenchymal	
	dc	iee	oee	er	pa	dp	ds
7–8	+++	+	++	/	/	++	++
9	+++	+	++	/	/	++	++
10	+	+	+	+	/	+++	+++
12	+	+	+	+	/	+++	+++
14	+	+	+	+	+	++	++
20	/	+	+	+	+++	++	++

dc dental crest, *iee* inner enamel epithelium, *oee* outer enamel epithelium, *er* enamel reticulum, *pa* preameloblasts, *dp* dental papilla, *ds* dental sac (dental follicle)

Reactivity: − (absent); + (mild); ++ (moderate); +++ (strong); / (absence of structure)

In the 10th developmental week, the incisor tooth germ is at the cap stage. The intensity of syndecan-1 expression decreases to mild in epithelial parts of the tooth germ whereas it reaches its peak in the future dental papilla and dental sac area. Thus, complete shift from epithelial-to-mesencyhmal parts of the tooth in syndecan-1 expression can be observed (Fig. 3c; Table 2).

In the 12th developmental week, the incisor tooth germ is at the late cap stage. The expression patterns of syndecan-1 remain mostly unchanged throughout the epithelial parts (enamel organ) of the tooth germ and dental papilla, while the area of dental sac expressing syndecan-1 widens (Figs. 3d, 4; Table 2).

Co-localization of Ki-67 proliferation marker with syndecan-1 by double immunofluorescence

Co-localization of Ki-67 and syndecan-1 was analyzed during the late cap (Fig. 4) and early bell developmental stages of the incisor tooth germ (Fig. 5). Precise expression of Ki-67 has been already reported elsewhere (Guven et al. 2007; Kalibovic Govorko et al. 2010).

In the 12th developmental week (late cap stage), numerous Ki-67 positive cells can be seen in inner enamel epithelium, cervical loops, parts of outer enamel epithelium closest to the cervical loops, enamel reticulum and dental papilla (Fig. 4a). The expression of syndecan-1 reflects its epithelial-to-mesenchymal shift, being mostly pronounced in the cells of dental papilla and dental sac (Fig. 4b; Table 2), mildly throughout the enamel reticulum and in the cervical loop epithelia (Fig. 4f). However, slight differences in intensity of expression of syndecan-1 between individual cervical loops can be observed. Merging of DAPI nuclear staining with Ki-67/syndecan-1 reveals their

Fig. 4 Co-localization of Ki-67 proliferation marker and syndecan-1 ▶ by double immunofluorescence. (**a–d**) Human incisor tooth germ in the 12th developmental week: outer enamel epithelium (*oee*), inner enamel epithelium (*iee*), cervical loop (*cl*), enamel reticulum (*er*), dental papilla (*dp*), dental sac (*ds*), cells expressing specific marker (*thin arrows*), forefront of mesenchymal-to-epithelial shift of syndecan-1 expression (*thick arrows*). **a** Cells positive to Ki-67 can be seen throughout the tooth germ. Nuclei of Ki-67 positive cells in inner enamel epithelium and dental papilla (*thin arrows*). Ki-67 immunofluorescent staining. Magnification: ×40, *scale bar* 25 μm. **b** Strong syndecan-1 expression in dental papilla and dental sac in contrast to moderate syndecan-1 expression in enamel reticulum. Varying forefronts of mesenchymal-to epithelial shift of syndecan-1 expression (*thick arrows*) in individual cervical loops. Syndecan-1 immunofluorescent staining. Magnification: ×40, *scale bar* 25 μm. **c** DAPI staining of nuclei in the tooth germ. DAPI immunofluorescent staining. Magnification: ×40, *scale bar* 25 μm. **d** Merging of A + B + C reveals co-localization of Ki-67 and syndecan-1 in dental papilla, both enamel epithelia, cervical loops and restricted regions of enamel reticulum. Magnification: ×40, *scale bar* 25 μm. **e–h** Enlarged detail of a single cervical loop of human incisor tooth germ in 12th developmental week: outer enamel epithelium (*oee*), inner enamel epithelium (*iee*), enamel reticulum (*er*), dental papilla (*dp*), dental sac (*ds*), cells expressing specific marker (*thin arrows*), forefront of mesenchymal-to-epithelial shift of syndecan-1 expression (*thick arrows*). **e** Cells positive to Ki-67 in the confluence region of outer and inner enamel epithelia (cervical loop). Nuclei of Ki-67 positive cells (*thin arrows*). Ki-67 immunofluorescent staining. Magnification: ×100, *scale bar* 10 μm. **f** Forefront of mesenchymal-to-epithelial shift of syndecan-1 expression (*thick arrows*) detained at the interface between cervical loop and mesenchymal parts of tooth germ. Syndecan-1 immunfluorescent staining. Magnification: ×100, *scale bar* 10 μm. **g** DAPI staining of the nuclei in the region of cervical loop. DAPI immunofluorescent staining. Magnification: ×100, *scale bar* 10 μm. **h** Merging of E + F + G reveals co-localization of Ki-67 and syndecan-1 in enamel reticulum and mesenchymal parts of tooth germ. Magnification: ×100, *scale bar* 10 μm

strong co-expression in dental papilla, as well as mild co-expression in other epithelial and mesenchymal parts of the tooth (Fig. 4d, h).

In the 14th developmental stage (early bell stage; Fig. 5a–d), inner enamel epithelium and cervical loops are part of the enamel organ that contain the highest fraction of Ki-67 positive cells (Fig. 5a). To a lesser extent, Ki-67 positive cells are also ubiquitously present in the dental papilla. The expression of syndecan-1, however, shows an interesting asymmetric pattern with respect to the dental papilla, outer enamel epithelia and enamel reticulum (in the enamel epithelia confluence region) (Fig. 5b). While both cervical loops express syndecan-1 with equal intensity, significant differences in reactivity to syndecan-1 are displayed by the outer enamel epithelia, portions of enamel reticulum and dental papilla mesenchyme (underlying cervical loops) on the opposite sides of tooth germ (Table 2; Fig. 5b). Merging of DAPI nuclear staining with Ki-67/syndecan-1 shows their co-expression in inner and outer enamel epithelia, cervical loops and diffusely in the dental papilla (Fig. 5d).
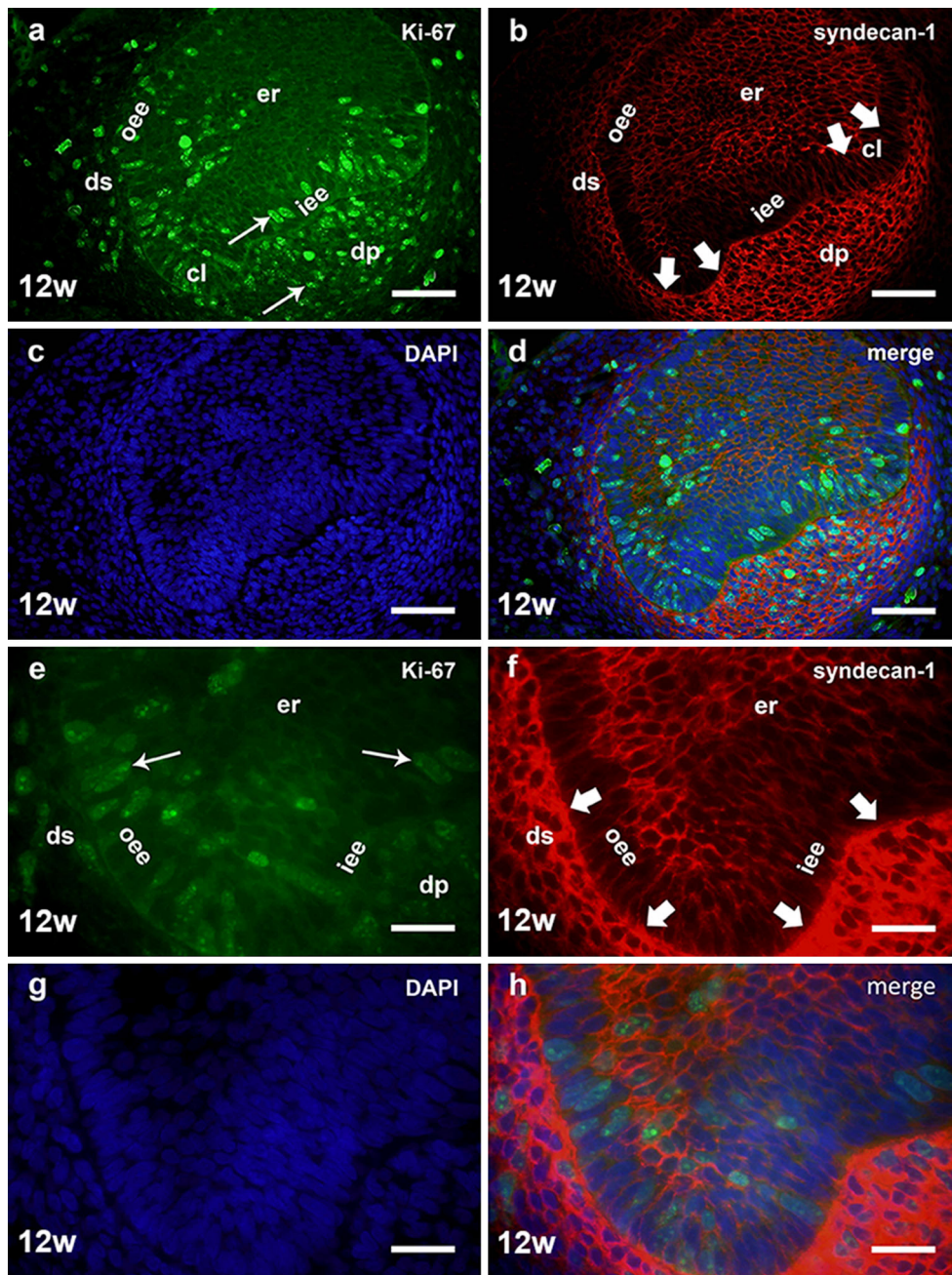
In the 20th developmental week, Ki-67 is still strongly expressed in the dental papilla and in the epithelia of the cervical loops, but not in the ameloblasts and stratum intermedium (Fig. 5e). Syndecan-1 is strongly expressed by the differentiating preameloblasts at the site of future cusp tip and moderately by the preodontoblasts of the dental papilla. Both differentiating preameloblasts and preodontoblasts express syndecan-1 in their apical poles. Significant reduction of reactivity to syndecan-1 can be observed throughout the dental papilla. (Table 2; Fig. 5f).

Discussion

During early odontogenesis, in contrast to mesenchymal parts of the tooth germ (dental papilla and dental sac), the epithelial components of the developing tooth undergo dramatic changes, influenced by the reciprocal epithelial-to-mesenchymal interactions. Those changes range from initial epithelial budding in restricted regions of oral epithelium with low profile stratification at the onset of tooth development, to morphohistologically distinctive outlook in the bell stage. The enamel organ is ultimately comprised

of various tissues displaying morphological features of simple epithelia (inner and outer enamel epithelium), stratified epithelia (dental crest), loose connective tissue (enamel reticulum) and dense connective tissue (stratum intermedium). Developing embryonic and fetal epithelia usually express a variety of different sets of CKs prior to their maturation into adult epithelia, associated with establishment of permanent and tissue specific CK expression patterns (Lindberg and Rheinwald 1990; Vaidya et al. 2000), which are prone to changes during malignant alteration (Heikinheimo et al. 1991; Lindberg and Rheinwald 1990; Raul et al. 2004). In our study, CK8 was expressed throughout the epithelial tooth part. During progression of development we noticed a population of respective cells expressing CK8 very strongly, which remained only in the dental crest and stratum intermedium at later developmental stages, while they disappeared in the rest of epithelial tooth parts. In the mesenchymal parts of the developing tooth, CK8 was expressed mildly or not at all, with the exception of respective scattered cells, displaying very strong CK8 expression. The only populations of cells which showed increase in CK8 expression from moderate to strong were preameloblasts, while preodontoblasts displayed only weak CK8 expression. The expression pattern of CK8 in epithelial parts of human tooth germ during the investigated developmental stages was certainly more dynamic in intensity, albeit as continuous as those of mesenchymal marker vimentin. During the 14th developmental week, the enamel reticulum and the underlying stratum intermedium differentiated into distinctive structures displaying dramatic changes of cell shape by acquisition of star-like or spindle-shape appearance. However, the outlook of loose and dense connective tissues which characterized those two tooth germ parts was associated with unexpected increase of CK8 in the stratum intermedium, in parallel with increased vimentin expression. This indicates that CK8 (together with vimentin) might have more influence on differentiation of enamel reticulum and stratum intermedium than previously thought. Another region of strong expression of CK8 was detected in future cusp tip area of the inner enamel epithelium (preameloblasts) during the late bell stage. Previous studies on expression of CK8 in the same regions of human tooth germs are confounding due to reporting either presence (Heikinheimo et al. 1989; Kasper et al. 1989) or complete absence (Domingues et al. 2000) of CK8 in those tissues. However, our data demonstrated that previously shown redistribution of simple epithelial CK19 in the preameloblasts (Domingues et al. 2000) is followed by the matching redistribution of CK8, displaying the same intracellular pattern. Therefore, decrease of stratified squamous marker CK14 (Domingues et al. 2000; Tabata et al. 1996) with increased expression of CK8 and CK19

Fig. 5 Co-localization of Ki-67 proliferation marker and syndecan-1 ▶ by double immunofluorescence. **a–d** Human incisor tooth germ in the 14th developmental week: dental crest (*dc*), outer enamel epithelium (*oee*), inner enamel epithelium (*iee*), cervical loop (*cl*), enamel reticulum (*er*), dental papilla (*dp*), dental sac (*ds*), forefront of syndecan-1 expression (*arrows*). **a** Stronger expression of Ki-67 in epithelial parts of the tooth germ in comparison to dental papilla and dental sac. Ki-67 immunofluorescent staining. Magnification: ×10, scale bar 100 μm. **b** Asymmetrically restricted strong expression of syndecan-1 in dental papilla to a region underlying single cervical loop (*thick arrows*). Equally strong expression of syndecan-1 can be seen in cervical loop (*thin arrows*), outer enamel epithelium and portion of enamel reticulum on the opposite side of tooth germ. Inset: details of individual cervical loops on the opposite sides of tooth germ with complete (1) and delayed (2) mesenchymal-to-epithelial shift of syndecan-1 expression. Syndecan-1 immunofluorescent staining. Magnification: ×10, scale bar 100 μm. **c** DAPI staining of nuclei. DAPI immunofluorescent staining. Magnification: ×10, scale bar 100 μm. **d** Merging of A + B + C reveals unilateral co-localization of Ki-67 and syndecan-1 in the enamel organ. Magnification: × 10, scale bar 100 μm. **e–h** Future cusp tip area of the human canine enamel organ in the 20th developmental week: preameloblasts (*pa*), stratum intermedium (*si*), enamel reticulum (*er*), predontoblasts (*po*), dental papilla (*dp*), forefront of syndecan-1 expression (*thick arrows*). **e** Moderate expression of Ki-67 can be observed in the dental papilla. Differentiating cells at the epithelial-mesenchymal interface (preameloblasts and preodontoblasts) do not express Ki-67 weakly. Ki-67 immunofluorescent staining. Magnification: ×40, scale bar 25 μm. **f** Absence of syndecan-1 expression in dental papilla. Apical poles of preameloblasts moderately express syndecan-1 (*thick arrows*). Syndecan-1 immunofluorescent staining. Magnification: ×40, scale bar 25 μm. **g** DAPI staining of nuclei. DAPI immunofluorescent staining, ×40, scale bar 25 μm. **h** Merging of E + F + G does not reveal any co-localization of Ki-67 and syndecan-1. Magnification: ×40, scale bar 25 μm

seem to be the signs of preameloblasts differentiation. Quite the opposite example of CKs expression changes has been reported in differentiating simple epithelium of developing mouse esophagus (Yu et al. 2005), additionally implying specific and important role of CK8 in differentiation of preameloblasts in inner enamel epithelium. Furthermore, we observed moderate expression of CK8 during the late bell stage of canine tooth germ in some cells of dental pulp opposing preameloblasts. Those cells, i.e. preodontoblasts, were undergoing initial differentiation, and the expression of CK8 could be just a brief occurrence heralding withdrawal from the cell cycle. Namely, similar phenomenon has been demonstrated for simple epithelial CK19, which was expressed in rat preodontoblasts during very short period in the late bell stage (Webb et al. 1995), but during further development it quickly disappeared. The very reason for such a short neo-expression of simple epithelial CKs in differentiating preodontoblasts still remains unknown. In contrast to CKs as epithelial markers, vimentin is considered to be a characteristic intermediate filament of mesenchymal (connective) tissues lineage (Kasper et al. 1989). In our study, vimentin expression was observed in both epithelial and mesenchymal tooth parts,
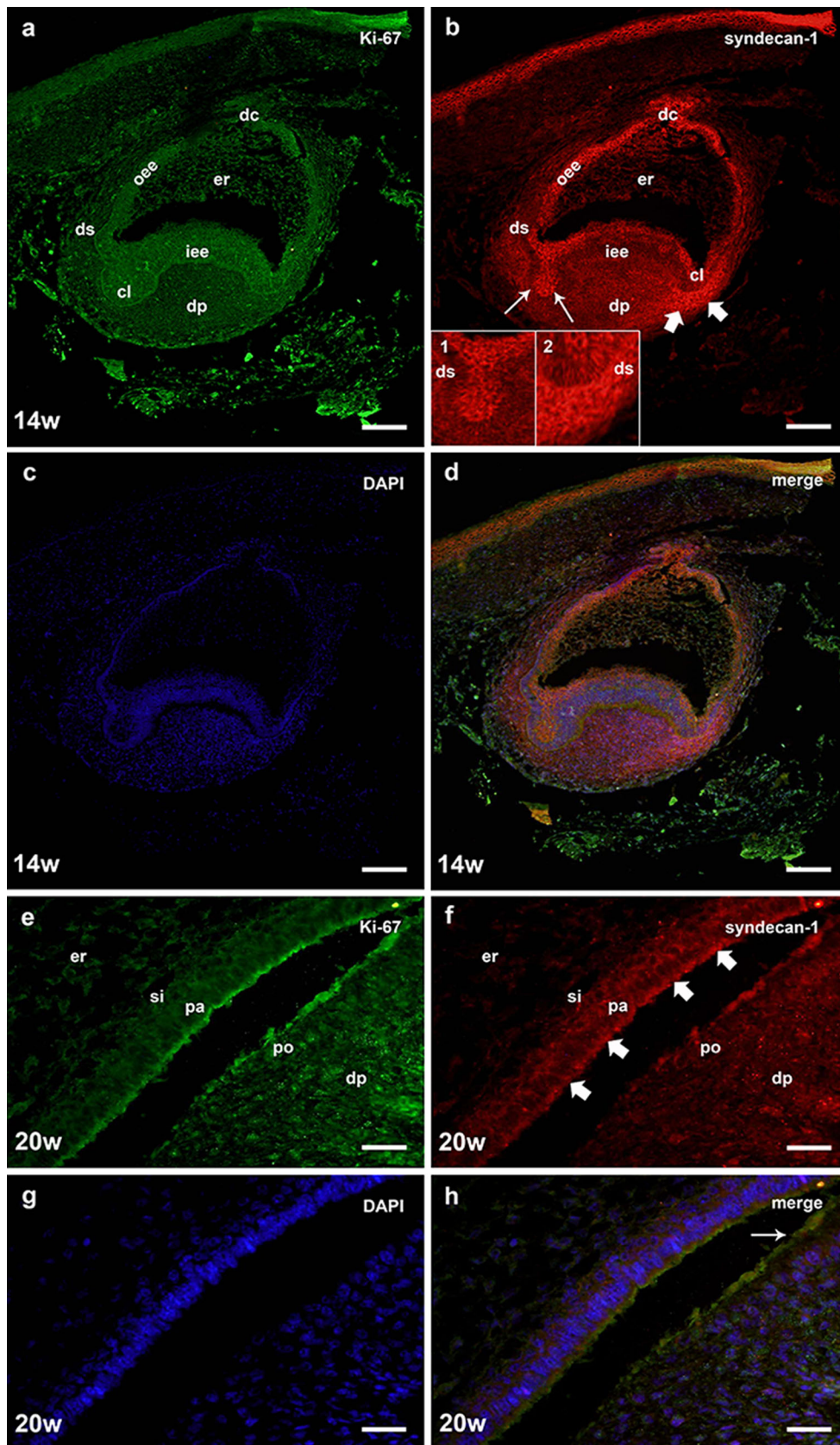
showing tendency to increase during development in the dental pulp and odontoblasts, but in the preameloblasts as well. Co-expression of CKs and vimentin seems to be a common phenomenon that occurs at brief periods of organogenesis with strictly regulated timeline, and it usually hallmarks epithelial-to-mesenchymal or mesenchymal-to-epithelial transformation of developing tissues (Carev et al. 2008; Wang et al. 2014; Vukojevic et al. 2012).

Co-expression of CKs and vimentin is also a feature of human tooth germ development (Heikinheimo et al. 1989; Kasper et al. 1989). What was also striking in our study was that the expression of vimetin was continuously stronger in epithelial than in the mesenchymal parts of the tooth germ. This might confirm the genuine IF pattern signature of embryonic oral epithelium cells which are committed to odontogenic lineage, if cellular properties associated with vimentin IF network such as motility and resistance to shear stress (Mendez et al. 2010) are taken into an account. Of course, both motility and mechanical resistance are critically needed by proliferating cells during the early embryonic development (Viebahn et al. 1988). It was also shown that in cultured epithelial cells, which normally express only CKs, vimentin expression was associated with acquisition of random and directional motility (Ivaska et al. 2005; Mendez et al. 2010). Furthermore, epithelial expression of vimentin has been observed during embryonic development of other ectodermal derivates such as human hair (Schirren et al. 1997), which have similar features such as chemotactically and/or haptotactically directed controlled ingrowth of epithelial tissue into the underlying connective tissue (Aznavoorian et al. 1990). Also, some epithelial cells in marginal areas of dental crest begin to express vimentin as they move away from dental crest, thus initializing its developmentally scheduled disintegration (Buchtova et al. 2012). Therefore, de novo expression of vimentin in epithelial cells might be necessary to propagate their migratory competence. Melanomas, however, offer an interesting example of how simple epithelial CKs might modulate proliferation and invasiveness (Raul et al. 2004), as highly aggressive and invasive types of melanomas strongly express simple epithelia CK8 and CK18 on top of characteristic vimentin expression (Si et al. 1993). Furthermore, differential expression of simple epithelial CKs and vimentin in post-implantation chick (Page 1989) and rabbit (Viebahn et al. 1988) embryos showed that expression patterns of these IFs might be connected to various (mechanical and non-mechanical) cellular functions. This cannot be applied to early mouse embryo, where IF expression patterns are more strictly associated with germ layer origin (Franke et al. 1982). Having these interspecies differences in mind, we shouldn't exclude the possibility that regions of embryonic oral epithelia destined for tooth development might need to express both simple epithelial CKs and vimentin even before the human tooth development begins.

Another important factor in human tooth development is syndecan-1, a cell surface proteoglycan whose expression was repeatedly reported in epithelial and mesenchymal tissues of various organs during development (Mitsiadis et al. 1995). Syndecan-1 seems to play several important roles including fine-tuning of various growth factors' activity (Mali et al. 1993; Su et al. 2007) and consequential maintenance of co-ordinated growth in developing tissues (Vainio and Thesleff 1992), modulation of epithelial-mesenchymal interactions (Vainio et al. 1989), as well as proliferation and differentiation (Hall and Miyake 1995). Reports from extensive research on syndecan-1 during tooth development on experimental animals (Bai et al. 1994; David et al. 1993; Muto et al. 2007; Salmivirta et al. 1991) and recombinant tissues (Vainio et al. 1989; Vainio and Thesleff 1992) are additionally confirmed by the present study. Generally, expression of syndecan-1 during cap and bell stages of human odontogenesis showed constant increase in mesenchymal parts of the tooth germ, followed by the steady decrease in epithelial parts (enamel organ), thus displaying a complete epithelial-to-mesenchymal shift by the late cap stage. Furthermore, increasing expression of syndecan-1 in mesenchyme of dental pulp and dental sac matched increase in the overall proliferation activity and advancement of the cell condensation forefront. However, these trends start to gradually reverse by the end of the late cap stage, shifting syndecan-1 expression back from mesenchymal towards epithelial parts of the tooth germ. An interesting feature of syndecan-1 expression pattern was observed during the early bell stage of human incisor development, when enamel organ begins to attain the definitive shape of incisor tooth crown. Namely, it seems that mesenchymal-to-epithelial shift of syndecan-1 expression does not occur simultaneously throughout the tooth germ. Strikingly similar features of mesenchymal-to-epithelial shift of syndecan-1 expression with matching developmental timetable were described during the mouse incisor and molar odontogensis (Bai et al. 1994), using an antibody against native heparan-sulfate (HS) chains of syndecan-1. Since we used the antibody to human syndecan-1 core protein, we could speculate that different level of modifications of syndecan-1 might be involved during human and mouse tooth development (Dore et al. 1998; Kokenyesi and Bernfield 1994; Su et al. 2007), displaying polymorphism associated with stage of cell differentiation (Brauker et al. 1991; Sanderson and Bernfield 1988). Nevertheless, the asymmetric feature of syndecan-1 expression pattern during the late cap and early bell stage of human and mouse tooth development strongly implies roles of syndecan-1 in determination of tooth crown morphology. Completion of mesenchymal-to- epithelial shift of syndecan-1 expression, followed by the disappearance of its

asymmetric pattern was finally observed in the late bell stage of human tooth development. Along with moderate syndecan-1 expression in apical portions of immature hard tissue secreting cells (preameloblasts and preodontoblasts), its reduction in dental pulp seems to be consistent with progression of mesenchymal cell differentiation.

In conclusion, our study on the developing human tooth germ demonstrated co-expression of CK8 and vimentin primarily in the epithelial tooth part and gradual increase of vimentin expression in mesenchymal tooth parts during progression of development. We suggest association of strong CK8 expression in some tooth germ cells with the onset of proliferation, and strong expression of vimentin with tooth germ cells proliferation and migration. Increase of expression of both intermediate filaments in preameloblasts and preodontoblasts accords with processes of cell differentiation and mesenchymal-to-epithelial transition. Spatiotemporal changes of syndecan-1 expression during odontogenesis were associated with development of crown and cervical loop morphology, and proper differentiation of preameloblasts and preodontoblasts.

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