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Original Article

Coexpression of *Notch3* and *Rgs5* in the pericyte-vascular smooth muscle cell axis in response to pulp injury

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ABSTRACT Recent studies have shown that the pulp of human teeth contains a population of cells with stem cell properties and it has been suggested that these cells originate from pericytes. Molecules of the Notch signaling pathway regulate stem cell fate specification, while Rgs5 represents an excellent marker for pericytes. Pathological conditions such as dental trauma and carious lesion stimulate pulp stem cells to elaborate reparative dentin. Previous studies have shown that genes involved in the Notch pathway are activated in response to pulp injury in rodent and humans. To demonstrate the importance of pericytes as a source of stem cells during dental repair, we have studied *Rgs5* and *Notch3* mRNA expression by *in situ* hybridization in developing, adult intact and injured rodent teeth. Furthermore, we have examined the distribution of Notch3 protein in carious and injured human teeth using immunohistochemistry. Overlapping expression patterns of *Rgs5* and *Notch3* were observed during rodent tooth development as well as immediately after injury. Both genes were expressed in vascular structures during development and in perivascular and single capillary cells of injured teeth. However, the expression patterns of *Rgs5* and *Notch3* were different during tooth repair, with relatively extensive *Rgs5* expression along the pericyte-vascular smooth muscle cell axis in central pulp arterioles. These results show co-expression of *Rgs5* and *Notch3* in pericytes of developing and injured teeth and furthermore indicate the importance of vascular-derived stem cells during pulp healing.

KEY WORDS: *pulp injury, stem cell, tooth, wound healing, vasculature, Notch signaling*

Introduction

Dental injury leads to the initiation of pulp repair through the activation of genes that are involved in stem cell fate determination (Mitsiadis *et al.*, 1999; Lovschall *et al.*, 2005). Formation of tissue patterns during development is somehow reiterated during regeneration of an injured tissue (Thesleff and Tummers, 2003; Martin and Parkhurst, 2004; Mitsiadis and Rahiotis, 2004). Pathological conditions, such as dental injuries and carious lesions, are often lethal to odontoblasts, which are then replaced by new cells that produce a reparative matrix called osteodentin (Fitzgerald *et al.*, 1990). Adult pulp stem cells are the reservoir of reparative cells after dental injury. They proliferate and migrate to the wounded site, where, in cooperation with local cells, participate in tooth repair (Løvschall *et al*., 2007). Stem cells isolated from the dental pulp are capable of forming osteodentin *in vitro* and ex vivo (Shi and Gronthos, 2003; Iohara *et al.*, 2006) and they have been suggested to originate from pericytes (Shi and Gronthos, 2003).

Pericytes are enclosed in the basement membrane of blood vessels and surround endothelial cells. The pericyte coverage of the endothelium is partial depending on the vascular bed (Shepro and Morel, 1993; Rucker *et al.*, 2000). There appears to exist a continuum of phenotypes ranging from the classical vascular smooth muscle cell (vSMC) to the typical pericyte distributed subjacent to the endothelium (Andreeva *et al.*, 1998). Pericytes modulate their phenotype along this pericyte-vSMC axis and several findings suggest they may transdifferentiate into other cell types, including osteoblasts, chondroblasts, fibroblasts, adipocytes (Nehls and Drenckhahn, 1993) and odontoblasts (Alliot-Licht *et al.*, 2001; Shi and Gronthos, 2003).

Abbreviations used in this paper: vSMC, vascular smooth muscle cell.

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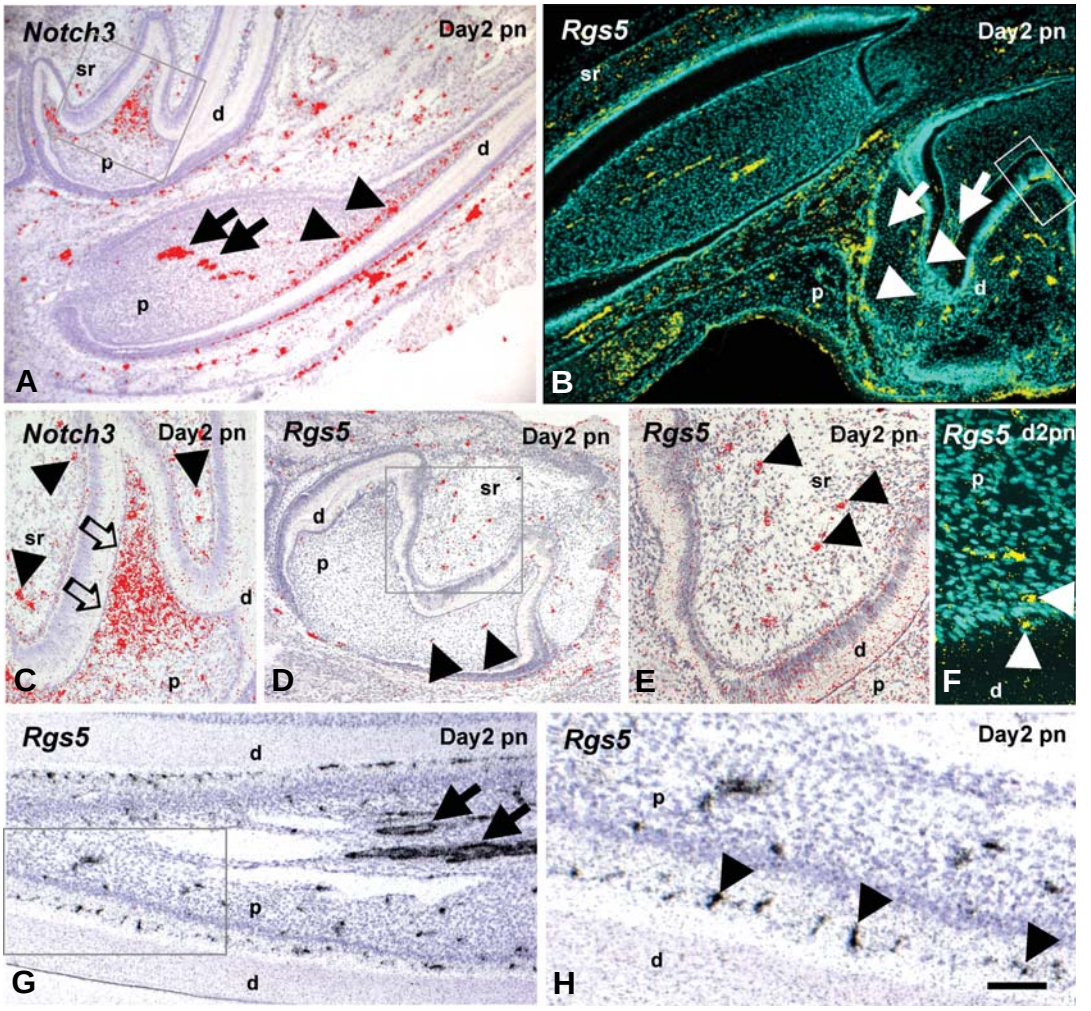

Fig. 1. Expression patterns of *Notch3* and *Rgs5* in developing post-natal mouse teeth. (A-H) *Radioactive* in situ *hybridization on sections of developing mandibular mice molars and incisors at post-natal day two.* **(A,C-E)** *Microphotographs of grains from dark-field superimposed in red color on bright-field (hematoxilin stain).* **(B,F)** *Grains in yellow color superimposed on fluorecence-field (Hoechst stain).* **(G,H)** *Grains in bright-field with black grains on hematoxilin stained background.* **(A,B)** *Overview of the incisor (down/right) and the first molar (up/left).* Notch3 *(A) and* Rgs5 *(B) hybridization signal in central arterioles (arrows) and peripheral capillaries near the odontoblastic layer (arrowheads).* **(C)** Notch3 *expression across the pulp horn (arrows) in developing molar (magnification of boxed area in A) and in pericytes along the blood vessels (arrowheads) that invade the stellate reticulum (sr).* **(D)** Rgs5 *expression in the stellate reticulum and pulp (arrowheads) in the developing molar.* **(E)** *Magnification of boxed area in (D) showing* Rgs5 *expression in pericyte-like locations (arrowheads) along blood vessels invading the mouse molar stellate reticulum after birth.* **(F)** *Magnification of boxed area in (B), showing the* Rgs5 *hybridization signal (arrowheads) along peripheral juxtaodontoblastic capillaries.* **(G,H)** Rgs5 *expression in the mural cells along central arterioles (arrows in G) and in cells along the juxtaodontoblastic capillary tree (arrowheads in H). (H) is a magnification of the boxed area in (G). Abbreviations: p, pulp; d, dentin; sr, stellate reticulum. The bar in (H) represents in A,B,D: 200 μm, C: 80 μm, E: 100 μm, F,H: 50 μm, G: 125 μm.*

Pericyte markers include proteins such as smooth muscle α-actin (SMA), NG2, PDGFR-beta, RGS5 (Armulik *et al.*, 2005) and 3G5 (Shi and Gronthos, 2003). However, the expression patterns of these molecules are tissue and time dependent. None of these markers are absolutely specific for pericytes and none recognizes all pericytes (Armulik *et al.*, 2005). *Rgs5* is the earliest gene which is activated in pericytes during neovascularization (Bondjers *et al.*, 2003). RGS5 protein stimulates GTPase activity, which accelerates G-protein inactivation and thereby inhibits signaling downstream of G-protein-coupled receptors (Anger *et al.*, 2004).

The Notch signaling pathway regulates the fate of stem cells in most tissues and organs (Gray *et al.*, 1999). Notch signaling operates through local cell-cell interactions and is involved in a wide range of developmental processes including odontogenesis (Mitsiadis *et al.*, 1995; 1997; 1998; 2005; Harada *et al.*, 1999; Tummers and Thesleff, 2003), vasculogenesis (Iso *et al.*, 2003), hematopoiesis (Kojika and Griffin, 2001) and formation of skin appendages (Thelu *et al.*, 2002). Notch signaling is reactivated during repair of injured tissues and organs (Mitsiadis *et al.*, 1999; 2003; Lindner *et al.*, 2001; Thelu *et al.*, 2002; Lovschall *et al.*, 2005). Studies on pulp-dentin repair after perforation of adult rat molars have shown activation of the Notch signaling pathway in teeth with both open perforations (Mitsiadis *et al.*, 1999) and pulp capping (Lovschall *et al.*, 2005). These studies have demonstrated that *Notch3* expression is mainly associated with perivascular cells, *Notch1* expression is restricted to pulp cells close to the lesion, whereas *Notch2* in the pulp is expressed much more widely (Mitsiadis *et al.*, 1999; 2003; Lovschall *et al.*, 2005).

Studies on vascular markers potentially involved in determination of pericyte and stem cell fates have become increasingly relevant. The aim of the present study was to demonstrate the importance of pericytes as a source of stem cells during dental repair. For this purpose we have studied *Notch3* and *Rgs5* expression in developing, adult healthy and injured rodent teeth and furthermore Notch3 protein expression in response to carious and traumatic pulp-dentin injury in human teeth both *in vitro* and *in vivo*.

Results

Rgs5 *and* Notch3 *expression in developing mouse teeth*

Hybridization signals using radiolabeled antisense riboprobes were detected for both *Rgs5* and *Notch3* genes on histological sections from developing post-natal mouse tooth germs. In the post-natal day 2 molars, *Notch3* and *Rgs5* transcripts were detected in pericytes of vascular structures of the dental pulp, as well as in the stellate reticulum where blood vessels enter after birth (Fig. 1A-E). A strong hybridization signal for *Notch3* was also localized across the pulp horns. In the incisor pulp, the *Rgs5* and *Notch3*£hybridization signals were found along the inner deep plexus of arterioles and the outer capillary tree (Fig.1A,B,F,G,H).

Rgs5 *and* Notch3 *expression after injury in rat teeth*

The hybridization signals for *Rgs5* and *Notch3* were up-regulated in blood vessel-related structures after pulp injury in adult rat molars (Fig. 2B,C,E,F,H-K,N and Fig. 3) when compared to the intact molars (Fig. 2D,G). The hybridization signals for *Rgs5* and *Notch3* were observed in perivascular locations near and at a distance from the injury site. *Rgs5* and *Notch3* hybridization signals were observed in single cells juxtaposing endothelial cells that correspond to capillary and arteriolar pericytes. *Notch3* expression was up-regulated in vascular structures the first days after the dental lesion (Fig. 2E,F,H-J), but expression decreased in following weeks after surgery (Fig. 3C,E). By contrast, vascular *Rgs5* expression was more extended and maintained in the weeks post-injury (Fig. 3A,B,D,F). *Rgs5* expression was more extensive along the pericyte-vascular smooth muscle cell axis in the central pulp arterioles when compared with *Notch3* expression. Control sections from intact adult molars showed weaker hybridization signals and minor perivascular expression (Fig. 2D,G). The hybridization signals were almost absent in sections using sense *Notch3* and *Rgs5* RNA riboprobes (not shown).

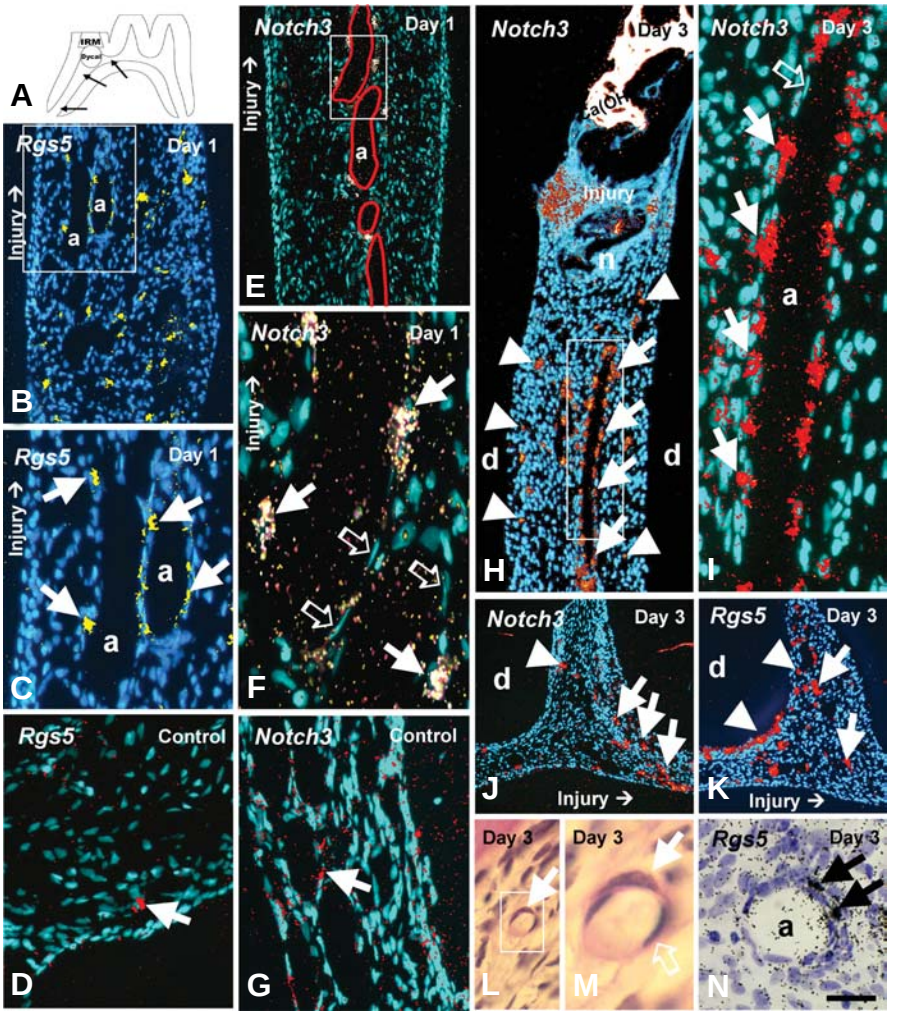

Fig. 2. Expression patterns of *Notch3* and *Rgs5* in injured rat teeth one and three days post-surgery. (A) *Schematic representation of a first adult rat molar after experimental perforation at the mesial cusp. Arrows indicate the regions of interest. The pulp horn was capped after perforation by Dycal and IRM. The mesial side is presented to the left on photomicrographs* **(B-I & L-N)**. **(B-N)** *Radioactive* in situ *hybridization on sections of intact rat molars* **(D,G)**, *injured rat molars at post-operation day one* **(B,C, E,F)** *and post-operation day three* **(H-N)**. *Grains, establishing cell specific hybridization signal, are superimposed as dark-field microphotographs of original white grains in* **(E,F)**, *as yellow color in* **(B,C)** *and as red/orange color in* **(D,G & H-K)** *on fluorescence field (Hoechst stain). Black grains on bright-field microphotographs (hematoxylin stain) are seen in (N).* **(B,C,E,F)** *Central part of the mesial root canal. The direction of injury is indicated by small arrows. Endothelial cells in F are indicated with open white arrows, hybridization signal in pericytes with white arrows.* **(B,C)** Rgs5 *hybridization signals (yellow color) are evident in pericytes around central arterioles (a).* **(E,F)** Notch3 *is also expressed in pericytes (white color) around central arterioles (outlined with a red line in E). Boxed areas in (B,E) represent magnifications in (C,F) respectively.* **(D,G)** Rgs5 *and* Notch3 *expression (white arrows) in intact molars.* **(H,I)** *Central and upper part of the mesial root canal including the injury and necrotic (n) areas.* Notch3 *expression in pericytes (white arrows) around the central arteriole (a) in close contact with endothelial cells (open white arrow).* Notch3 *is also expressed in single cells along juxta-odontoblastic capillaries (arrowheads). Boxed area in (H) represents magnification in (I).* **(J,K)** *Central coronal pulp, the injury (small arrows) to the right. Similar expression patterns for* Notch3 *in (J) and* Rgs5 *in (K) are detected in pericytes (white arrows) around arterioles and in cells along juxta-odontoblastic capillaries (arrowheads).* **(L,M)** *Coronal pulp area (PAS stain). Boxed area in (L) represents magnification in (M), where endothelial cells (open white arrow) are in part covered by pericyte (white arrow).* **(N)** Rgs5 *expression in pericytes (black arrows) around a coronal arteriole (a) (hematoxylin stain). Abbreviations: a, arteriole; d, dentin; n, necrotic pulp area. The bar in (N) represents in (B,E): 100 μm, C,D,G,L: 50 μm, F: 20 μm, H: 120 μm, I,N: 40 μm, J,K: 200 μm, M: 15 μm.*

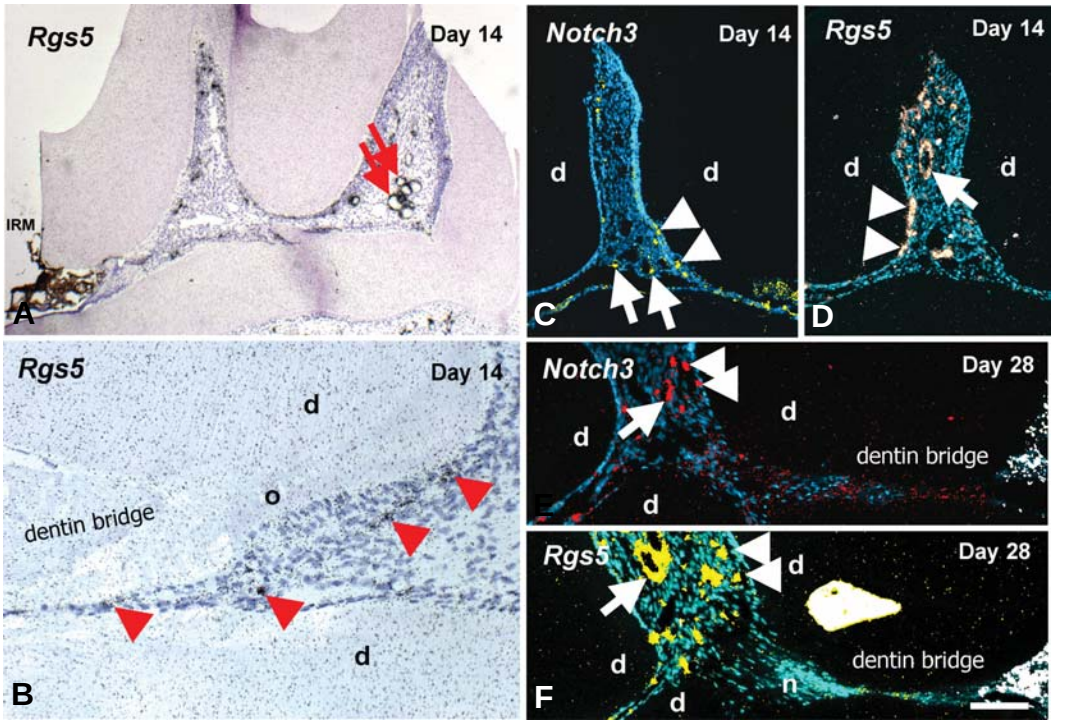

Fig. 3. Expression patterns of *Notch3* and *Rgs5* in injured rat teeth 14 and 28 days post-surgery. *The mesial injury is represented to the left in (A,B) and to the right in (C-F).* **(A-F)** *Radioactive* in situ *hybridization on sections of injured rat molars at post-operation day 14* **(A-D)** *and post-operation day 28* **(E,F)**. **(A,B)** *bright field with original black grains (hematoxylin stain).* **(C-F)** *Fluorescence field with grains in yellow, white or red color superimposed (Hoechst stain).* **(A)** *The cavity with IRM and* $Ca(OH)_2$ *to the left. Specific* Rgs5 *is expressed in the central pulp arterioles along the pericyte-vSMC axis (arrows).* **(B)** *Dentin bridge to the left with polarized bright field photo superimposed.* Rgs5 *expression in single cells along newly formed juxta-odontoblastic capillaries (arrowheads).* **(C,E)** Notch3 *expression along central arterioles (arrows) and in single cells of the peripheral capillaries (arrowheads).* **(D,F)** Rgs5 *expression mainly in the pericyte-vSMC axis (arrow) and in single cells of the peripheral capillaries (arrowheads). Dentin bridge area to the right* **(E,F)**. *Abbreviations: d, dentin; n, necrotic pulp area; o, odontoblasts. The bar in (F) represents in (A,C,D): 150 μm, B: 75 μm; E,F: 100 μm.*

Notch3 protein expression in injured, carious and cultured human teeth

Immunohistochemistry, by using an antibody that recognizes the intracellular domain of the Notch3 protein, showed a similar expression pattern of the Notch3 protein in vascular structures of pathological human teeth. In injured teeth, nine weeks after cavity preparation ('drilling'), as well as in teeth with advanced carious lesions, the Notch3 immunostaining was observed in the walls of dilated blood vessels (Fig. 4A-D). Staining was also detected in isolated pulp cells of carious teeth (Fig. 4D). In cultured human tooth slices, Notch3 staining was intense in cells of the putatively new-formed vessels (red arrows; Fig. 4E,G). Notch3 protein was also detected in odontoblasts of cultured tooth slices (green arrows; Fig. 4E,F).

Discussion

The present study demonstrates that expression of *Notch3* and *Rgs5* is activated in response to dental injury. *Notch3* and *Rgs5* were strongly expressed in the pericyte-vSMC axis of the vascular wall both in the developing and in adult injured teeth. *Notch3* expression was highly activated as an early response to pulp injury and expression decreased with time during pulp healing. *Notch3* was expressed in single cells of vessels juxtaposing endothelial cells that correspond to capillary and arteriolar pericytes, which are localized either close to or inside the odontoblast layer (Josephsen *et al.*, 1974). These results are in agreement with recent findings demonstrating a similar expression pattern of *Notch3* expression in pericytes of the retina (Claxton and Fruttiger, 2004). Activation of *Notch3* expression during tooth repair might be important for the regulation of the fate of pericyte-derived stem cells. It has been shown that the Notch signaling pathway is essential for stem cell fate regulation and appropriate differentiation of many cell types during development (Lewis, 1998).

Notch receptors and ligands are involved in a variety of pathological conditions (Gridley, 2003), including dental pathology in humans (Mitsiadis *et al.*, 2003) and rodents (Mitsiadis *et al.*, 1999; Lovschall *et al.*, 2005). In injured teeth, two of the Notch ligands, *Delta1* and *Jagged1*, are expressed in small groups of vascular and perivascular cells (Mitsiadis *et al.*, 1999; Lovschall *et al.*, 2005). The present findings are in agreement with the concept that activation of the Notch signaling pathway plays a central role during tooth repair and neovascularization. Both *Notch3* transcripts and protein were upregulated in vascular structures of the injured and carious teeth. However, in cultured human tooth slices Notch3 protein was also upregulated in odontoblastic cells. Potentially, these cells are not able anymore to maintain the odontoblastic fate and they start to dedifferentiate, adopting thus another fate, or die. Another possible explanation is that the Notch3 protein is expressed in pericytes, loses its extracellular domain by proteolysis in response to the injury and retains the intracellular domain in cells that finally differentiate into odontoblasts. The upregulation of *Notch3* in vascular structures during pathological conditions suggests that signaling through the Notch3 receptor play a pivotal role in the control of vascular-derived cell fates. A similar role could be suggested for Notch3 during tooth development, where the present and previous studies have showed that *Notch3* expression is mostly correlated with cells located in the walls of blood vessels in continuously erupting

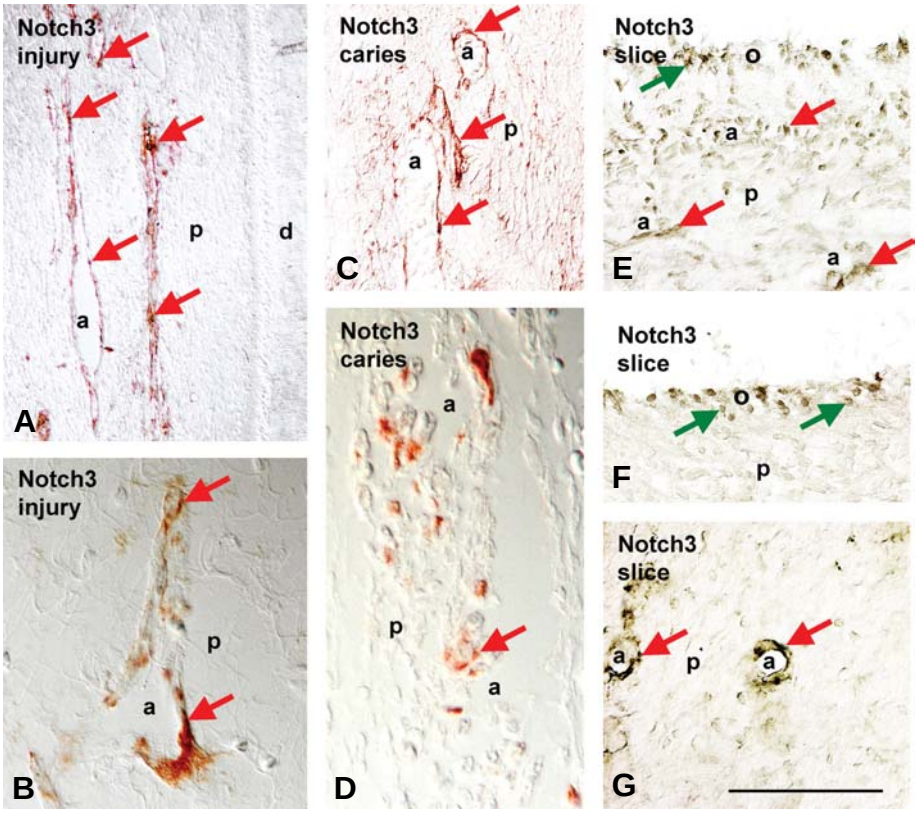

Fig. 4. Distribution of the Notch3 protein in injured, carious and cultured human teeth. *Immunodetection of the intracellular Notch3 domain in injured* **(A,B)**, *carious* **(C,D)** *and cultured* **(E-G)** *human teeth.* **(A,D)** *Notch3-positive cells (red color) around the arterioles (a, arrows). Staining is also observed sporadically in pulp cells.* **(E-G)** *In cultured slices of intact human teeth, Notch3 protein is observed in odontoblasts (green arrows in E,F) and in putatively new-formed arterioles (a) (red arrows in E,G). Abbreviations: a, arteriole; d, dentin; o, odontoblasts; p, dental pulp. The bar in (G) represents in (A,C): 50μm, B,D,E,F,G: 30 μm.*

mouse incisors and vole molars (Tummers and Thesleff, 2003).

In developing and injured teeth, *Rgs5* was expressed in capillary walls and in single cells around central arterioles, which often were observed juxtaposing endothelial cells. The *Rgs5* expression extended from pericytes to the vascular smooth muscle cells in the tunica media. Pericytes may change their phenotype along the pericyte-vSMC axis (Nehls and Drenckhahn, 1993; Andreeva *et al.*, 1998). Previous reports have suggested RGS5 as a marker for pericytes (Bondjers *et al.*, 2003; Cho *et al.*, 2003), vSMCs (Li *et al.*, 2004) and activated pericytes during wound healing and vascular remodeling (Berger *et al.*, 2005). The present results show that *Rgs5* and *Notch3* are co-expressed in the pericytes and along the vSMC axis. RGS proteins are regulators of the G-protein and have been implicated in the control of chondroblast (Appleton *et al.*, 2006) and osteoblast (Thirunavukkarasu *et al.*, 2002) differentiation, as well as in the differentiation of mural cells during embryonic vascular maturation (Cho *et al.*, 2003) and peripheral artery function (Li *et al.*, 2004). Notch3 is crucial for tissue homeostasis since mutation of this gene leads to CADASIL, a systemic disease in the arterioles (Brulin *et al.*, 2002). Recent findings have shown that Notch3 is involved in the specification and control of arterial identity during angiogenesis (Claxton and Fruttiger, 2004; Armulik *et al.*, 2005).

The present results suggest that Notch3 is also involved in cell fate regulation during dental pulp remodeling. It has been suggested recently that residual *Rgs5* expression may be present in sporadic and rare pericytes, which might adopt a non-vascular cell fate (Bondjers *et al.*, 2003). During pulp repair, *Notch3* and *Rgs5* expression in the healing pulp is correlated with sporadic cells of the vascular structures, but not with proliferating and migrating new cells that border the injury interface during repair (Feit *et al.*, 1970; Fitzgerald, 1979; Dahl, 1983; Fitzgerald *et al.*, 1990). *Notch3* and *Rgs5* were frequently expressed in single cells distributed along this vascular tree. Up-regulation of *Notch3* and *Rgs5* expression in pericytes is seen around vessels near and distant from the injury site, including new invading vessels, as well as during tooth repair with increased expression around vessels close to the injury site. In few cases *Notch3* and *Rgs5* expression was down-regulated around the wounded area, indicating that tooth perforation and pulp capping may occasionally occlude and impair the adjoining microvascular system. *Notch3* and *Rgs5* hybridization signals were not expressed in cells that border the injury interface during repair and prior to neovascularization.

Dental injury stimulates recruitment of cells that differentiate into odontoblast-like cells and possess a reparative capacity (Fitzgerald *et al.*, 1990). The environment in the granulation tissue and proliferation phase of pulp healing provides a niche, which allows for generation of odontogenic progeny from the stem cells. Recent studies based on *in vitro* isolation and transplantation of STRO-1 positive pulp cells have suggested that pericytes may be odontogenic precursors (Gronthos *et al.*, 2004). Our studies indicate that *Notch3* and *Rgs5* expression are involved in regulation of the fate of pericytes or recruitment of new cells during angiogenesis and neovascularization. Dental injury is expected to provide networks of complex epigenetic signals (Mitsiadis and Rahiotis, 2004; Løvschall *et al.*, 2007), including cascades of cytokines, redistribution of extracellular matrix molecules and growth factors (Silva *et al.*, 2004) and putative homotypic or heterotypic cell-to-cell interactions (Thesleff *et al.*, 1996; Mitsiadis and Rahiotis, 2004), which may influence the fate of vascular-derived dental stem cells.

Materials and Methods

Animal experiments

Experiments were approved by the Danish Experimental Animal Board. Teeth of two-months old male Wistar rats (Møllegaarden, Eiby, Denmark) were disinfected and mesio-buccal pulp horn in first upper molars were perforated as previously described (Lovschall *et al.*, 2001). Pulp capping was made using hard setting calcium hydroxide (Dycal Cement, LD Chaulk Company, Del, USA). Cavities were filled with IRM-

cement (Dentsply, DeTrey, Germany) (Figure 2A).

Processing of tissues

For *in situ* hybridization analysis, experimental and control rats were anaesthetized and sacrificed 1, 3, 7, 14 or 28 days after pulp treatment by vascular perfusion fixation with pre-rinsing in saline. For perfusion fixation, 4% paraformaldehyde (PFA) in PBS at 4°C was used for 10 min, followed by over-night immersion fixation of the rat molars and incisors (Lovschall *et al.*, 2002). Developing teeth from postnatal mice were immersed in the same fixative. All specimens were decalcified in 12.5% EDTA (ethylenediamine tetraacetic acid) containing 2.5% PFA for 4-6 weeks, dehydrated, embedded in paraffin and serially sectioned at 6 μm (Lovschall *et al.*, 2005). Hoechst 33342, hematoxylin, or PAS was used as background stain.

Probes and* in situ *hybridization

In situ hybridization on paraffin sections was performed as described previously (Vainio *et al.*, 1993). The *Rgs5* and *Notch3* plasmids that were used for making the ^{35}S-UTP-labeled riboprobes were kind gifts from Dr Christer Betsholtz (Karolinska Institute, Sweden) (Cho *et al.*, 2003; Bondjers *et al.*, 2003) and Prof. Urban Lendahl (Karolinska Institute, Sweden) (Larsson *et al.*, 1994) respectively. The plasmids were transformed into *Escherichia coli* TOP10 (Invitrogen) and plasmid DNA was purified using the Plasmid Maxi Kit (Qiagen). For preparing the antisense and sense (for control experiments) riboprobes the plasmid DNA was linearized by cleaving with restriction enzymes prior to labelling with [35S]-UTP by using T3 or T7 polymerase. Fluorescence-, dark-, or bright-field microscopical fields were photographed digitally to capture the full dynamic range of image without exceeding the capacity of the camera (Axiocam MRc5) in similar locations of section. Grains from the dark-field were selected, colored and added to the bright-field pictures in Photoshop CS.

Permanent intact, carious and injured human teeth

The human teeth used in this study were mature, intact, carious, or injured teeth. Cavity preparation in intact first premolars scheduled for extraction was performed as previously described (Heymann *et al.*, 2002). The cavities were restored with IRM. After a post-operative interval of 9 weeks, the teeth were extracted with the patient's informed consent. Teeth were fixed in 10% neutral-buffered formalin for 24 hours, demineralized in sodium formiate for 3 weeks and then embedded in paraffin wax. Teeth were serially sectioned (6 μm thick sections) and then processed for immunohistochemistry.

Culture of human dental slices

Human premolars and molars extracted for orthodontic reasons were cut into 750 μm slices. These slices were cultured as previously described (Mitsiadis *et al.,* 2003).

Antibodies and immunohistochemistry

Rabbit antiserum against the intracellular domain of the mouse Notch3 protein (Mitsiadis *et al.,* 1999) were used. Vector Vectastain ABC kit with a biotinylated secondary antibody and peroxidase-conjugated avidin was purchased from Biosys (Compiègne, France). Immunohistochemistry on paraffin sections was performed as previously described (Mitsiadis *et al.,* 2003).

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