Fig. 8

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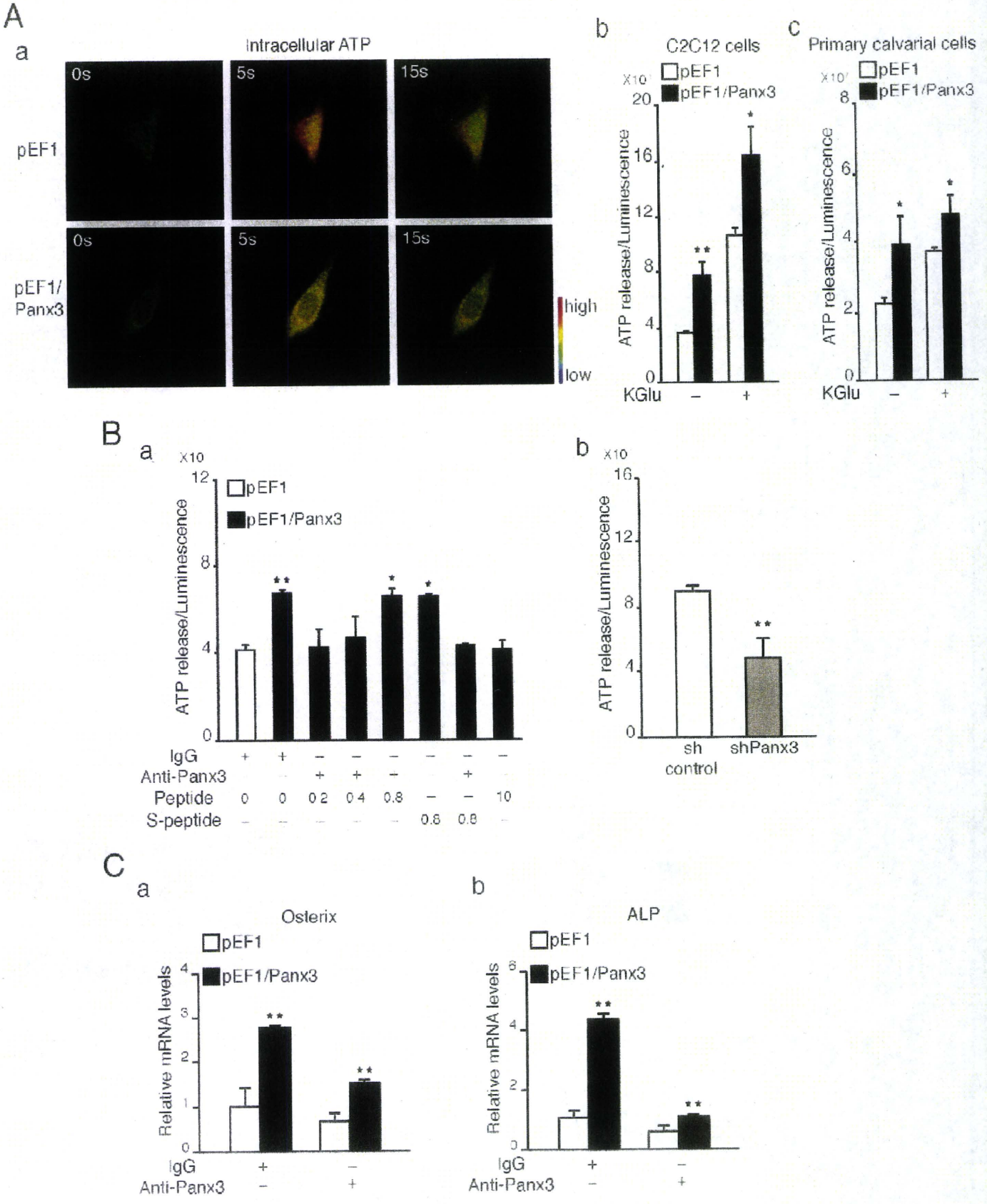

Fig. 9

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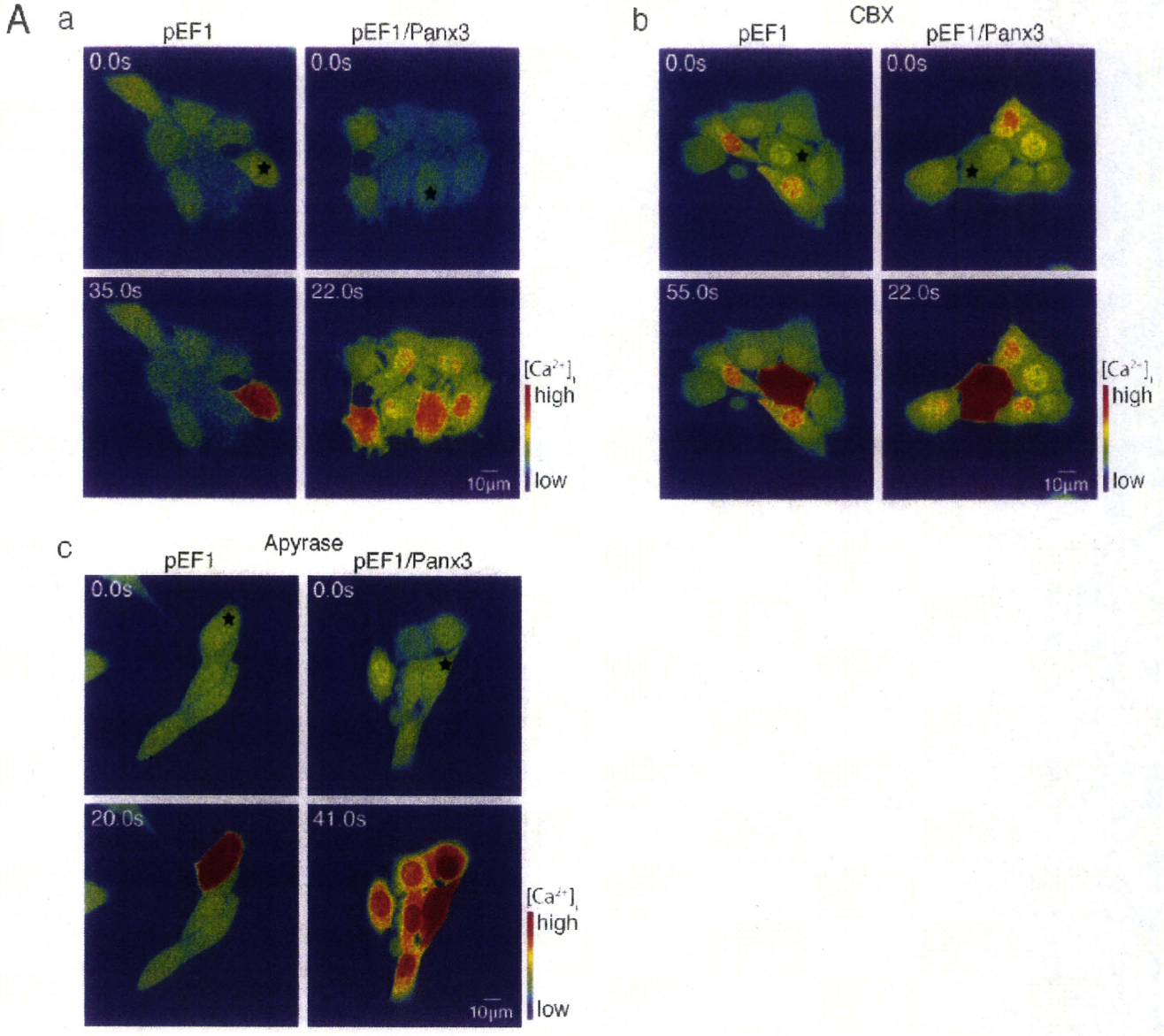

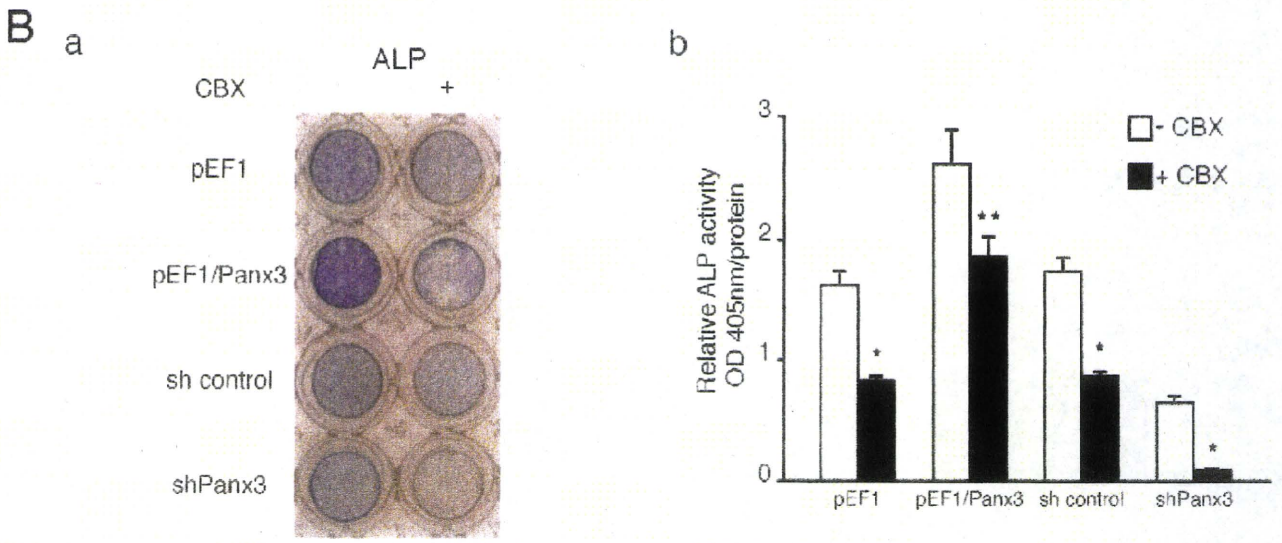

Fig. 10

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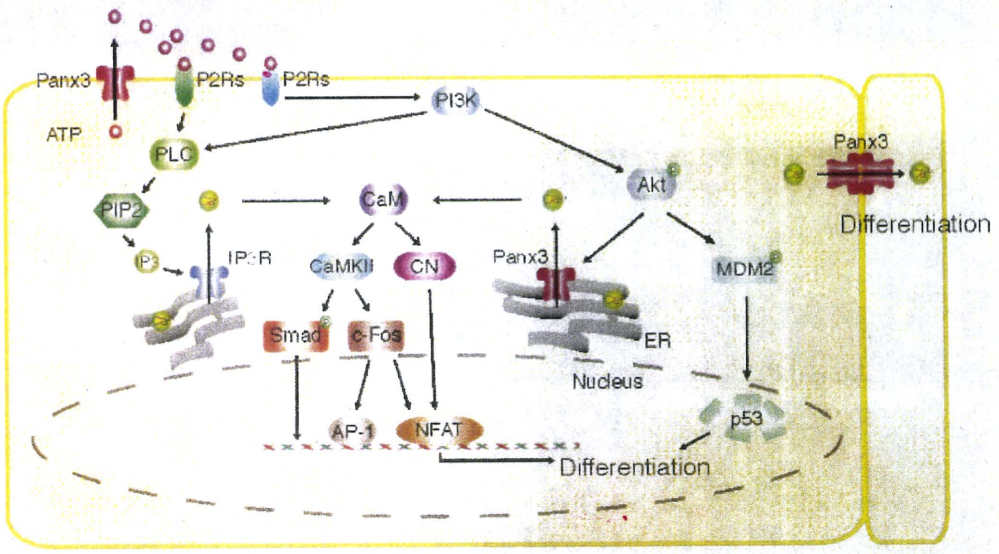

Supplemental Fig. 1

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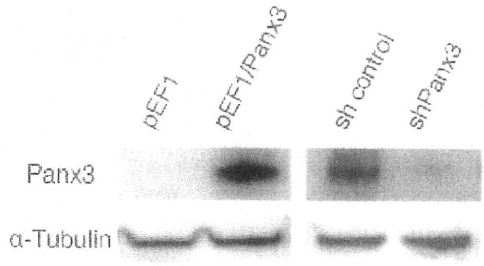

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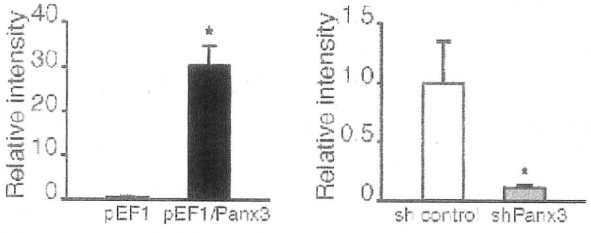

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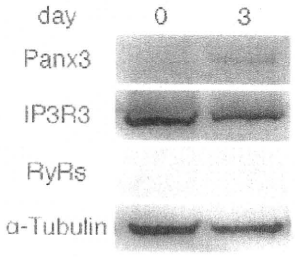

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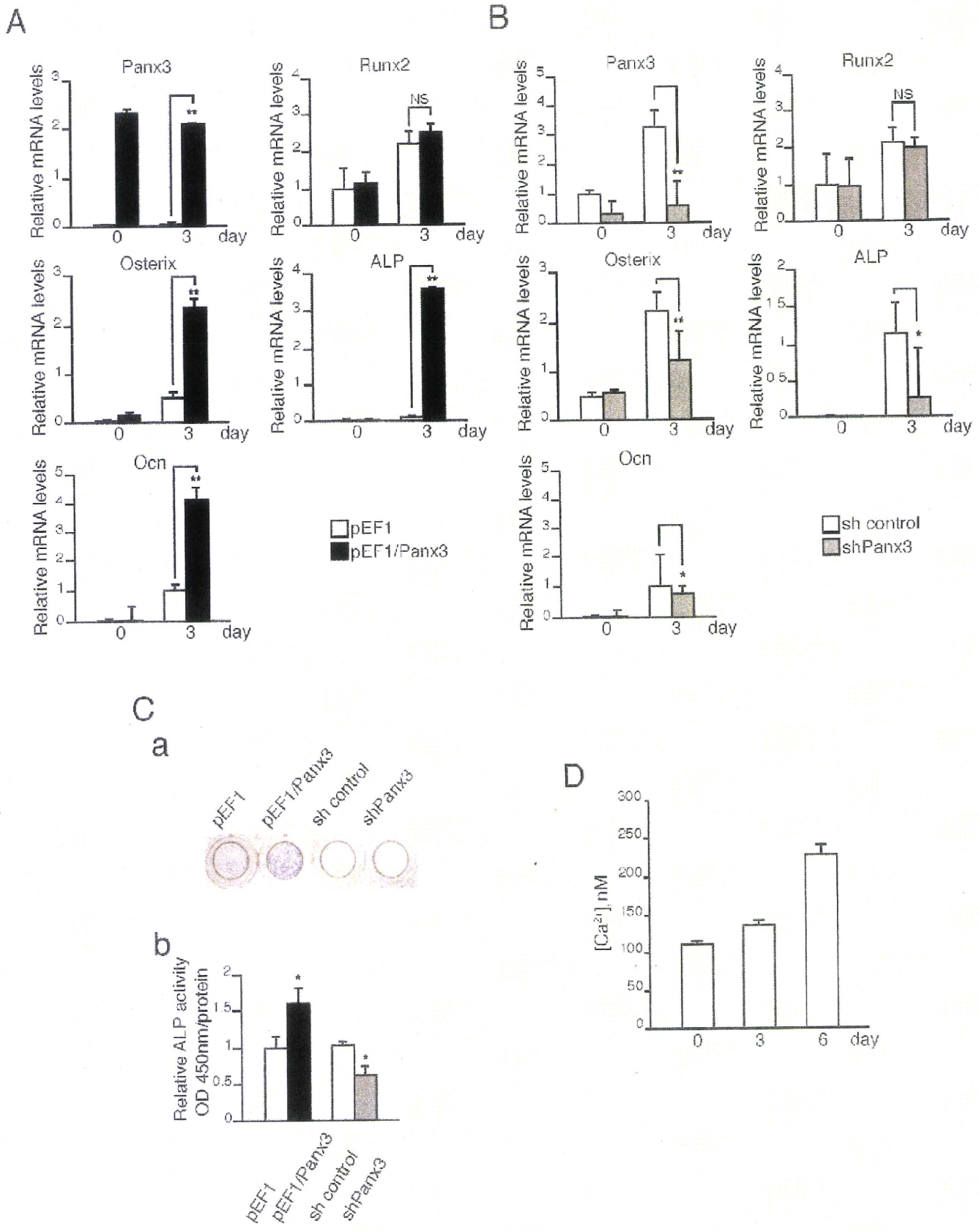

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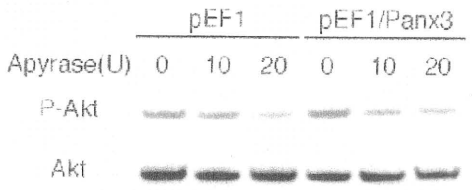

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REVIEW (New Aspects of Molecular and Genetic View on Mammalian Tooth Development)

Expressions and Functions of Neurotrophic Factors in Tooth Development

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Abstract : Neurotrophic factors are soluble growth factors predominantly expressed in vertebrate nervous systems and have been well-characterized for their critical roles in neural tissues. Recent studies have revealed that neurotrophin factors and their receptors are also expressed in multiple non-neural tissues, and play a role in a wide range of biological functions, such as regulation of cellular proliferation, survival, migration, and differentiation. The neurotrophic factor family is defined by its structural and functional similarities to 4 ligands ; nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4, also known as NT-5). They activate 2 different receptors, trk tyrosine kinase and p75, the latter of which is a member of the tumor necrosis factor receptor superfamily. During tooth development, observations of dynamic changes of specific expression patterns of neurotrophic factors and their receptors imply their important functions in odontogenic processes. In addition, our recent study demonstrated that NT-4 regulates proliferation and differentiation of dental epithelium, and promotes the production of enamel matrixes. In this review, we describe the expression patterns and functions of neurotrophic factors in the tooth germ, and discuss the relationships with tooth development.

Introduction

The complex interactions between epithelial and mesenchymal cells play crucial roles in tooth development. Initial signaling from the oral epithelium induces neural crest-derived ectomesenchyme development, and then both processes lead to tooth formation. Many of these interactions are facilitated by various paracrine and autocrine growth factors such as transforming growth factor β, fibroblast growth factor, Hedgehog and Wnt families and their receptors, while genetic programs are regulated by transcription factors[1,2]. These are dynamic systems that operate precisely in cellular events and molecular mechanisms.

Neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4), play significant roles in the regulation of neuronal cell fate[3,4]. Neurotrophic factor signalling occurs through 2 types of receptors, which have distinct ligand affinitis and specificitis on the cell surface. One is the tropomyosin receptor kinase (trk) family, consisting of trkA, trkB, and trkC, which are tyrosine kinase receptors that bind to neurotrophic factors with a high level of affinity[5]. Of those, trkA is a signaling recep-

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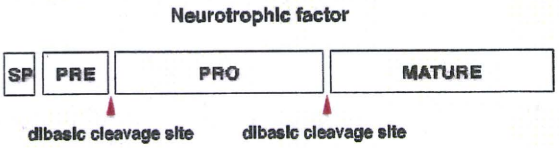

Fig. 1 Characteristic structures, and cellular and extracellular processing sites of neurotrophic factors
Shown are proteolytic processing sites for pro- and mature-neurotropic factors (red arrowheads). SP ; signaling peptide, PRE ; precursor, PRO ; pro-neutrophic factor, MATURE ; mature-neurotrophic factor.

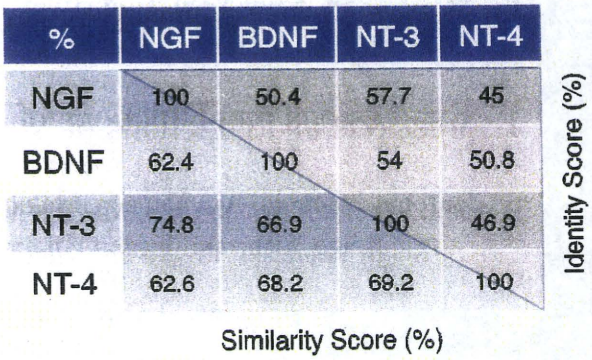

Fig. 2 Scores for protein similarity and identity between mature neurotrophic factors
The amino acid sequences of mature neurotrophic factors were analyzed using ClustalW (v. 1.83) and multiple sequence alignment was analyzed with MacVector software (v. 10.6).

tor for NGF[6]. BDNF and NT-4 bind to trkB as a primary receptor[7], while trkC mediates the biological responses of NT-3[8,9]. The other receptor is p75, a member of the tumor necrosis factor superfamily, which binds to all neurotrophic factors with a low level of affinity[9]. Although neurotrophic factors have important roles in neuronal cell development, survival, and apoptosis, the expressions of neurotrophic factors and their receptors are also observed in non-neuronal cells and tissues, such as those of the lungs and kidneys. Therefore, they may have multiple functions related to organogenesis of not only neuronal tissues, but also non-neuronal tissues including those related to tooth development.

Neurotrophic Factors and Their Receptors

The neurotrophic factor family, composed of NGF, BDNF, NT-3, and NT-4, is preferentially expressed in the nervous system, and has various critical roles in neurogenesis and the pathogeneses of neurodegenerative disorders. Structural similarities have been observed among neurotrophic factor family members and individual neurotrophic factors are remarkably conserved across vertebrate species[10]. All neurotrophic factor proteins are initially translated as a precursor protein containing a signal peptide, typical glycosylation sites, and pairs of dibasic protein cleavage sites[11]. Precursor neurotrophic factor proteins are cleaved intracellularly at pairs of dibasic cleavage sites by the calcium-dependent serin protease furin and the family of prohormone convertases (Fig. 1). A pro-neurotrophic factor is produced after single site proteolytic processing of the precursor form, while the mature-neurotrophic factor is the shortest isoform among the various neurotrophic factors. Interestingly, the amino acid sequences of the mature-neurotrophic factor are highly conserved in vertebrate species, indicating that mature-domain sequences are responsible for the biological functions of neurotrophic factors. Mature-neurotrophic factors have a protein identity of approximately 50%, while identity of 60—70% exists between other neurotrophic factors. NGF and NT-3, as well as BDNF and NT-4 have high scores for protein similarity, as shown in Fig. 2. In fact, BDNF and NT-4 have similar levels of receptor affinity with p75 and trkB. Recent studies suggest that pro-neurotrophic factors are also cleaved by extracellular proteases such as matrix metalloprotease 7 and plasmin[12—14]. Furthermore, pro- and mature-neurotrophic factors cause distinct biological actions by binding to specific receptor complexes, *e.g.*, pro-neurotrophic factors preferentially bind to p75, while mature-neurotrophic factors interact with trk receptors. Together, these observations indicate that the diverse biological functions of neurotrophic factors are tightly regulated multiple mechanisms related to variations of ligand-receptor complex formation, the morality ratio of pro- and mature-neurotrophic factors, and extracellular protease activi-

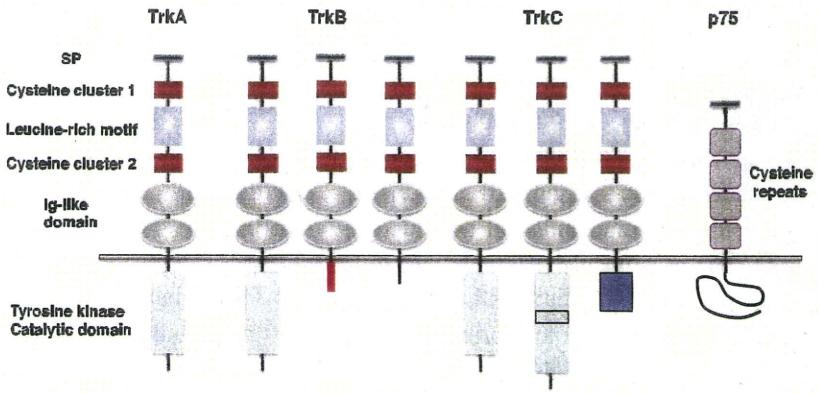

Fig. 3 Receptors for neurotrophic factors
Schematic comparison of the protein motifs of trk and p75 receptors. The extracellular structures are well-conserved among members of the trk receptor family, while a variety of protein motifs are observed in the intracellular regions of their receptors. p75, a member of the TNF receptor family, is shown localized with intracellular death domains in the cytoplasmic region. SP ; signaling peptide.

ties, which regulate the conversion of pro-neurotrophic to mature-neurotrophic factors.

Neurotrophic factor receptors have been classified into 2 types, the trk family and p75. Furthermore, the trk family of tyrosine kinase receptors has been found to have three subtypes (Fig. 3). Trk family neurotrophin receptors contain a common functional domain including a signal peptide, two sets of a cysteine cluster, a leucine-rich motif, immunoglobulin-like domains, a transmembrane domain, and a tyrosine kinase catalytic domain (Fig. 3). Two splicing variants have been identified in the trkA receptor, while the trkB receptor encodes three different isoforms, a full-length and two different splicing forms, which lack a tyrosine kinase catalytic domain. Basic analysis of cDNA clones has shown that four different splicing variants of the trkC receptor may also exist. In addition to full-length trkC, 2 trkC isoforms are missing a tyrosine kinase catalytic domain and one isoform contains additional amino acid residues in a tyrosine kinase catalytic domain. p75 has a relatively simple structure containing a signal peptide, cysteine repeats, a trans-membrane domain, and a cytoplasmic domain. The cysteine repeats motif is considered to posses the same low affinity as all neurotrophins. p75 is a member of the tumor necrosis factor (TNF) receptor family, although its function and structure are unique compared to other TNF receptors[15]. The intracellular domain of p75 contains death domains, which are different from those observed in other TNF receptor family members. Also, the cytoplasmic region of p75 does not have a characteristic ligand-inducible kinase motif, which is distinctly different from trk neurotrophic receptors.

Roles of Neurotrophic Factors in Organogenesis

Neurotrophic factors and their receptors are expressed by non-neuronal tissues where they regulate tissue innervation, and are also involved in cell proliferation, migration, and differentiation in multiple organogenic processes. TrkA is expressed in the kidneys, testes, and thymus, while trkB is found in the lymph nodes and spleen[16,17], and both trkA and trkC are expressed in the salivary glands and lungs[18]. Nerve growth factor was shown to affect Ca^{2+} currents via the p75 receptor to enhance prolactin mRNA levels in GH_3 rat pituitary cells, indicating that this induction reflects the conversion of GH_3 cells into lactotrope-like cells[19]. In a study of Leydig cells, NGF increased cellular steroid production and those results also suggested potential roles of neurotrophic

factors during testis morphogenesis[20]. In normal skin development, neurotrophic factors play roles in keratinocyte proliferation, melanocyte development and apoptosis, and hair growth, and also have pathological functions in skin, such as stress-induced hair loss and psoriasis[21]. In another study, lungs in trkB deficient mice showed thinner bronchial epithelia, apparent larger air spaces, and a lack of neuroepithelial bodies, suggesting that trkB is essential for normal development of the lungs as well as the nervous system in the lungs[22]. However, the physiological functions of neurotrophic factors in tooth development have not been clearly demonstrated.

Expression of Neurotrophic Factors in Teeth

The process of tooth development can be divided into the following stages : initiation, bud, cap, and bell. During the initiation stage, a specific area of oral epithelium thickens and invaginates into the neural crest-derived mesenchyme. Continuation of this invagination forms the tooth bud, then a cap-shaped tooth germ structure is formed. After the cap stage, the morphology dramatically changes into a bell-shaped tooth germ. During the bell stage, epithelial and mesenchymal cells differentiate into enamel-secreting ameloblasts and dentin-secreting odontoblasts, respectively. The expressions of neurotrophic factors in this process have been investigated in rat and human studies[23—27].

NGF is expressed in dental epithelium and mesenchyme during the initiation stage of tooth development[25,26]. During the bud stage, NGF is located around dental epithelium and condensed mesenchyme, while it becomes localized in restricted areas of the inner dental epithelium and also in the stratum intermedium during the bell stage. During later embryonic and postnatal tooth development, NGF is restricted to the cervical part of the enamel organ and intense expression has been observed in dental papilla mesenchymal cells[25,26].

BDNF is also considered to be involved in tooth development, though its expression is significantly low at that time. During the bud stage, weak BDNF expression is observed in dental mesenchyme surrounding the dental epithelium. During the late embryonic period, low levels of BDNF are expressed in the inner and outer dental epithelia, as well as in dental papilla cells[26]. Then, during postnatal tooth development, a weak expression of BDNF is restricted to the middle part of the dental pulp of the cusp[26].

NT-3 is initially expressed in the mesenchyme of the mandibular process, then its expression is observed in dental epithelium and mesenchyme at the initiation stage[25,26]. During the cap stage, NT-3 is expressed in the epithelial cervical loops, while in later stages that expression is restricted to the cervical parts of the inner enamel epithelium and disappears from P4 tooth germs[25,26].

NT-4 expression is observed in oral and dental epithelial cells during tooth development. In the late embryonic stage, the expression of NT-4 in oral epithelium decreases, whereas it is increased in inner dental epithelium. During postnatal tooth development, NT-4 is observed in ameloblasts and outer enamel epithelium[25,26]. NT-4 is the only neurotrophin detected in differentiated ameloblasts (Fig. 4), indicating that it might be involved in dental epithelium differentiation and maintenance of ameloblast functions.

Expressions of Neurotrophic Factor Receptors in Teeth

At the initiation stage of tooth development, p75 is absent in both thickened dental epithelium and adjacent mesenchyme[23,24]. However, its expression can be observed in inner dental epithelium and condensed mesenchyme during the transition between the bud and cap stages. p75 is then found in parts of the inner dental epithelium except for the enamel knot, stratum intermedium, and dental papilla in the cap stage. During the bell stage, p75 is expressed in the inner dental epithelium and stratum intermedium, while it is absent from pre-ameloblasts and differentiated ameloblasts[23,24]. In dental papilla mesenchyme, the expression of p75 is slightly decreased. Then, during postnatal tooth development, p75 is observed in the subodontoblastic layer and proliferating cells of the

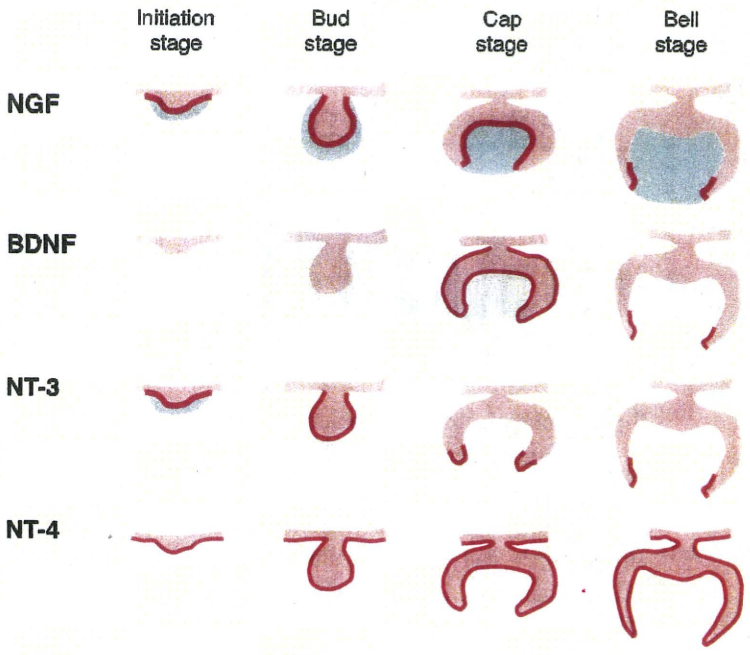

Fig. 4 Neurotrophic factors expression during tooth development
NGF, BDNF, and NT-3 are expressed in inner dental epithelium around the cervical loop. NT-4 is expressed in oral and dental epithelia, including pre-ameloblasts and ameloblasts. In mesenchyme, both NGF and BDNF are expressed in dental papilla, while the expression of BDNF is significantly low in dental papilla and epithelium at the bell stage. Red line and blue area indicate neurotrophin expression in epithelium and mesenchyme, respectively.

inner dental epithelium[23,24].

Mitsaadis *et al.* suggested that trkA can be found in matured ameloblasts[23]. However, no trkA mRNA was observed in rat first molars at all developmental stages, though *in situ* hybridization demonstrated its expression in the trigeminal ganglia in the head region[24]. Therefore, the expression of trkA during tooth development remains controversial. On the other hand, it was reported that trkA immunoreactivity was observed in the epithelial rests of Malassez of the periodontal ligament[28].

The trkB gene generates 3 variant transcripts ; full length mRNA, trkB-FL, and two C terminus truncated splicing forms that lack the tyrosine kinase domain, trkB-T1 and -T2[29,30], indicating that trkB and its isoforms serve multiple distinct cellular physiologies. However, only trkB-T1, and not trkB-FL or -T2, is expressed during tooth development[23–25]. During the early stage of tooth development, trkB-T1 is expressed in both dental epithelium and mesenchyme during the initiation and bud stages. During the cap and bell stages, trkB-T1 is located in the cervical part of the inner dental epithelium, and then becomes concentrated in the cervical loops and dental papilla mesenchyme, as well as the mesen-

Table 1 Expressions of neurotrophic factor receptors during the bell stage of tooth development

Bell stage	TrkA	TrkB	TrkC	p75
Epithelium	−	+	−	+
Mesenchyme	−	+	+	+

Both trkB and p75 are expressed in epithelium, which indicates that their signaling is involved in tooth development and morphogenesis.

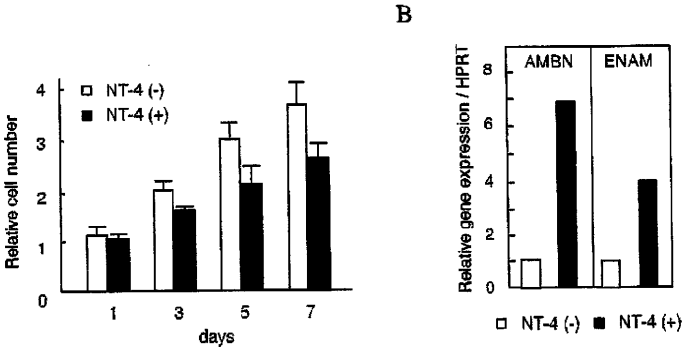

Fig. 5 Inhibition of cell proliferation and promotion of cell differentiation in HAT-7 cells cultured with NT-4

(A) HAT-7 cells were cultured with or without NT-4, and cell numbers were determined with a trypan blue exclusion method after 1, 3, 5, and 7 days of culture. NT-4 inhibited HAT-7 cell proliferation. (B) HAT-7 cells were cultured with 100 ng/mL of NT-4 for 48 h. Total mRNA was analyzed for the expression of Ambn or Enam using a semiquantitative RT-PCR method. Hprt was used as the internal control. NT-4 promoted the expressions of Ambn and Enam.

chyme lining the outer dental epithelium and dental lamina. During postnatal tooth development, trkB-T1 is expressed in the cervical part of dental papilla mesenchyme and in mesenchyme adjacent to the cervical loop area. We analyzed the expressions of trkB-FL, -T1, and -T2 in P3 mouse molars using RT-PCR with specific primers. All trkB transcripts were expressed in dental epithelium, while trkB-T1, but not trkB-FL or trkB-T2, was detected in dental mesenchyme. Our findings suggest that trkB-FL and -T2 are also involved in dental epithelial cell differentiation.

No expression of trkC including all different transcripts was found in embryonic rat tooth germs, while it was seen during postnatal tooth development in the central parts of dental papilla mesenchyme[23,24].

During the process of tooth development, the bell stage is believed to be the most important for tooth development and morphogenesis, which define tooth shape and size. In this stage, trkB and p75, but not trkA or trkC, are expressed (Table 1) in dental epithelium, suggesting that trkB and p75 signaling is important for regulation in the late stages of tooth development and morphogenesis.

Functions of Neurotrophic Factors in Tooth Development

Since regulation by neurotrophic factors determines various neuronal progenitor cell lineages, and also promotes neural cell differentiation and maturation, those factors are thought to only play roles in tissue innervation and dental pulp formation during tooth development. In fact, the expression pattern of NGF suggests that it is involved in local sprouting and establishment of the final innervation pattern of dental papilla and dentin, indicating that NGF is required for tooth innervation[25]. BDNF and or NT-4 could be target-derived factors involved in sensory trigeminal tooth pulp nerve fiver development, differentiation, or regeneration[31]. A study of BDNF heterozygous mice demonstrated that neuronal density in the periodontal ligament was reduced by roughly 18% as compared to that in wild-type mice[32]. Also, NT-4 5 gene depletion caused a delay in formation and maturation of the periodontal Ruffini endings in mice by inhibiting the growth of the periodontal nerves at an early stage[33]. Meanwhile, it was

reported that neurotrophins function in non-neuronal tooth cells. NGF increased DNA synthesis and expressions of mRNA for bone-related proteins, alkaline phosphatase (ALPase), and osteopontin (OPN) in human periodontal ligament (HPL) cells[34]. In another study, NT-4/5 elevated the mRNA levels of ALPase, OPN, osteocalcin, and BMP-2, and also enhanced the amount of mineral deposits in cultures of HPL cells[35].

Neurotrophic factors and receptors are also broadly expressed during tooth development (Fig. 4, and Table 1). Among the neurotrophic factors, NT-4 is only expressed in pre-ameloblasts and ameloblasts, which prompted us to assess the potential biological role of NT-4 with ameloblast proliferation and differentiation. We examined the effect of NT-4 on mediation of dental epithelial cell proliferation[36]. NT-4 decreased the cell number of HAT-7, a dental epithelial cell line (Fig. 5A)[37]. This result suggests that NT-4 negatively regulates dental epithelial cell proliferation. We also tested whether NT-4 affects dental epithelial cell differentiation[36]. RT-PCR demonstrated that ameloblastin (Ambn) and enamelin (Enam), markers for ameloblast differentiation, were strongly induced in NT-4 treated HAT-7 cells (Fig. 5B), indicating that NT-4 positively promotes ameloblast differentiation. Furthermore, trkB-FL overexpression in HAT-7 cells also enhanced Ambn expression as compared to untransfected HAT-7 cells, whereas trkB-T1 overexpression in those cells was blocked by Ambn expression. These results suggest that induction of Ambn by NT-4 is regulated by trkB-FL, but not by truncated trkB-T1, indicating that TrkB-T1 might be involved in cell proliferation. The phenotypes of tooth development in NT-4 deficient mice strongly support our *in vitro* analysis of dental epithelial cells. P3 molars in NT-4 deficient mice had a thinner enamel matrix layer than those in the control mice, although there were no differences in regard to size, shape, and polarization of ameloblasts between the mutant and heterozygote molars[36]. Furthermore, Ambn expression in NT-4 null tooth germs was reduced as compared with that in heterozygotes[36]. These findings suggest that NT-4 has important roles in Ambn expression and enamel layer formation.

Conclusion

During the process of tooth development, dynamic changes are observed in the distributions of neurotrophic factors and their receptors. Further, NT-4 plays crucial roles in dental epithelial cell proliferation and differentiation, as well as enamel matrix gene expression via trkB-FL, but not truncated trkB forms. Based on these results, neurotrophic factors have functions in the development of tooth innervation and also contribute to tooth development.

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REVIEW(New Aspects of Molecular and Genetic View on Mammalian Tooth Development)

Diverse Function of Epiprofin in Tooth Development

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Abstract : The developing tooth is a good model for studying the mechanism of organogenesis. Tooth development begins as the placode formation with the thickening and invaginating of the oral ectoderm into the dental mesenchyme. A series of reciprocal interactions between these two cell types give rise to differentiation into various cell types including epithelial-derived enamel-secreting ameloblasts and neural crest-derived dentin-secreting odontoblasts. A number of transcription factors control tooth development in order to form unique structures specialized for tooth function with optimized shapes and sizes. We identified Epiprofin (Epfn) as a transcription factor preferentially expressed in teeth. *Epfn* KO mice display profound embryonic and postnatal growth retardation and develop impaired hair follicle and whisker formation. The drastic phenotypes of *Epfn* KO are supernumerary tooth formation and lack of enamel in teeth. These observations suggest that Epiprofin plays critical roles in ectodermal organ development and regulates the number of teeth. This review summarizes the roles of Epiprofin in tooth development.

Introduction

At the end of 1990, the human genome project began as an international initiative project and discovered all the predicted 30,000—40,000 human genes. Following the human genome project, we initiated the Oral and Craniofacial Genome Anatomy Project (OC-GAP) to identify novel genes important for tooth and craniofacial development. The primary goal of the OC-GAP is to discover and characterize previously unknown genes to help understand how tooth and craniofacial tissues develop and to define the molecular defects underlying anomalies of these tissues or oral cancer. We dedicated all our effort and time to search for and identify the genes involved in tooth development, and to characterize their functions to accomplish the OC-GAP. The initial phase of the OC-GAP was to prepare a cDNA library using mRNA from tooth germ molars of embryonic day (E) 19.5 mice. Differentially screening using tooth cDNA microarrays containing about 12,000 clones from this library, we identified 197 cDNA clones that were preferentially hybridized to E19.5 molar mRNA. The majority of these clones encode enamel matrix pro-

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Table 1 Expression pattern of Sp transcription factors in vertebrate embryos

Factors	Expression	Chromosomal Locations	Major phenotypes in knockout mice
Sp1	Ubiquitous Dental epithelium	Human : 12q13.1 Mouse : 15	Growth retardation, prenatal lethality[9]
Sp2	Ubiquitous	Human : 17q21.32 Mouse : 11	Growth retardation, prenatal lethality Lost autonomous cell proliferation[8]
Sp3	Ubiquitous Dental epithelium	Human : 2q31 Mouse : 2	Growth retardation, death at birth, defects in tooth, lung, bone and hematopoietic development, cardiac malformation[6,10,11]
Sp4	CNS, liver, lung, kidney, heart, gonads, intestine Dental papilla and dental sac	Human : 7q15.3 Mouse : 12	Higher incidence of postnatal mortality ; smaller body size ; reproductive sterility[12,13]
Sp5	Mesoderm precursors and derivatives ; posterior neuroectoderm and derivatives	Human : 2q31 Mouse : 2	No morphological changes enhanced frequency of taillessness[14]
Epiprofin Sp6	Developing teeth ; caudal neuropore ; limb buds ; hair follicles ; skin Dental epithelium	Human : 17q21.32 Mouse : 11	Enamel defect, supernumerary teeth Defective cusp and root formation Abnormal hair and skin development[25]
Osterix Sp7	Developing bone and teeth Odontoblasts and dental follicle cells	Human : 12q13.13 Mouse : 15	Death at birth ; failure in ossification[15]
Sp8	CNS, limb buds	Human : 7q15.3 Mouse : 12	Neural tube closure failure ; shorter tail ; shorter limbs ; death at birth[16]
Sp9	In specific domain of the CNS ; limb[17]	Human : 2q31 Mouse : 2	Unknown

CNS : central nervous system

teins, such as *ameloblastin* (*Ambn*), *amelogenin*, and *enamelin*, indicating the feasibility of microarray analysis. One of the clones, which we named Epiprofin (Epfn), encodes a member of the Sp/Krüppel-Like Factor (KLF) family containing three characteristic C_2H_2-type zinc-finger motifs[1]. In situ hybridization analysis shows that *Epfn* mRNA is expressed in the proliferating dental epithelium in addition to the hair follicle matrix epithelium. Whole-mount in situ hybridization shows transient expression of *Epfn* mRNA in cells of the apical ectodermal ridge (AER) in developing limbs as well as at the genital ridge in genital development.

Regulation of Sp Transcription Factors in Organ Development

Epfn is a zinc finger protein that belongs to the Sp transcription factor super-family. The family comprises of nine members (Sp1—Sp9) in mammals[2], and Epfn corresponds to Sp6. Each member contains three C_2H_2 type zinc finger motifs at the C-terminus and binds to GGGCGG motifs or related GC-rich sequences. The proteins share a high degree of structural conservation even outside the zinc-finger domain[3]. Sp1 to Sp4 are expressed widely, while Sp5 to Sp9 are expressed in specific tissues and developing stages[4]. The proteins can act as transcriptional activators or repressors and are able to influence

diverse biological processes including cell proliferation, cell cycle progression, apoptosis, tumorigenesis, and differentiation (Table 1)[5—7]. Gene targeting approaches reveal that Sp transcription factors play diverse roles in organ development as described in Table 1. Sp1 and Sp2 knockout mice die in the embryonic stage[8,9]. Sp3 knockout mice die after birth due to respiratory failure[10]. Although Sp3 is ubiquitously expressed, mice lacking Sp3 show interesting phenotypes such as defect of enamel, impaired ossification, and cardiac malformation[6,10,11]. Sp4 knockout mice survive with growth retardation and infertility[12,13]. Sp5-null mice show no obvious abnormalities[14]. Sp7 Osterix knockout mice lack entire bone, but cartilage is normal[15]. Sp8 knockout mice die after birth with defects of limb outgrowth and failure of neural tube closure[16]. Sp9 knockout mice have not been reported while the function of Sp9 in limb development is similar to that of Sp8[17]. During tooth development, Sp1, Sp3, and Epfn Sp6 are expressed in dental epithelium and Sp4 and Osterix Sp7 are expressed in dental mesenchyme. The genetic deletion of Sp3 showed defective tooth enamel formation due to a lack of the enamel matrix proteins, amelogenin and ameloblastin[10]. The amino acid sequence similarity of the zinc-finger domain between Epfn and Sp3 is approximately 80%. This suggests that a possible functional redundancy could exist between Epfn and Sp3. The N-terminal portion of amino acid sequences in the Sp transcription factor family is unique except for the Sp-box and the Buttonhead (Btd)-box[18]. The Sp-box consists of a 13 amino acid motif located at the N terminus of the protein, whereas the Btd-box has been described as a 10 amino acid box situated immediately at the N terminal to the zinc-finger domain. At the moment, the roles of these motifs in the function of the Sp protein are unknown. They are suggested to be involved in transactivation activity (Btd-box) or as an interaction-site with a repressor (Sp-box). Interestingly Epfn lacks SP Box whereas the Btd-box is conserved. We are interested in the roles of the N-terminal part of Epfn in tooth development.

Epiprofin Expression in Tooth Development

Mouse tooth development is initiated at embryonic day (E) 11.5, when the oral epithelium thickens and invaginates into the underlying neural crest-derived mesenchyme. The dental epithelial invagination forms epithelial tooth buds at E13.5. During the cap stage of development, the condensed dental mesenchyme diverges into two different pathways : the dental papilla that give rise to dentin-forming odontoblasts and dental pulp fibroblasts, and the dental follicle that contains progenitors for cementoblasts and osteoblasts, and periodontal ligament fibroblasts[19]. After the cap stage, the tooth germ progresses to bell stages, and epithelial cells differentiate into enamel-forming ameloblasts. Dental epithelium differentiates into ameloblasts mainly through five distinct stages, *i.e.*, 1. proliferative stage : 2. differentiation stage : 3. secretory stage : 4. early maturation stage, and 5. late maturation stage[20]. At the proliferative stage, dental epithelium proliferates rapidly. At the differentiation stage, the cells stop proliferating (cell cycle exit) and differentiate into preameloblasts, which show cellular polarity and begin to secrete enamel matrix proteins. At the secretory stage following dentin mineralization, differentiated ameloblasts secrete enamel matrix proteins including amelogenin, ameloblastin, enamelin, and tuftelin, and additionally amelotin is secreted in later stages. During the maturation stage via the transitional stage when ameloblasts eventually undergo apoptosis, the enamel matrix is almost completely replaced by calcium and phosphorous, and ameloblasts eventually give rise to reduced enamel epithelium at the regressive stage[21].

Expression of Epfn is detected at the initiation stage of tooth development. Epfn is clearly expressed in dental epithelium of dental lamina and is not expressed in dental mesenchyme at the early stage of tooth development. During the bud stage, Epfn is expressed widely in dental epithelial cells and tooth buds develop rapidly by dental epithelial cell proliferation. At the cap stage, dental epithelial cells determine their cell fate into several lineages such as stellate reticulum, and inner and outer enamel

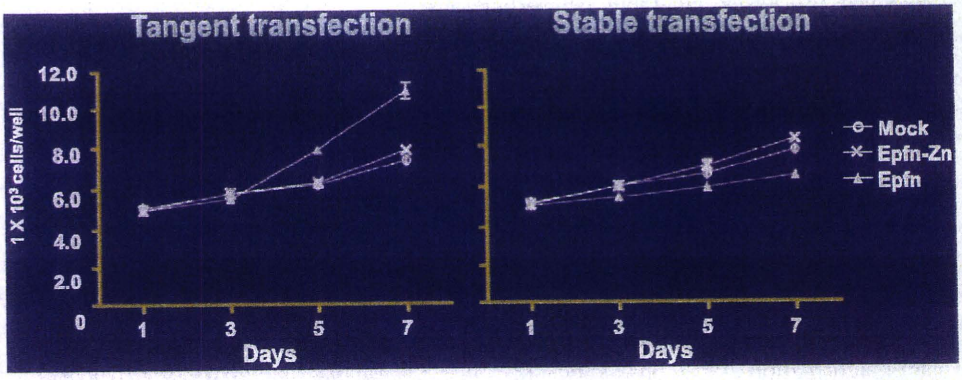

Fig. 1 Distinct roles of Epfn in dental epithelial cell proliferation
Primary mouse dental epithelial cells were transfected with Epiprofin (Epfn), Epiporfin Zinc finger domains (Epfn-Zn) expression or mock vector (Mock). Five $\times 10^3$ cells were seeded in 24-well culture plates and the numbers of cells were counted at 1, 3, 5, and 7 days. To establish stable transfected dental epithelial cells, primary mouse dental epithelial cells were transfected with either Epfn, Epfn-Zn expression or mock vector and cultured with 400 μg mL of G418 in DMEM F-12 medium supplement with 10% fetal bovine serum for 10 days. After G418 selection, 5×10^3 dental epithelial cells were seeded in 24-well culture plates and the numbers of cells was counted at 1, 3, 5, and 7 days.

epithelium. At this stage, the expression of Epfn is limited to the inner enamel epithelium and it is not expressed in other dental epithelial cell types. At the bell stage, Epfn is continuously expressed in pre-ameloblasts and ameloblasts. Interestingly odontoblasts, which derived from dental mesenchyme, start expressing Epfn.

Epiprofin Regulation in Cell Proliferation and Cytodifferentiation during Tooth Development

The multiple cellular function of Epfn is demonstrated by transfection assay using Epfn expression vector into primary dental epithelial cells. Over-expression of Epfn exerts different roles in cell growth in transient or stable expression in dental epithelial cells[1]. By transient expression of Epfn, primary dental epithelial cells showed strongly stimulated cell mitogenic activity (Fig. 1A). It can be considered that transient expression of Epfn in dental epithelial cells might mimic the progenitor cell types of dental epithelium, which give rise to differentiation into ameloblasts. On the other hand, stable expression of Epfn inhibits cell proliferation (Fig. 1B). We also demonstrated that over-expression of Epfn promotes dental epithelial cell differentiation into ameloblasts, which in a terminal differentiated phase of dental epithelial cells and stops cell proliferation[1]. Continuous expression of Epfn could induce cell cycle exit due to the rapid promotion of dental epithelial cell differentiation into ameloblasts. It is also known that up-regulation of *Ambn* gene expression, which is a marker of ameloblasts, directly inhibits cell growth in dental epithelial cells and ameloblastoma cells[22]. In addition, *Ambn* gene deletion mice develop odontogenic tumors due to sustained mitogenic activity in dental epithelial cells[23]. Therefore Ambn is a regulator for dental epithelial cell proliferation. A couple of Sp1 binding elements have been identified in the promoter region of *Ambn* and EMSA assay showed direct binding of Sp3 at the Sp binding element upstream of *Ambn* promoter[24]. Because of the amino acid similarity between Sp3 and Epfn, Epfn could share the Sp binding sequences in *Ambn* promoter with Sp3. In fact, overexpression of Epfn strongly induces *Ambn* gene expression in primary dental epithelial cells and dental epithelial cell lines. The negative regulation of cell proliferation in stable-

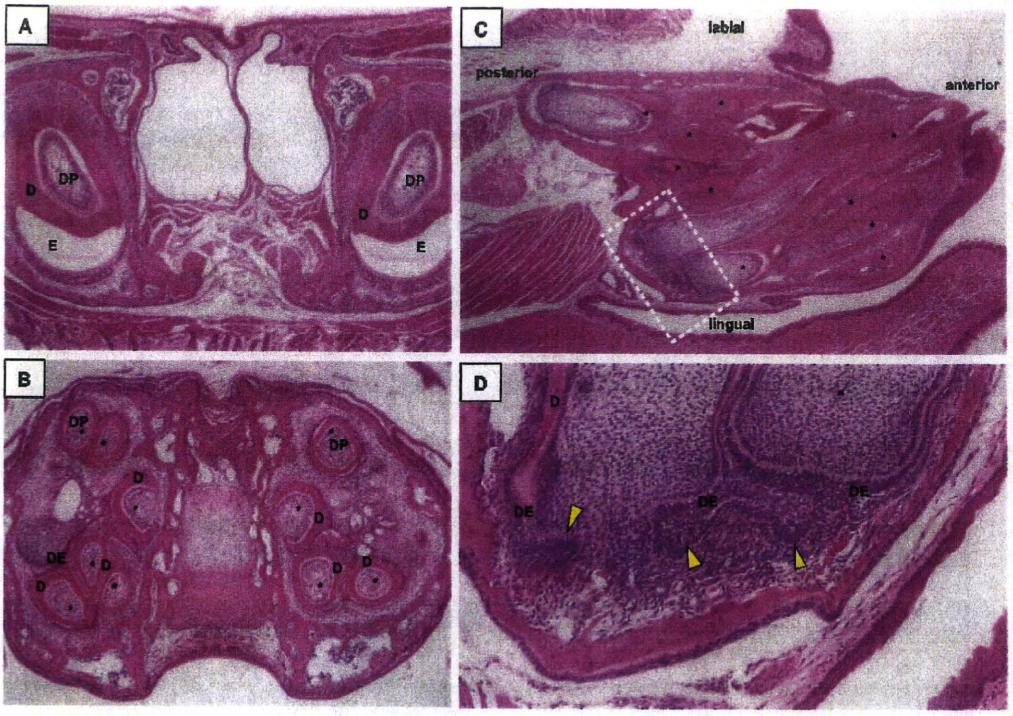

Fig. 2 Supernumerary tooth formation in *Epfn* KO mice
Hematoxylin-Eosin (H-E) staining of incisors in *Epfn* KO 3-week-old mice (B) and control heterozygous mice (A) (frontal section). Supernumerary tooth formation in *Epfn* KO at 6-week-old (C) (Saggital section) and the lack of ameloblast layer. Enlarged images of the square in Fig2C. Yellow arrowheads indicate undifferentiated dental epithelium in *Epfn* KO, which keep invaginating into mesenchymal tissues. * ; supernumerary teeth, D, dentin ; E, enamel ; DP, dental pulp ; DE, dental epithelium

transfectant Epfn dental epithelial cells could be involved in the induction of *Ambn*. On the other hand, we are investigating the mechanism of the possible direct regulation of cell cycle exit by Epfn in dental epithelial cells.

Interestingly, the enamel organ of the tooth bud in *Epfn* knockout (*Epfn KO*) mice consists of a large number of dental epithelial cells compared to tooth buds in counterpart heterozygous mice[25]. However the proliferation activity of dental epithelial cells in *Epfn* KO is drastically reduced. We found that the enamel organ of *Epfn* KO mice is occupied by slowly proliferating dental epithelial cells. In addition, *Epfn* KO mice fail to form enamel in incisors and molars and show impaired cusp formation. In general organogenesis, undifferentiated cells give rise to transit amplifying (TA) cells which subsequently rapidly divide a couple times as a mitotic progenitor and are committed to differentiate[26]. During the mitogenic phase TA cells are determined into their lineages and eventually induced to make their cell cycle exit on the way to functional maturation. Thus, the rapidly proliferating cell induction by Epfn is the initial differential step of dental epithelial cells. Epfn could modulate the balance between cell proliferation and cyto-differentiation to form a functional shape and size of tooth crown. Once the regulation by Epfn, is removed dental epithelial cells continuously proliferate at a slow rate and lose control of cell fate determination into[25] ameloblasts.