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# Molecular study on gene and protein expression analysis of TGF beta1 - An in vitro model to develop drugs towards dentin tissue remodelling

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## ABSTRACT

**Introduction:** TGF-1–3 are one-of-a-kind multifunctional growth factors.TGF-beta1 is active in the reconstruction of the dental pulp tissue, according to recent reports. Furthermore, dental pulp cells can divide into odontoblast-like cells, which will then develop reparative dentine.

**Materials and Methods:** DEMM were used to culture and preserve 3T3 cells. The cells were incubated with LPS after differentiation. 3t3 Cells were used to extract total RNA. It was necessary to amplify cDNA. Using specific reagents, Rt PCR was used to measure the genes of interest.

**Results:** TGF-beta1 mRNA has a role in protein expression in lipopolysaccharide (LPS) induced macrophage-like cells.TGF-β signals are largely related to the magnitude and duration and careful regulation of their ligand activation, both temporally and spatially. If appropriately activated, TGF-β signalling plays an important function in tissue remodelling.

**Conclusion:** TGF-beta1 mRNA has a role in protein expression in lipopolysaccharide (LPS) induced macrophage-like cells.TGF-β signals are largely related to the magnitude and duration and careful regulation of their ligand activation, both temporally and spatially. If appropriately activated, TGF-β signalling plays an important function in tissue remodelling. The evidence suggests that hVEGF has a therapeutic potential in regeneration and other disorders of the pulp.

**Keywords:** *Gene expression,Protein expression,TGF beta1, Dentin tissue remodelling*

## INTRODUCTION

The precision time-spatial expression of glycoprotein-based bioactive molecules referred to as growth factors (GF) controls the crosstalk from the epithelial to the mesenchymal germ layers, contributing to the distinction between the odontoblast. Terminally specialised hard tissue is formed by the odontoblast. These cells are responsible for the dentine extracellular

matrix (DECM) secretion, as well as the reconstruction of the dentine extracellular matrix (DECM). The GF fossils in the tissue matrix become a rich source of bioactive molécules after dentin mineralisation.To develop a multicellular organism into ever more complex life forms, coordination and control between individual cells must be established to ensure that the organism remains in order.(1)(2) Fundamental physiological processes, such as replication, differentiation, metabolism and apoptosis, are regulated by a thick, cytokine generating signal network, growth

factors or polypeptide hormones.(3)The family of transforming growth factor-β (TGF-β) is especially significant among the polypeptide/hormone-induced signals. (4,5)

TGF-β 1–3 is a rare multi-functional growth factor since it is only found in mammals that are primarily latently secreted and retained in the extracellular matrix immediately (ECM). Only after ligand activation can the biological activity of a TGF-β be provided.(6) Most notably, activation of TGF-β is required to recruit stem/progenitor cells for tissue regeneration/remodelling at the right time.(7)(8)Our knowledge of these mechanisms, it is essential for the characterisation of molecular and cellular events necessary for dental tissue injury and recovery. However, there is no specific molecular basis for differentiation in odontoblast but signalling molecules have been involved in controlling aspects of tooth growth and tissue repair, particularly those in the superfamily transforming the growth factor-beta (TGF-b) .The involvement of TGF-b receptors I and II is observed by odontoblasts and dental pulp cells, which could modulate the activity of the dental pulp and odontoblast. TGF-b1 is also used to improve the formation of reparative dentine in rat molars as a pulp-capping medicine. (9)The regulation of TGF-b1 in dental pulp cells is not well understood, however, as a response to external stimuli.

Vascular endothelial growth factor (VEGF) is a glycoprotein that has the capacity to improve microvascular permeability.(10) In rheumatoid arthritis, delayed hypersensitivity, salivary glands, corpus luteum, and endometrial cells, VEGF has been identified. VEGF has recently been linked to the initiation and progression of gingivitis to periodontitis.(11)(12)VEGF-A (also known as VEGF) is a member of the VEGF family of growth factors that regulates its effects by binding to its primary receptor, vascular endothelial growth factor receptor-2 (VEGFR2). VEGF promotes endothelial cell (EC) proliferation, increased pulpal blood flow, and capillary hyperpermeability when bound to the receptor. VGEF influences other angiogenic factors and is released mainly by EC's, pulp inflammatory cells and fibroblasts. Several inflammatory mediators, including prostaglandins, interleukin-6, and interleukin-1, have been found to induce VEGF expression in the dental pulp.(13–17)(15,18)

The broad-scale profiling of pulp and odontoblasts gene expression makes it possible to gain an overview of the cellular processes during health and disease in these tissues. It also reveals possible biomarkers of pulp protection potential for diagnosis and estimation and allows identifying potential treatment target proteins.

## MATERIALS AND METHODS

### *Procurement and culture of cell line*

The NCCS Pune, India and cultivated 3T3-L1 cells were derived in 5% CO2/95 percent of humidified air, at 37 NCC. The cells had been held at 25 mM HEPES, 100 U / mL (100 U/ m L; 100 μg / mL) and 10 percent calf serum, in the Dulbecco Eagle Modified Medium (DMEM). The cells were segregated in 10% Fetal Bovine Serum (FBS), 0.25 μM dexamethasone, 0.5 mM 3-isobutyl 1-methylxanthine, 5 μg/mL insulin, 2 days after confluence (daily 0) in DMEM, and 10% FBS/DMEM with 72 h insulin. Cells were incubated for 10 per cent FBS/DMEM. Finally, every 2 days 10% of FBS/DMEM is updated.

### *Cell treatment of T3-L1*

On the eighth day Following differentiation, the colonies of cells were pretreated for 24 hours with a 0.1 mM alliin-containing medium (Sigma Chemical Co.). Cells grown in the same environment (100 ng/mL) were then incubated for 1 hour prior to harvesting. The medium

cultivated was gathered and deposited in pipes at -80 C.

***Analysis of gene expression by Rt PCR***
***tRNA Isolation***

The Total RNA extracted with the TRIR package (total RNA reagent insulation) from 3T3 cells. In short, cells with 1 ml TRIR were sonicated, and homogeneous material was immediately moved to the microfuge tube at –80 ° C for 60 minutes such that nucleoproteins could be dissociated fully.Add 0,2 ml chloroform, whirl for 1 minute, and drop it on ice for 5 minutes at 4°C. Centrifugation of homogenates took place at 12000 x g for 15 min at 4°C. In a fresh microfuge tube, an equivalent amount of isopropanol has been carefully transported, spurred for 15 sec and placed on ice for 10 minutes at 4 degrees Celsius.At 12,000 g x 10 min at 4°C, the samples were centrifuged. RNA pellets have been washed with 1 mL of 75% ethanol by vortexing and subsequent centrifuge for 5 minutes at 7.500 x g (4°C). Supernatant has been discarded. The supernatant was separated, and the RNA pellets were dissolved in 50 l of autoclaved Milli-Q water by heating in a water bath for 10 minutes at 60°C.

***RNA Quantification***

The absorbance (A) at 260/280 nm of a diluted RNA sample was measured spectrophotometrically.One absorbance at 260 nm is produced by 40 g of RNA in 1 ml. As a result, the concentration of RNA in a particular sample may be calculated by multiplying its A260 by 40 and adding a dilution factor. The purity of an RNA preparation may be estimated by dividing its absorbance at 260 and 280 nm by its absorbance at 260 and 280 nm. A 260/280 nm absorbance ratio greater than 1.8 is called high quality RNA.The received RNA purity was 1.8.

***Reverse Transcriptase – Polymerase Chain Reaction (RT – PCR)***

RT-PCR is a method for translating and Increasing the number of copies of a single-stranded RNA template into a large amount of double-stranded DNA.

1.First strand reaction: Using OligodT, dNTPs, and reverse transcriptase, complementary DNA (cDNA) is synthesised from the mRNA template.

2. Following the reverse transcriptase reaction, normal PCR (also known as the "second strand reaction") is started.

***Principle***

RT-PCR is a technique for amplifying cDNA copies of RNA. Enzymatic translation of mRNA into a single cDNA template. An oligodeoxynucleotide primer is hybridised to the mRNA and then expanded by an RNA-dependent DNA polymerase to create a cDNA clone.

***First strand DNA synthesis***

The RT kit was purchased from Eurogentec (Seraing, Belgium).

***Procedure***

The total reaction volume of 20 µl contains the following.

| Components | Volume for 20 µl reaction |
|---|---|
| 10 X RT buffer | 2 µl |
| 25 mM MgCl2 | 4 µl |
| 2.5 mMdNTP | 4 µl |
| OligodT | 1 µl |
| RNase Inhibitor | 0.4 µl |
| Euroscript RT | 0.5 µl |
| RNase free water | Varies according to RNA template volume |
| RNA Template (2 µg) | Varies |

The tubes were mixed gently and spun briefly and kept in the thermocycler programmed as initiation step at 25°C for 10 min, Reverse transcriptase step at 48°C for 30 min, inactivation of RT enzyme step at 95°C for 5 min. After the reaction, samples were stored at -20ºC or proceeded to the PCR.


**FIG 1:** RNA Conversion Kit

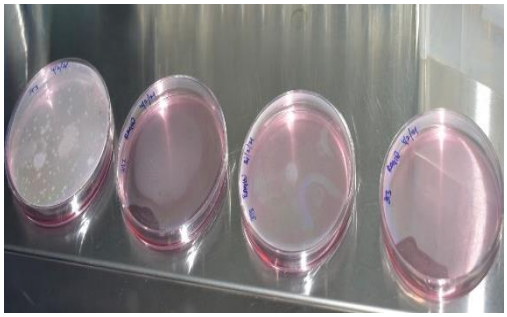

**FIG 2A:** Cell seeding

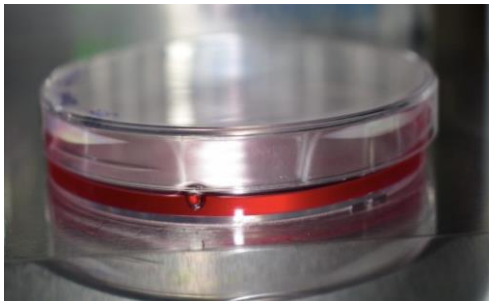

**FIG 2B:** Cell culture plates

***Quantitative Real-Time PCR***

***Principle***

The objective of a PCR is to produce a high number of transgene. The Polymerase Chain Reaction. A PCR consists of three main stages, which are as follows: 3 minutes of denaturation at 94°C: Both enzymatic reactions cease as the double strand of DNA breaks, producing single-stranded DNA. at 94°C for 2-5 minutes. Apply for 30 sec at 54°C-65°C.To ensure the extension phase, ionic bonds consistently form and break between the basement and the individual stranded template.Extension at 72°C for 30 sec: Primers which are in positions that don't match exactly (due to the higher temperature) and don't extend the fragment. The foundations (complementing the template) are combined with the 3' sides first (polymerase adds 5' to 3' to dNTP, reading 3' to 5' on the side of the template; the basis is applied in addition to the template). Due to the copying of two strands during the PCR, the number of copies of the gene increases exponentially.


**FIGURE 3:** Centrifuge

1.2X Reaction buffer: The PCR master mix kit was purchased from Takara Bio Inc., Japan. Contains TaKaRa Ex Taq HS (a hot-start PCR enzyme) dNTP Mixture, Mg2+, TliRNase H (a heat-resistant RNase H that reduces the inhibition of PCR by residual mRNA), and SYBR Green I.

2. Forward primer (10µM)

3. Reverse primer (10µM)

4.cDNA- Template

5.Autoclaved milli Q water

6. Primers: The following gene-specific oligonucleotide primers were used.

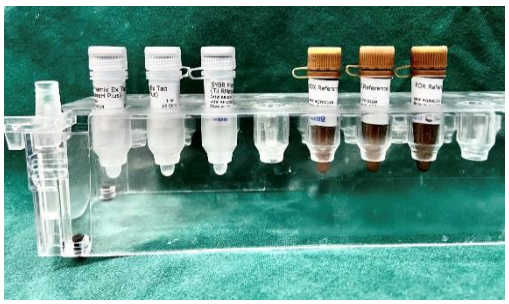

**FIGURE 4:** Reagents used

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**FIGURE 5:** Rt-PCR

## RESULTS

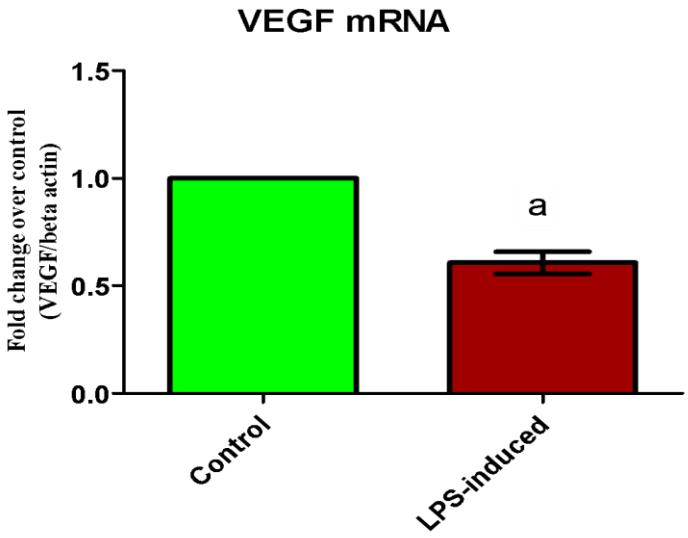


**FIGURE 6:** Effect of TGF beta1 on mRNA expression of VEGF.The mRNA expression levels were determined using RT-PCR.. Each bar represents mean ± SEM (n=6). Significance at P <0.05, Significantly different from untreated 3t3 fibroblast cells.

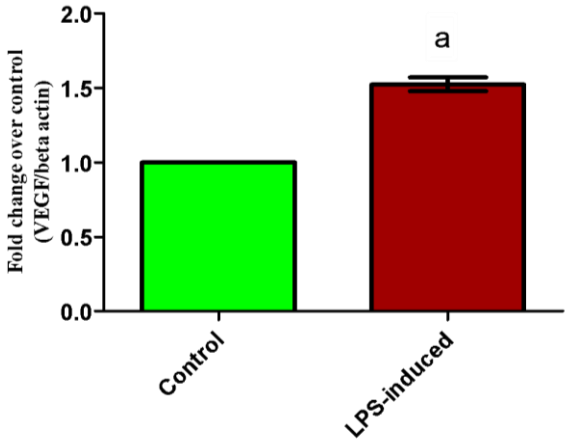


**FIGURE 7:** Effect of LPS induced cells on mRNA expression of IL-1β. The mRNA expression levels were determined using RT-PCR.Each bar represents mean ± SEM (n=6). Significance at P <0.05, aSignificantly different from untreated 3t3 fibroblast cells.

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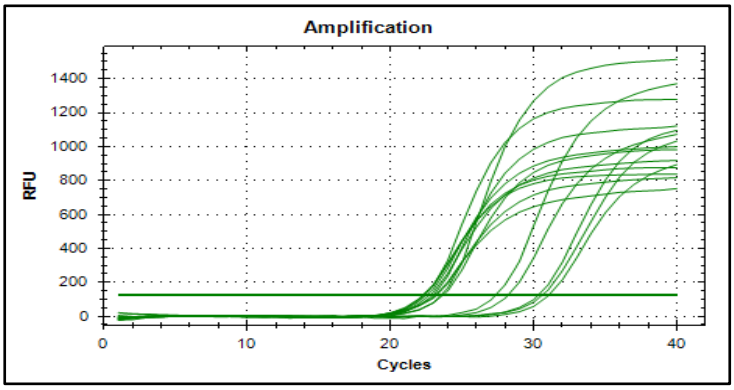


**FIGURE 7: Amplification** plots showing mRNA expression of Il-1β in LPS-induced 3T3-fibroblast cells

**TABLE 1:** Effect of LPS induced cells on mRNA expression of IL-1β.Statistical analysis done using unpaired t-test,shows a mean of 0.523±0.04 Significance at P <0.05.

| | | |
|---|---|---|
| Mean | 1.000 | 0.523 |
| Std. Deviation | 0.000 | 0.08083 |

**TABLE 2:** Effect of LPS induced cells on mRNA expression of VEGF-1 .Statistical analysis done using unpaired t-test,shows a mean of 0.6067±0.09 Significance at P <0.05

| | | |
|---|---|---|
| Mean | 1.000 | 0.06067 |
| Std. Deviation | 0.000 | 0.09018 |

## DISCUSSION

Odontoblasts are the cells that form dentine and provide the teeth with a natural immunological barrier. (19) Odontoblasts have as their major purpose the synthesis and secretion of collagen, along with other non-collagenous proteins that constitute the dentin biological matrix. By influencing the composition of the cell matrix and the inflow of odontoblasts, mineral ions govern the mineralization of the dentin matrix. (20) Odontoblasts are the secretive elements of a dentine matrix that contain a storage of physiologically active molecules, such as platelets, endothelial growth, fibroblasts, metalloproteinases as well as TGF-β super-family members.

The diffusion of bacterial metabolic acids during cavities may solubilize such bioactive compounds from the dentine matrix's soluble tissue body and expose them to the insoluble fabric body. Further bacterial proteolytics may help to and maybe control this process at more advanced phases of cavities. Furthermore, these chemicals establish a healing process that provides chemotactic indications for recruiting inflammatory cells and undifferentiated pulp cells to the damage area, stimulates angiogenic reaction and initiates tissue mobility for repair thereafter. (21,22) Releasing TGF-β from the dentin matrix will need its diffusion to the pulp cells for signalling events, predominantly through dentinal tubules. A number of factors may affect the agent's capacity to engage in such occurrences, e.g. its release into biologically active form, its contact with the dentinic material in solubilised form or by immobilising the insoluble matrix, and the dentinal tubule diffusion distance.

The vascular system of the body serves the vital tasks of nutrition supply and disposal of waste materials for cells and tissues. In chronic and acute inflammations such as pulpitis, vascular

permeability is dramatically enhanced (23,24). Endotoxins generated by caries promoting bacteria help in dental pulp cells' synthesis of VEGF, (25)and VEGF is a crucial regulator for pulp damage, which leads to increased permeability of the vascular tissues and angiogenesis. The results of this study show that VEGF is capable of supporting in vitro and in vivo differentiation and proliferation of pulpal tissue cells.

Much TGF-β in the dentine matrix seems to be active and is linked to the components of the dentine matrix, which can control its availability and biodiversity. (26) A research by Lucchini et al;(27) has shown that differentiated odontoblasts produce and place TGF-β in the dentine matrix, which can react, permitting autocratic action modalities. Detection on odontoblast or other pulpal cells of receptor TGF-β I and II reveals the existence of an active signal complex on those cells for signal transduction. During the carious demineralisation, the target cells for signalling molecules will change, but odontoblast will be of key relevance in terms of healing. (24)TGF-β commences its effects with the signal pathway Smad by engaging two distinct subtypes of serine/threonine kinase receptors killed in odontoblast and pulp cell types Type I and Type II. (28) The type I receptor produced by Ligand-I receptor activation leads to type I receptor kinase activating, which are phosphorylated in turn by receptor-controlled Smads 2/3.

The phosphorylated Smad2/3 will then connect Smad4, an ordinary Smad mediator. These heteromeric complexes are sent to the nucleus where gene transcript is regulated by either DNA binding protein association or by a direct binding to enhance gene sequences. (29,30) Inhibitors Smad6 and Smad7 belong to the Smad's inhibitor and operate as TGF-β inhibitors, maybe by competitive contact with the type I or Smad4 receiver. (31) The solubilized TGF-βs may spread to odontoblast and pulpal cells located underneath a damaged region during reactionary dentinogenesis and indicate an up-regulation of the secretive activity of those cells(32,33)This activation of the odontoblasts survivors will cause new matrices to be focally secreted from the pulp-dentin contact. (34)

A few investigations have shown that TGF-β increases extracellular matrix production and also causes in vitro (35) and in vivo odontoblastic cytodifluency. (35,36) In an in vivo investigation using transgenic mice overexpressing TGF-β2 of DenBesten et al.;(37) their mineral dentine was increased in comparison with their fungal litterates. It was demonstrated that TGF-β2 was present in mature dentin; hence TGF-β2 also appears to enhance odontoblast development in the matured dentin, hence increasing the rate of dentine apposition in minerals. (38) An in vitro work by Sloan and Smith41 has shown that in the region immediately surrounding agarose beads containing those growth factors, TGF-β1 and TGF-β3 enhance predentin thickness of the cultivated tooth. The localisation of predentin thickness shows reactive dentinogens. TGF-β also plays a major part in module soft and hard tissue regeneration after a carious lesion, which increases the development of remediated dentine. The production of reparative dentine is caused by the recruitment and multiplication of stem cell pulp cells. Pasavant et al. (39) earlier study The creation of secreted protein acidic, high in protein is shown to have been induced by TGF-β, which in turn causes migration of dental pulp cells towards the odontoblastic layer.(40) The dental pulp stem cells are lured to the site of an injury to replace permanently wounded odontoblasts into second generations of odontoblasts and odontoblasts.(41,42)Pulp cells may be distinguished from pre odontoblast and odontoblast by TGFβ1 and in reparatory dentinogenesis following tissue damage.(43)In a recent in vivo investigation, TGF-β indicated that pre-odontoblasts are differentiated and functional odontoblastic cells are formed. (44,45)

Several studies show that TGF-β1 and TGFβ3 increase subodontoblastic layer proliferation and the production of cells similar to odontoblast. (46)(47)(45) Furthermore, TGF-β1 promotes the formation of collagen type I by the odontoblastic pulp cells.(48) TGF-β3, by elevation of osteocalcin and collagen type I expression, has been observed by Huojiaet al.(49) and may govern differentiating the dental pulp stem cells to odontoblasts. During the dentin-pulp complex

repair processes, after tissue damage, such activities might be crucial.(50)

Human Deciduous teeth which were exfoliated showed that there was an expression of the VGEF receptors which are membrane bound (VEGFR-1) and 2, vascular endothelial cadherin and CD31(51) In Another study it was stated that VGEF stimulated the increase and proliferation of ALP in pulp tissue.(52)It has been extensively documented that Runx-2 and ALP gene expression levels play essential roles in the early and intermediate phases of differentiation during bone formation, whereas BSP, OPN, and OCN expression in the late phases help in differentiation of osteoblasts, they play crucial roles.(53)

Two key dentin proteins which are non collagenous are produced by the DSPP gene which are the dentin phosphoprotein and the dentin sialoprotein which is necessary for the remineralisation of the dentin.(54)(55)DMP-1 gene is linked to dentinogenesis imperfecta and is expressed by the odontoblasts.(56)The current study's findings showed that VEGF can stimulate hDPC calcification and segregation and significantly enhance gene expression of Runx2,SP7, ALP, Col 1, DMP1,OCN, BMP2, BMP3, and DSPP in pulp cell culture in vitro. As a result, the findings of this study show that VEGF can promote calcification and osteoblast/odontogenic development in human DPCs in vitro.(57)

Reparative dentin is formed in response to any severe injury and it is formed by the replacement odontoblasts.It is a type of tertiary dentin formation which consists of the reparative and reactionary dentin.(58) In tissue regeneration plays a key role as it helps in supplying nutrients and removes waste and helps in proper functioning of the vascular network(59)

Pulpal tissue typically gets only one-ended blood supply, hence the root apex of the tooth is prone to infection,injury and permanent pulpitis..(59) Local application of angiogenic growth factors has been proposed to promote local angiogenesis at the site of dental tissue regeneration following tooth root fracture.(60) Applying different techniques have been used in various research to promote angiogenesis for their disease model.(61)(62)(63) Mullane et al (64) showed that recombinant hVEGF enhanced the new vascularization of the pulp tissue. Our present study in vivo results showed that hVEGF increases the growth of the dental pulp and in the tooth pulp, neovascularization and the creation of reparative dentin occur.

In conclusion, the present study shows that, hVEGF has beneficial effects on pulp tissue proliferation, differentiation, mineralization, neovascularization, and the development of the reparative dentin formation in vitro and also in vivo.Our team has a plethora of research and knowledge that has resulted in high-quality publications.(65–80) The evidence suggests that hVEGF has a therapeutic potential in regeneration and other disorders of the pulp. In addition, the current study shows that a gene therapy method might be beneficial in the treatment of dental pulp disorders. In the following phase, researchers will utilise hVEGF and inflammatory inhibitors to see if reversible and irreversible pulpitis can be treated, with an emphasis on irreversible pulpitis.Depending on the strength of the initial reaction and the circumstances surrounding the freshly deposited dentin matrix. was generated, the dentin-pulp complex can respond to a carious lesion by localised deposition of a tertiary dentin matrix that can be described as either reactive or regenerative in nature. Molecular signalling to induce tertiary dentinogenesis is provided by the TGF-super family, which is trapped in the dentin matrix and may be solubilized or exposed during carious demineralization. TGF-s boost the upregulation of odontoblast cells' synthetic and secretory activity in reactionary dentinogenesis, resulting in the secretion of a reactionary dentin matrix.TGFs can also stimulate the proliferation, migration, and differentiation of odontoblast-like cells, resulting in the production of reparative dentin.

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