EVIDENCE FOR A ROLE OF MAP KINASES (ERK1 and pERK) IN PROLIFERATING AND DIFFERENTIATING ODONTOGENIC EPITHELIA

BY

BRENT R. NICKOLAYCHUK

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Faculty of Dentistry
Department of Preventive Dental Science
University of Manitoba
Winnipeg, Manitoba

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1. ABSTRACT

The epithelial rests of Malassez (ERM), remnants of odontogenic epithelium, have been implicated in maintaining the periodontal space by stimulating osteoclastic bone resorption and interstitial matrix degradation. While the binding of epidermal growth factor (EGF) to its receptor (EGFr) on ERM appears to play a central role in the functional activation of these cells, the nature of intracellular signaling pathways downstream of EGFr remains unclear. Mitogen-activated protein (MAP) kinases (or extracellular signal-regulated kinases – ERKs) have been described as downstream mediators of EGF-induced cellular activation in other tissues. The purpose of this study was to determine the expression pattern of ERK1 and its phosphorylated form (pERK) in proliferating and differentiating odontogenic epithelia, including ERM, in the presence or absence of inflammation. To this end, immunohistochemistry for ERK1, pERK, PCNA (marker for cycling cells), and PAN-cytokeratin (marker for epithelial differentiation) was performed on paraffin-embedded sections of dental follicles and odontogenic cysts. Statistically significant differences in specific binding of ERK1 and pERK antibodies were observed to correlate with proliferation (PCNA-positive) and differentiation (PAN cytokeratin-positive). Actively proliferating (>75% PCNA positive) epithelial cells were more likely to express both ERK1 and pERK than relatively quiescent (<25% PCNA positive) cells (ERK1: 8.4 ± 3.0% (<25% PCNA) and 45.4 ± 14.0% (>75% PCNA), $P < 0.05$; pERK: 17.7 ± 4.5% (<25% PCNA) and 66.0 ± 18.5% (>75% PCNA), $P < 0.05$). Resting epithelial islands and nondifferentiated epithelial cyst lining had significantly fewer

positively stained cells as compared with vacuolated islands and differentiated squamous epithelium (resting islands and nondifferentiated epithelial cyst lining: 11.5 ± 3.9% (ERK1) and 15.3 ± 4.4% (pERK); vacuolated and differentiated squamous epithelium: 40.7 ± 4.2% (ERK1) and 64.2 ± 4.3% (pERK), $P < 0.01$). Elevated pERK specific binding to epithelial cells correlated significantly with the presence of inflammation (56.7 ± 4.9% (inflamed) and 28.5 ± 9.2% (uninflamed), $P < 0.05$) while ERK1 binding exhibited no correlation with the presence or absence of inflammation. These data suggest that pERK plays a role in stimulating the epithelium of dental follicles and odontogenic cysts to proliferate and differentiate in response to inflammation.

2. REVIEW OF THE LITERATURE AND RATIONALE FOR THE STUDY

2.0 Epithelial Rests of Malassez (ERM) and Suggested Pathways of Activation

The epithelial rests of Malassez (ERM) are distinct groups of cells found in the periodontal ligament (PDL). They represent remnants of Hertwigs root sheath, a component of the developing tooth germ. During tooth morphogenesis, as root formation procedes, Hertwigs root sheath fragments, but its remnants, the ERM, persist as epithelial strands throughout the PDL (reviewed by Spouge 1980). It is well established that the epithelial lining of odontogenic developmental and inflammatory (periapical) dental cysts is derived from the ERM. Epithelial rests are contained also in the connective tissue wall of developmental odontogenic cysts such as the dentigerous cyst.

Histochemical (Ten Cate 1965) and ultrastructural (Valderhaug & Nylen 1966) studies indicate that the ERM cells are generally quiescent and, in the inactive state, they are characterized by poorly developed organelles, high nuclear-cytoplasmic ratio, low ribonucleic acid content, the presence of glycogen, and the absence of neutral lipids (Torabinejad 1983). In addition, they are functionally unique in their ability to persist throughout life as individual, tightly packed clusters of epithelial cells, each complete with an outer lining of basal lamina (Hamamoto *et al.* 1991) and surrounded by connective tissue.

While a role for their existence remains unclear, it is accepted that ERM cells can be stimulated to a more active state (Valderhaug & Nylen 1966). Immunohistochemical studies have revealed that porcine ERM and basal cells of human gingival epithelium express Cytokeratin 5, 6, 14, 16, and 19 in a manner

similar to basaloid cell-like, undifferentiated and hyperproliferative cells (Gao *et al.* 1988, Moll *et al.* 1982, Oda & Watson 1990). Grupe *et al.* (1967) observed that proliferated rest cells *in vitro* and *in vivo* exhibited little succinic dehydrogenase activity, contained no glycogen, accumulated lipids, and exhibited lactic dehydrogenase and glucose-6-phosphate dehydrogenase activity. Ten Cate (1972) suggested that increased lipid synthesis and protein production in these cells is associated with utilization of glycogen stores. This would account for the apparent exhaustion of glycogen stores in epithelial clusters. In view of these observations, it was assumed that a distinct synthetic or proliferative state of epithelial rest cells could be readily identified by intracellular features, such as low glycogen content. Also, these early histochemical findings led to the theory that a decreased oxygen/carbon dioxide tension was the initiating signal for epithelial cell proliferation via their ability to undergo anaerobic glycolysis. Lipid synthesis was linked to pentose shunt activity in protein synthesis necessary for cell multiplication. Further, it was suggested that in periapical inflammation (granuloma), the increase of cellular elements in the periapical tissue competes for available oxygen and causes increased carbon dioxide levels. This low O_2/high CO_2 tension may be the initiating factor for epithelial proliferation and apical cyst formation (Grupe *et al.* 1967).

Upon activation, the ERM may have a protective function in maintaining the periodontal space (Lindskog *et al.* 1988, Ten Cate 1996) by preventing ankylosis (Löe & Waerhaug 1961, Lindskog *et al.* 1988) and root resorption (Waerhaug

1958, Wallace & Vergona 1990). Spouge (1980) proposed that epithelial rests achieved this by acting as a physical barrier preventing the encroachment of bone. It has also been suggested that epithelial rest cells may be involved in the apical migration of the junctional epithelium during periodontal inflammation (Spouge 1986).

Yamasaki & Pinero (1989) showed that proliferating epithelial rests were characterized by more rough endoplasmic reticulum and free ribosomes, newly formed actin-containing microfilaments, less prominent tonofilaments and desmosomes and loss of gap junctions (normally present in epithelium during wound healing to coordinate the activities and responses of individual cells). In addition, they demonstrated a propensity of epithelial rest cells to differentiate into squamous epithelium. The proliferating cell clusters consisted of two cell types: outer basaloid cells and inner tonofilament-rich cells reminiscent of the prickle cells of a squamous epithelium.

More than a decade ago, the ERM have been shown to produce bone resorbing factors *in vitro* (Birek *et al.* 1983, Brunette *et al.* 1979) and thus they may have a role in the stimulation of osteoclastic bone resorption. This would be significant in bone remodeling during orthodontic tooth movement. Other studies indicate that the ERM may also contribute to interstitial matrix degradation (Birek *et al.* 1980, Salonen *et al.* 1991). While prostaglandins appear to be the most promising candidate for an osteolclast-stimulating factor, indomethacin (a prostaglandin inhibitor) does not entirely inhibit bone resorption (Birek *et al.*

1983). This suggests that factors other than prostaglandin may account for the osteolytic effects of epithelial rest cells.

Reitan (1961, 1994) suggested that ERM are not present in the PDL adjacent to areas of orthodontic root resorption and repair because the initial hyalinization induced by orthodontic forces would cause the epithelial cells to atrophy and never to reappear. Brice *et al.* (1991) challenged this view and noted that the use of transmission electron microscopy would be required for the positive identification of tonofilaments and desmosomes as small cell clusters of ERM cells are often overlooked at the light microscopy level.

Brice *et al.* (1991) compared the ultrastructural features of epithelial rests on freshly extracted premolars that had served as resorbing and nonresorbing root surfaces of anchoring teeth in rapid orthodontic maxillary expansion. The authors described two distinct cell types related to root surfaces: pale-stained and dark-stained cells. At the electron microscopy level, the pale cells had few ribosomes and tonofilaments and a highly vacuolated cytoplasm. The dark-staining cell clusters (containing from 2-8 cells and variably located between 10 to 100μm from the repairing root surface) were true epithelial cells as they contained both tonofilaments and true desmosomes. The epithelial cell clusters associated with orthodontic root resorption did not contain any pale cells. Dark cells, on the other hand, were found to be located preferentially in the repairing resorption bays. Their cytoplasm contained many polyribosomes but lacked an apparent endoplasmic reticulum or Golgi apparatus. Earlier, Fawcett (1981) had suggested that such characteristics are indicative of a proliferating cell that is

synthesizing protein as part of cytoplasmic renewal. From these electron microscopic observations, Brice *et al.* (1991) concluded that following orthodontic tooth movement, a regrowth of epithelial cells into resorption bays occurs, and that this process might be associated with the regeneration of the periodontal ligament as well as repair of the resorptive root defect. However, as Ten Cate (1996) pointed out, the changes observed by Brice were not the result of tooth movement, but rather reflected the involvement of anchoring teeth in rapid maxillary expansion.

Three dimensional computer reconstructions of serial sections confirmed the findings of Brice *et al.* (1991) regarding repairing resorption bays and showed that the relationship of epithelial cells and blood vessels to the resorption lacunae appears to depend on the level of resorptive activity in the bay (Kittel & Sampson 1994). However, it is still unclear whether epithelial cells maintain the PDL by producing bone resorbing factors, by acting as a physical barrier to the encroachment of alveolar bone, or by inhibiting the proliferation of blood vessels on one side while stimulating reparative cementum on the other.

Earlier studies of PDL regeneration (Andreasen 1988) and apicoectomy repair (Andreasen 1973) also questioned the role of ERM in tooth resorption. In both of these repair processes, cementum and PDL repair were found without evidence for regeneration of ERM. In one experiment, a window was made through the buccal PDL and a 4 X 4 mm wide cavity was made in the root surface. Removal of the remaining PDL from the socket wall during wound healing was accomplished by reversing the buccal bone plate. Three months later a new

periodontal ligament had grown into the cavity area with direct apposition of new cementum and no evidence of root resorption. This repair process occurred universally in the absence of ERM cells. Likewise, cementum and PDL repair after apicoectomy in humans occurred without regeneration of ERM.

It is this type of disparity in the interpretation of earlier observations that still remains to be addressed by fresh studies.

Other investigators have shown that proliferating ERM have a strong capacity for interstitial matrix degradation due to secretion of collagenolytic enzymes (Harris & Goldhaber 1973, Brunette *et al.* 1977, Brunette *et al.* 1979), collagenolytic inhibitors (Brunette *et al.* 1979), gelatinase, tissue inhibitor metalloproteinases, and the presence of acid collagenolytic cathepsins (Salonen *et al.* 1991). The signal for these cells to release these degrading enzymes was believed to be an interrupted basal lamina. In pathologic conditions the disrupted basal lamina may allow abnormally stimulated epithelial cells to extensively destroy the adjacent connective tissue and allow proliferation of the epithelial cells as often seen in initial cyst formation.

While all the possible mechanisms that may stimulate epithelial rest cell proliferation remain unclear, it is widely believed that inflammation and local accumulation of immune cells may direct the rest cells to proliferate and differentiate (Torabinejad 1983). Activated immune cells produce various soluble mediators that regulate multiple signal transduction cascades of other immune cells and nonimmune cells as demonstrated in numerous *in vivo* and *in vitro* models. It is possible that these mediators influence activation of the ERM,

promote differentiation, and alter keratinization in ERM cells as well. Gao *et al.* (1988) observed that the proliferation of rest cells to form the lining of inflammatory dental cysts was associated with a change from a simple, nondifferentiated epithelial phenotype to that of a more complex, stratified non-cornifying epithelium in which several epithelial keratins are co-expressed. Brunette (1984a, 1984b) has shown that the growth of epithelial rests occurs in response to mechanical stretching as well as subsequent to elevations of intracellular cAMP. It has also been suggested that growth factors such as epidermal growth factor (EGF), cell-to-cell, and cell-to-matrix interactions may play an important role in epithelial rest cell proliferation (Windnell & Pfenninger 1990). The studies of Thesleff (1987) have provided strong evidence that the binding of EGF to its receptor on epithelial rest cells plays a central role in the functional activation of these cells.

2.1 The Term "Activated" Odontogenic Epithelium

In the context of this study, as in many previous studies, proliferating and/or differentiating epithelial clusters arising in the epithelium of the odontogenic apparatus are referred to as "activated". The use of the term is borrowed from Ten Cate's work demonstrating a strong association between cell proliferation and the morpholoigical, functional, and histopathological features of differentiation in odontogenic epithelia (Ten Cate 1996). The term "activated" has been used previously in reference to a variety of *in vitro* and *in vivo* differentiated functions of cells derived from the ERM, including osteolytic activity

(Birek *et al.* 1983, Brunette *et al.* 1979), matrix degradation (Birek *et al.* 1980, Salonen *et al.* 1991), collagen phagocytosis (Birek *et al.* 1980), and EGF-receptor-mediated functional activation (Thesleff 1987).

2.2 Epidermal Growth Factor (EGF) and ERM Activation

The effects of EGF were first observed in an animal model of wound healing (Niall *et al.* 1982). The ability of EGF to accelerate incisor eruption and eyelid opening (Cohen 1962) had been demonstrated earlier. EGF is a single-chain acidic polypeptide containing 53 amino acid residues (reviewed by Shah 1998). In humans, it is secreted primarily by salivary glands, the duodenum, and the pancreas (Konturek *et al.* 1989). EGF is a chemotactic factor for fibroblasts and endothelial cells, a mitogen in keratinocytes, fibroblasts and endothelial cells, and an inducer of angiogenesis in wound healing (Shah 1998). Buckley *et al.* (1985) reported increased formation of granulation tissue when exogenous EGF was added to subcutaneously implanted polyvinyl alcohol sponges.

Thesleff (1987) observed that very intense binding of ^{125}I-labelled EGF was localized in the ERM of the dental follicle, and noted that in many cells (i.e. basal epidermal cells) the number of EGF receptors is related to cell proliferation. These observations led to speculation that activation of the epithelial rest cells in various pathologic conditions and inflammation may be associated with EGF. Several possible sources of EGF in the periodontium have been proposed, among them an elevated EGF synthesis in the residual tissues and/or exposure of the rest cells to salivary EGF following tissue injury. While it is known that

many different cells are capable of synthesizing EGF, little is known at present as to how that synthesis is controlled.

EGF exerts its effects on cells through binding to a high affinity cell surface receptor (EGFr) which leads to activation of a tyrosine kinase in the intracellular part of the receptor (Brown *et al.* 1994, Carpenter 1987, Carpenter & Cohen 1990). Many of the activating molecular phosphorylations that occur in response to other extracellular cues are not on tyrosine but on serine/threonine residues. Using immunolocalization, Irwin *et al.* (1991) found that EGF receptors were detectable in the epithelial cells of inflamed gingival tissue biopsies while they were not present in uninflamed specimens. Similar to Thesleff (1987), they postulated that, in inflammation, cell surface receptors are upregulated to increase the cellular response to EGF. Nordlund *et al.* (1991) provided evidence that seemed to contradict the suggestion that the number of EGF receptors is related to cell proliferation. Using monoclonal antibody labeling, they showed that quiescent ERM in normal periodontium expressed high quantities of EGF receptors on their surface. Li *et al.* (1993) also provided evidence that would appear to contradict the correlation of inflammation with the level of EGF receptors. Their results demonstrate that EGFr are expressed with greater intensity in the so-called developmental odontogenic cysts (i.e. odontogenic keratocyst and dentigerous cyst) than in inflammatory cysts (i.e. radicular cysts) or in proliferating ERM within periapical granulomas. ***In view of these seemingly contradictory results, the growth factor requirements of various***

cysts and epithelial rests and the role of inflammation in EGF receptor expression remain to be investigated further.

2.3 ERK1 and ERK2 (Extracellular Signal-regulated Kinases)

In vitro studies suggest that cell proliferation requires continuous exposure to EGF (Shah 1998); therefore, it is logical to suggest that known mediators downstream of the EGFr would be detectable in proliferating and/or differentiating ERM. Upon binding of EGF to the epithelial cell membrane receptor, signal transduction involving activation of common protein kinases, phosphorylation of regulatory proteins, upregulated gene transcription and protein synthesis would lead to cell proliferation, as proposed previously in other systems (Fig. 1).

Within the last decade, mitogen-activated protein (MAP) kinases (or extracellular signal regulated kinases – ERKs) have been shown to respond to growth and differentiation factors, in pathways mediated by receptor tyrosine kinases, heterotrimeric G protein-coupled receptors, or cytokine receptors (reviewed by Lewis *et al.* 1998). MAP kinases were initially described in cells that were stimulated with growth factors, hence the name "mitogen-activated".

ERK1 and ERK2 and their upstream regulators MKK1 (MAP kinase kinase) and MKK2 are activated at two regulatory phosphorylation sites with the sequence T(P)-X-Y(P) located in the "activation lip" between subdomains 7 and 8 of the conserved kinase core sequence (Lewis *et al.* 1998). Several different MAP kinase kinase kinases (i.e. the Raf family, c-Mos, NEK kinases, and

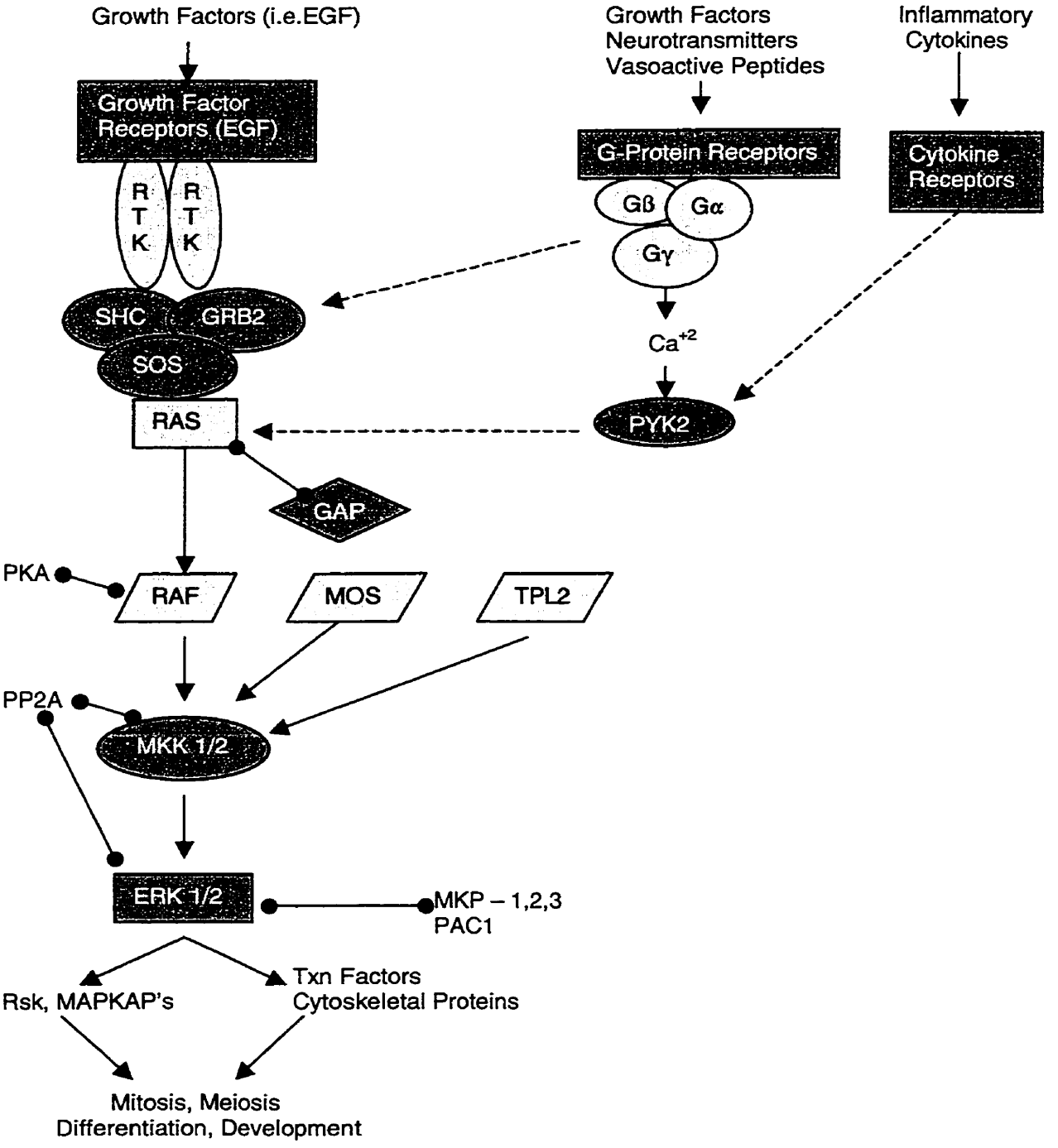

Fig. 1. Downstream and upstream mediators of the ERK 1/2 pathway. Positive signaling events are represented by solid pointed arrows (→) and inhibitory signaling is represented by blunted arrows (●—●). Signaling events that are indirect or mechanistically uncharacterized are denoted by dashed arrows. (Lewis *et al.* 1998)

multilineage protein kinases) can phosphorylate and activate each MKK (Lewis *et al.* 1998).

It is presently uncertain as to why two forms of ERK exist; however, unlike MKKs, each ERK is recognized specifically by its respective MKK (Lewis *et al.* 1998). ERK1 and ERK2 are 44- and 42-kDa enzymes, respectively, with 90% sequence identity (Boulton *et al.* 1991). Both enzymes are "robustly" activated on cell stimulation (Lewis *et al.* 1998) and are ubiquitously distributed in cells, although some variation in expression between various tissues has been noted (Boulton & Cobb 1991). Activation occurs via Tyr phosphorylation preceding phosphorylation of Thr within the activation lip at Thr_{183}–Glu-Tyr_{185} (Haystead *et al.* 1992). In general, both phosphorylation events are required to fully activate wild-type ERK1 and ERK2 (Boulton & Cobb 1991, Robbins *et al.* 1993). Boulton & Cobb (1991) identified at least three forms of ERK1 in stimulated cells, two of which are inactive: unphosphorylated and inactive, tyrosine phosphorylated and inactive, and tyrosine activated and active (due to serine/threonine and/or additional tyrosine phosphorylations). Inactivation of ERK with phosphatases has provided direct evidence that both ERK 1 and ERK2 are regulated by phosphorylation on both tyrosine and serine/threonine (Boulton & Cobb 1991).

Activation of ERK1/2 is mainly cytosolic (Ben-Levy *et al.* 1998) and the phosphorylated enzyme (pERK) translocates to the nucleus (Chen *et al.* 1992, Sandy 1998, Traverse *et al.* 1992) where it can phosphorylate various nuclear transcription factors (Hill *et al.* 1993). A new and potentially interesting area

involves the possibility that integrins may be upstream regulators for MAP kinase production. Integrins act as bridges between the extracellular matrix and the actin filaments of the cytoskeleton and they have been shown to have effects on tyrosine kinases that are similar to those activated by growth factors (Sandy 1998). Pece & Gutkind (2000) found that E-cadherins stimulate the MAP kinase pathway through the engagement of tyrosine kinase receptors for EGF. This ligand-independent activation of EGF receptors provides a novel molecular mechanism where E-cadherins can stimulate MAP kinases.

2.4 Mechanism of EGF-induced MAPK Activation in the A431 Epithelial Cell Line

The A431 epidermoid carcinoma cell line (Giard *et al.* 1973) has been used extensively as a positive control in studies of EGF-induced MAPK activation. Silvy *et al.* (1998) proposed a mechanism of signal transduction induced by high and low EGF concentrations in A431 cells. They note that p42 MAPK is activated by both low and high EGF doses. Low mitogenic doses of EGF induce MAPK activation without c-Raf, MEK1, and MEK2 activation. Under these conditions, the intermediate signaling components are unknown. The persistent and sustained activation of MAPK via this mechanism is accompanied by its translocation into the nucleus to activate transcriptional factors. In the presence of high doses of EGF, MAPK activation is driven by Ras, c-Raf-1 and MEK1/2; however, MAPK kinase remains in the cytosolic compartment. In this case, the intervention of an unidentified component (X) that drives the signal from MAPK to transcription is proposed. Both MAPK pathways result in differential regulation of

the expression of genes involved in cell proliferation or differentiation. Another theory hinges on the notion that additional events (i.e. calcium fluxes, phopholipase Cγ activation, cytoskeleton reorganization, etc.) activated by high doses of EGF might act by interfering with the signaling cascade induced by low EGF concentrations.

A431 cells do not express MAP kinases solely in response to growth factors. Xue & Lucoq (1997) demonstrated that ERK2 can be activated in A431 cells exposed to low pH media. Surprisingly, low pH caused a parallel activation of ERK2, JNK and p38. The effects of low pH on MAPK pathways is not yet known. Acidic conditions may trigger a cellular response that acts via integrated signals of multiple MAP kinase pathways.

3. OBJECTIVE OF THE STUDY AND HYPOTHESES

3.0 Objective of the Study

- In light of the above, the overall, long-term objective of the research is to determine if MAP kinases (i.e. ERK1 and the phosphorylated/activated form (pERK)) play a role in proliferating and/or differentiating ERM and other related odontogenic epithelia.
- The primary purpose of the study was to determine the distribution of ERK1- and pERK-expressing cells in tissues derived from odontogenic epithelium and to correlate ERK-expression with: a) PCNA expression (marker of cell proliferation), b) histomorphology, and c) PAN cytokeratin expression (marker of epithelial differentiation).
- A secondary purpose of the study was to determine whether ERK1 and/or pERK expression is associated with inflammation.

3.1 Hypotheses

We hypothesize that ERK1 and its phosphorylated form, pERK, are overexpressed in proliferating and differentiating odontogenic epithelia of dental follicles and odontogenic cysts and that the presence of inflammation is associated with elevated expression of ERK1 and pERK.

The principle hypotheses for this study are stated as follows:

1. There is a statistically significant difference in the expression of ERK1 and/or pERK between activated (proliferating and/or differentiating) and resting odontogenic epithelial cells.

2. There is a significant difference in ERK1 and/or pERK expression between uninflamed and inflamed odontogenic epithelia.

4. Materials and methods

4.0 Tissue Samples and Tissue Classification

A total of 30 biopsy specimens were selected from the archives of the Division of Oral Pathology of the University of Manitoba. Five of these specimens were later deleted from the statistical study due to high background immunoperoxidase staining or the absence of comparable cell clusters in serial sections. Tissues specimens of follicles (n = 8), dentigerous cysts (n = 4), radicular cysts (n = 8), lateral radicular cyst (n = 1), odontogenic keratocyst (n = 1), and developmental odontogenic cysts (not otherwise specified) (n = 3) were examined. For each tissue type, the following microscopic features were examined: the general architecture of the tissue, the histological appearance of the lining epithelium, the features of the epithelial rest cells and the presence or absence of inflammatory cell infiltrates.

All specimens had been fixed in 10% neutral buffered formalin (18-24 hours.) and processed routinely paraffin embedding. Serial 6μm sections were mounted to Colorfrost Plus slides (Fisher Scientific Co.) by floating the sections in a preheated water bath filled with distilled water. The mounted specimens were then dried overnight at 37°C in preparation for immunohistochemistry.

4.1 Histomorphology

Our primary criteria for the classification of epithelial differentiation were histomorphological:

1) Squamous differentiation in an epithelial cluster was defined as the presence of large cells with abundant eosinophilic cytoplasm, grouped together in clusters exhibiting intercellular bridging. By contrast, "resting" epithelial islands consisted of small cells, exhibiting very little cytoplasm arranged in typical rosettes or clusters with no apparent intercellular bridging (Fig. 2A). Similarly, differentiated cyst epithelium exhibited squamous cell differentiation and stratification (Fig. 2B) similar to that normally seen in the epidermis, while the nondifferentiated cyst lining was represented by a thin (one to two layer thick) simple cuboidal epithelium comprising small cells with scanty cytoplasm. Occasional differentiation into tall, columnar, ciliated epithelium (reminiscent of a respiratory type of epithelium) was also observed (Fig. 3, pg. 31). These histomorphological features were readily distinguished at the light microscope level.
2) Intracellular vacuolation (Fig. 4A, pg. 36) either in the presence or absence of squamous cells within the same epithelial islands was also considered as a sign of differentiation. As reviewed in Torabinejad (1983) and Shear (1992), such activated ERM cells exhibit significantly increased cytoplasm and accumulation of neutral lipid.

Fig. 2. Histomorphological features of "inactive" or "resting" and "activated" cells derived from odontogenic epithelia:

A: Section from a dentigerous cyst wall containing small islands of epithelial rest cells exhibiting some peripheral palisading of basaloid cells, but the inner cells remain undifferentiated.

B: Photomicrograph of a section from a dentigerous cyst, showing squamous cell differentiation of the surface epithelium (arrow); the underlying connective tissue is infiltrated by chronic inflammatory cells (the nuclei are stained with hematoxylin).

4.2 Immunohistochemistry

4.2.a Method

From each specimen, four parallel (serial sections), 6μm thick, were processed for immunohistochemistry with affinity purifed anti-ERK1, -pERK, -PCNA, and PAN cytokeratin antibodies, respectively (Table 1). A streptavidin-biotin immunoperoxidase labeling- (LSAB) and detection- (DAKO) kit was employed according to the specifications of the suppliers. The DAKO LSAB+ Peroxidase kit is specific for primary antibodies (monoclonal or polyclonal) from rabbit, mouse and goat.

In brief, sections were deparaffinized in xylene and rehydrated through a graded series of alcohols. Endogenous peroxidase activity was blocked with 1.0% hydrogen peroxide for 30 minutes. Nonspecific antibody binding was blocked by treating the sections with 0.1% pepsin in 0.01N HCl (pH 2.3) for 30 minutes prior to immunostaining. Diluted antibodies were applied to the sections and incubated for 2 hours at 37ºC (listed by source and dilution factor in Table 1).

Table 1. Antibody specifications

Antibody	*Specificity*	*Type*	*Dilution*	*Manufacturer*
ERK 1 (C16)	ERK 1 p44 > ERK 2 p42	polyclonal goat	1:500 PBS	Santa Cruz Biotechnology, Inc.
p-ERK (E-4)	Tyr-204 phosphorylated ERK 1 and ERK2	monoclonal mouse	1:500 PBS	Santa Cruz Biotechnology, Inc.
PCNA	PCNA	monoclonal mouse	1:50 PBS	DAKO
PAN Cytokeratin	NCL-5D3 Keratins 8 and 18 NCL-LP34 Keratins 5,6 and 18	monoclonal mouse	1:25 PBS	Novocastra Laboratories, Ltd.

After rinsing in PBS buffer (0.01 M phosphate-buffered saline, pH 7.6), the primary antibodies were detected with an enzyme-linked (horseradish peroxidase) avidin-biotin detection system (DAKO) using diaminobenzidine as the chromogen. The sections were overlaid with biotinylated immunoglobulin (DAKO) for 30 minutes. Unbound conjugate was removed by washing in PBS buffer at room temperature. The sections were then overlaid with peroxidase-labelled streptavidin (DAKO) for 30 minutes. Excess peroxidase-bound streptavidin was removed by washing in PBS. Then the antibody-bound peroxidase was visualized using diaminobenzidine (DAKO) as the chromogen reagent for 5 minutes. After washing in water, the specimens were counterstained with hematoxylin for 1 minute. Excess hematoxylin was removed by rinsing gently with distilled water and dipping briefly 10 times in 37mM ammonia water. The specimens were dehydrated through graded alcohols. They were coverslipped with a nonaqueous mounting medium (Permount). Demasking of the antigen substrates by microwave exposure was not performed as this technique is known to create false positive results.

4.2.b Negative Control

For each batch of sections processed for immunohistochemistry, two parallel sections of each tissue was included as a negative control in which primary antibody was omitted and replaced with normal mouse and goat IgG (10 μg/ml; Santa Cruz Bio.).

4.2.c *Rationale for use of the A431 Cell Line as a Positive Control*

Previous studies have shown that both ERK1 and pERK are overexpressed in epidermoid carcinoma A431 cells stimulated with EGF (Chen *et* al. 2000, Silvy *et al.* 1998, Zi & Agarwal 1999). For example, Chajry *et al.* (1996) have demonstrated, in the A431 cell line, the dual stimulatory/inhibitory effects of EGF on the activity of a serine/threonine protein kinase (p42 MAP kinase) that occupies a pivitol position in the signal transduction triggered also by a variety of other growth effectors. In response to growth stimulatory doses of EGF (10^{-12} M) this MAP kinase was shown to be activated and this persisted throughout cell treatment. Conversely, a sharp, but transitory activation of MAP kinase followed by its rapid decrease to basal levels was observed under conditions of EGF (10^{-9} M) growth inhibition. In this study, we chose to activate the A431 cells with the lesser concentration of EGF (10^{-12} M) in order to take advantage of both growth stimulation and the persistent MAP kinase activation.

PD98059 has been shown to act *in vitro* as a highly selective inhibitor of MEK1 activation and the MAP kinase cascade (Pang *et al.* 1995). It binds to the inactive forms of MEK1 and prevents activation by upstream activators such as c-Raf. Hence, incubation of A431 cells with PD98059 prior to EGF induction would demonstrate decreased pERK expression; further indicating that the EGF-stimulated A431 cell line was a good choice for positive control. Since PD98059 inhibits MEK1, which prevents phosphorylation of ERK1, the expression of ERK1 would be equivalent in the presence or absence of PD98059. However, ERK1 is expressed with less intensity and in a fewer proportion of A431 cells that are not

stimulated with EGF. In order to verify the validity of A431 cells as controls, we have conducted a preliminary study of ERK1 and pERK expression in these cells, under conditions of EGF stimulation or in the absence of EGF, and in the presence or absence of PD98059.

In this study A431 cells were incubated with EGF for 5 min based on a previous study (Chen *et al.* 2000) where it was observed that EGF stimulated both ERK1 and JNK activities in a time-dependent manner. Activation of ERK1 and JNK could initially be observed in cells treated with EGF for 0.5 min, and the maximum response was observed in cells treated with EGF for 5 min.

EGF-stimulated A431 (human epidermoid carcinoma; American Type Cell Culture Collection, ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% streptomycin-penicillin, and 0.25 μg/ml fungizone. The cells were grown at 37°C under 5% CO_2 in air. Subcultures were performed by lifting of the cells by trypsinization according to the protocols provided by the supplier of the cells (ATCC) and seeding onto sterile microscope glass slides by cytospin centrifugation at a density of 5 X 10^5 cells/mm^2. 70-80% confluent cultures were serum starved for 36 h, and during the last 2 hours of starvation, they were treated with either 0.1% DMSO or 50 μM PD98059 (MEK1 inhibitor) in 0.1% DMSO (Pang *et al.* 1995); EGF (10^{-12} M) was then added to the cultures and incubated for 5 min at 37°C. The cells were fixed with 10% neutral buffered formalin (18-24 h.) and dehydrated with a series of graded alcohols. In order to further reproduce the processing conditions of the

experimental tissue sections, the slides carrying the control cells were paraffinized by dipping in melted paraffin at 50°C.

4.2.d Rationale for the Use of PAN-cytokeratin as a Marker of Differentiation

NCL-PAN cytokeratin is a broad spectrum anti-cytokeratin for general use to identify differentiated, complex, keratinized epithelia. It is commonly used in conjunction with a wide panel of antibodies in the differential diagnosis of malignant tumors. The rationale for use of NCL-PAN cytokeratin in this study was to confirm the state of differentiation previously determined by histomorphological characteristics. We assumed that, as with all anti-cytokeratin antibodies, poorly differentiated epithelia will fail to stain.

Cytokeratins are filaments containing keratin-like proteins that are characteristic of epithelial cells. They show biochemical and immunological relationships of various degrees of overlap and are expressed in different epithelia, in different combinations of polypeptides ranging in their isoelectric pH values from 5 to 8 and in their apparent molecular weights from 40,000 to 68,000 KD (Moll *et al.* 1982).

4.2.e Rationale for the Use of PCNA as a Marker of Proliferation

PCNA is a 36 kDa acidic non-histone nuclear protein that is intimately involved in DNA synthesis as an auxiliary protein for DNA polymerase δ (Hall *et al.* 1990). It was originally detected with serum from patients with systemic lupus erythematosus, which was found to contain an antibody against a nuclear

antigen present in proliferating cells (Miyachi *et al.* 1978, Yu & Filipe 1993). A number of monoclonal antibodies to PCNA have been produced, including PC10 (Waseem & Lane 1990) which was used in this study. The anti-PCNA antibody PC10 has been most widely used (reviewed by Yu & Filipe 1993) as a marker of active proliferating cells in clinical applications of formalin-fixed paraffin-embedded tissue in the recent literature.

Two populations of PCNA are present at S-phase (Bravo & Macdonald-Bravo 1987, Hall & Woods 1990). One form is nucleoplasmic, representing PCNA which is highly soluble and present at low levels in quiescent cells (but cells that are capable of division) not involved in replication. The other form is insoluble and associated with sites of DNA synthesis. Morris & Mathews (1989) have shown that there is only a small change in the total level of PCNA during the cell cycle but there is a dramatic alteration in the insoluble fraction associated with DNA replication. In long-term quiescent cells, PCNA is undetectable by immunoblotting methods, but it is present at low levels (10 percent of that found in cycling cells) in cells capable of division (Hall *et al.* 1990).

PC10 immunoreactivity has been found to correlate with bromodeoxyuridine labeling in methacarn-fixed paraffin-embedded rat mammary tumors (Wijsman *et al.* 1992). However, it was found that the percentage of cells detected by PC10 was always higher than the bromodeoxyuridine labeling indices and it corresponded better to the growth fraction. It is in light of these facts that we have selected PCNA as a marker of proliferation in odontogenic epithelia.

4.3 Quantification and Statistical Analyses

The intensity of nuclear and/or cytoplasmic positive staining with ERK1, pERK, and PCNA was determined. Two parameters were evaluated: 1) the overall staining pattern (focal or diffuse) and 2) the staining intensity, scored on a scale of 0 (no staining above background) to "mild", "moderate" or "strong" (Fig 3). A cell was considered as positive if it exhibited at least moderate staining. In

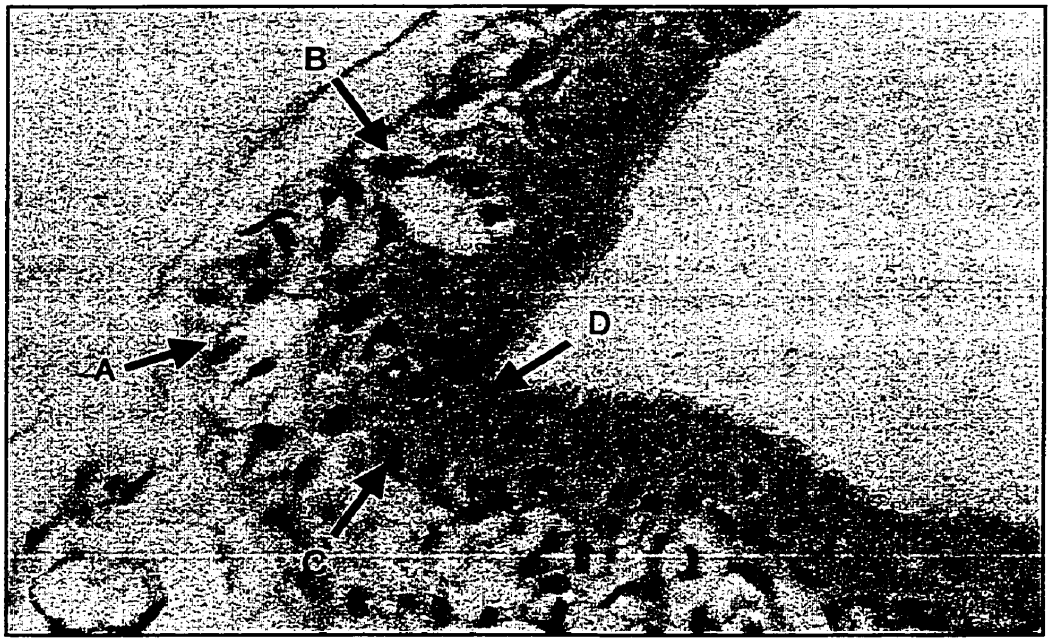

Fig. 3. Immunoperoxidase staining intensity classification in a representative odontogenic epithelial specimen (follicle). A) no staining above background, B) mild, C) moderate, D) strong.

order to increase the validity of the comparisons between antigens, epithelial cell clusters (islands or lining) under observation had to be present in all serial sections with minimal background staining. So as not to overestimate ERK1 and pERK expression in the samples, each section was counted twice on separate occasions and if there discordance between the two observations, the lower of

the two scores was considered in the final evaluation. Only the presence of nuclear staining was considered positive for PCNA. All scoring of staining was performed in a blind fashion without prior knowledge of section treatment.

Within each cell cluster, 100 cells were observed and the number of positively stained cells was counted in this manner. The counting was facilitated by a grid incorporated in the ocular lens.

The original histopathological diagnoses were reconfirmed by careful examination under a light microscope. Squamous cell differentiation and intracellular (perinuclear) or intercellular vacuolation within ERM-cell clusters was interpreted as representing functional differentiation or "activation". The location of the epithelium was noted as either cyst lining or islands embedded in the cyst wall or connective tissue. The degree of inflammation was interpreted by the density and nature of the inflammatory cell infiltrate (chronic and/or acute) under light microscopy and this was confirmed with the original pathology report from the Oral Pathology Dept. at the University of Manitoba Faculty of Dentistry. Various degrees of differentiation were determined based on histomorphological characteristics of the specimen and this was later confirmed by noting the expression of PAN cytokeratin (<25% staining was considered (-) differentiation and >25% staining was considered (+) differentiation). Intracellular vacuolation of individual epithelial cells within the clusters was interpreted as a feature of differentiation, as it is generally observed in initial cyst development.

The data were expressed as the mean proportion (%) of cells exhibiting positive staining per tissue group ± SE (classified as shown in Figs. 6-9). For

each tissue group, the data were derived from at least 100 cells, distributed over at least three random areas of the samples (each containing a total of at least 100 odontogenic rest cells and/or lining epithelial cells).

Multiple comparisons of the proportion of ERK1 and pERK expressing cells were made between groups classified by type of odontogenic epithelium (histopathological diagnosis), presence or absence of inflammation, the proportion of epithelial cells actively proliferating, and between resting and differentiated epithelia. The significance of differences between the groups was determined by Chi square analysis (Yates corrected). Fisher's exact test (two way) was applied in comparisons between groups that involved expected results below 5.0, as predicted by the null hypothesis of no association.

5. Results

In general, the immunoperoxidase staining obtained with pERK and ERK1 antibodies appeared as brownish deposits in the cytoplasm and/or nucleus of the tissue sections (Fig. 4B). Positive staining was limited to the epithelium, some fibroblasts and endothelial cells whereas inflammatory cells were devoid of staining (Fig. 4B). ERK1 was expressed predominantly in the cytoplasm (Fig. 4D) whereas pERK was expressed in both the cytoplasm and nucleus (Fig. 4A and 4B). Specimens in which the primary antibody was omitted and replaced with nonimmunized mouse/goat serum did not exhibit immunoperoxidase staining (Fig. 4E), however, it was observed for occasional nonspecific binding in the keratinizing cornified layer.

As expected, all of the vacuolated and differentiated squamous epithelia expressed NCL-PAN cytokeratin in the cytoplasm similar to the positive control (skin)(Fig. 4F). While most of the resting islands did not express PAN cytokeratin, a small percentage of epithelia that had been classified on histological grounds as nondifferentiated (as they lacked both vacuolation and the typical eosinophilic cytoplasm of squamous cells, but which exhibited more cytoplasm), did express cytokeratin. It appears that, in the latter, cytokeratin expression was simply associated with the epithelial phenotype rather than with advanced functional differentiation. Consequently, our primary criteria for classification of epithelial differentiation was based on histomorphological characteristics.

PCNA staining was confined almost entirely to the nucleus in most odontogenic epithelial cells (Fig. 4C). In proliferating islands of ERM, PCNA expression was generally limited to the outer basaloid epithelial cells while quiescent islands were mostly devoid of immunostaining for PCNA. PCNA expression in stratified epithelium was observed more in the proliferating basal layers. A consistent and unexpected finding was the presence of cytoplasmic and nuclear PCNA expression in differentiated stratified squamous epithelium and hyperplastic ERM islands.

The positive control cell line (A431) exhibited brownish deposits predominantly in the cytoplasm for ERK1 and in both the cytoplasm and nucleus for pERK. Immunoperoxidase staining for ERK1 and pERK in A431 cells was consistent with previous studies (as reviewed in the introduction). ERK1 was expressed in a similar proportion of cells and with similar intensity in the presence or absence of PD98059 (Fig. 5B). However, in the absence of EGF, ERK1 expression was significantly reduced, albeit still present at basal levels (Fig. 5A). pERK was expressed in both the cytoplasm and nucleus of EGF-induced A431 cells in the absence of PD98059 (Fig. 5C). Expression of pERK was significanctly reduced when the cells were incubated with the MEK1 inhibitor (PD98059) prior to EGF induction (Fig. 5D). The A431-EGF cell line cultured in the presence or absence of EGF and PD98059, therefore, served as an appropriate positive control for the ERK1 and pERK antibodies.

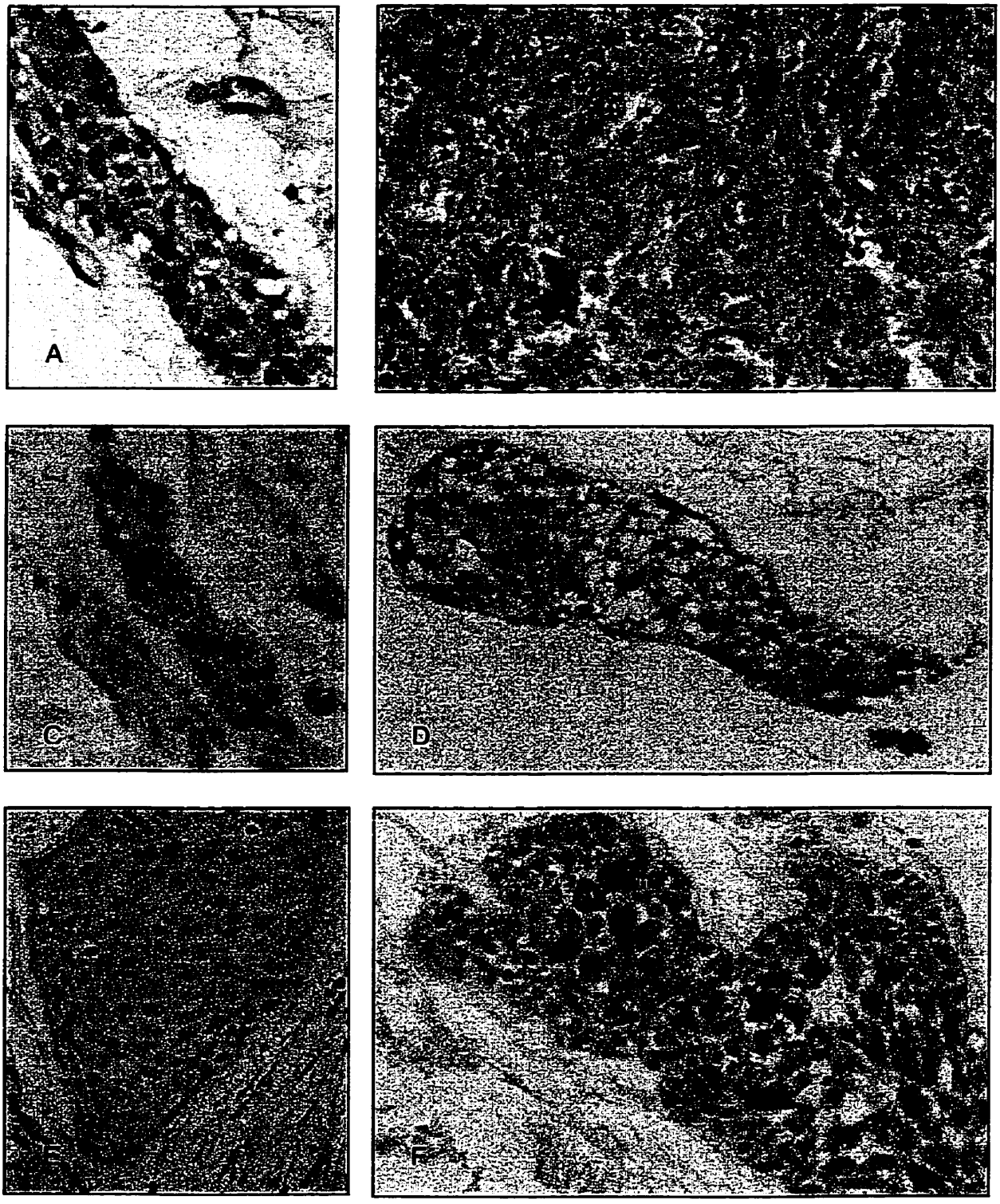
A
C
D
F

Fig. 4. Immunohistochemistry. Representative photomicrographs (original magnification = 40X).

A. *island of odontogenic epithelial cells in a dentigerous cyst treated with anti-pERK antibody* - notice intense pERK expression in the peripheral basaloid cells
B. *stratified squamous surface epithelium in a radicular cyst* - shows cytoplasmic and nuclear pERK expression
C and D. *islands of odontogenic epithelial cells in a dentigerous cyst stained with PCNA and anti-ERK1, respectively* – note intracellular and intercellular vacuolation in (D)
E. *negative control* - primary antibody was omitted and replaced with normal mouse IgG
F. *squamous differentiation in a dentigerous cyst with intense cytokeratin expression* in a large sheet of ERM cells

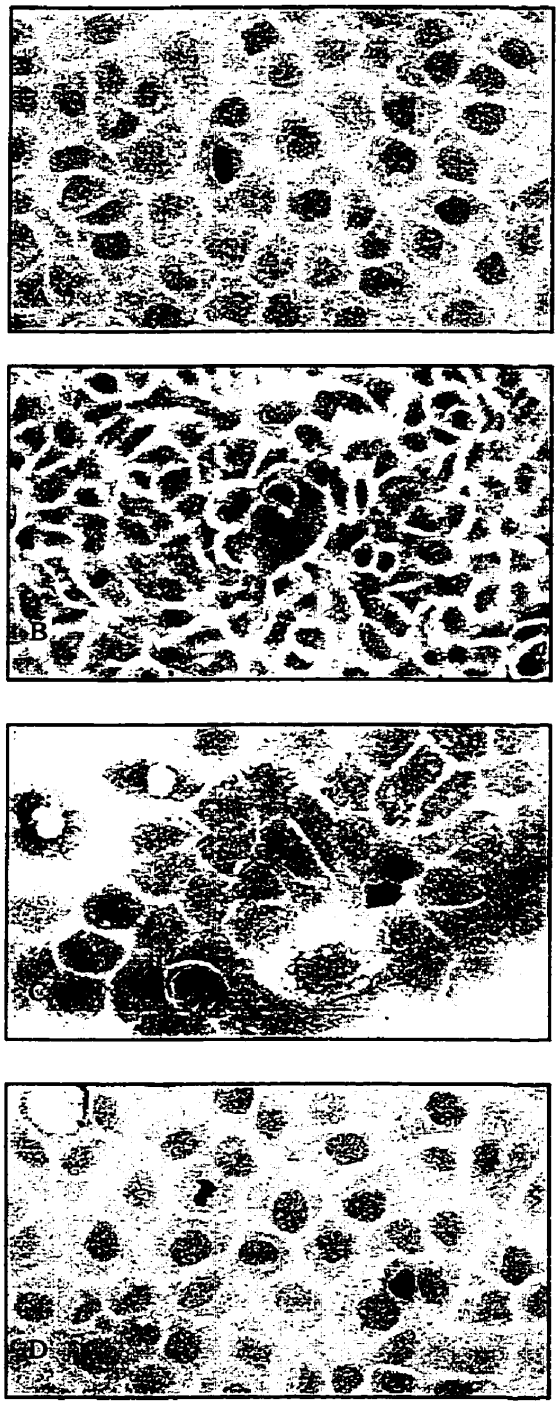

Fig. 5. Expression of ERK 1 and pERK in A431 cells. (A) ERK1 expression is significantly lower in the absence of EGF. (B) ERK1 is expressed predominantly in the cytoplasm of EGF-stimulated cells in the presence of PD98059. (C) pERK is expressed in both the cytoplasm and nucleus of EGF-stimulated cells in the absence of PD98059 (D) pERK expression, especially nuclear, is significantly decreased in the presence of PD98059. (original magnification = 40X objective)

5.0 ERK Expression in Odontogenic Epithelia

In this set of experiments, we have examined and compared ERK1 and pERK expression in the epithelia of various odontogenic tissues: radicular cysts, dentigeous cysts, and follicles. The apical radicular cysts consisted of multiple strips of fibrous connective tissues and granulation tissue and all were focally infiltrated by inflammatory cells (predominantly chronic, but with aggregates of polymorphonuclear leukocytes within the granulation tissue). pERK was expressed strongly in both the nucleus and cytoplasm of the epithelial lining of the apical radicular cysts. ERK1 was observed predominantly in the cytoplasm.

The follicles consisted of strips of loose, uninflamed fibrovascular connective tissue containing ribbons of small islands of odontogenic epithelia, and covered in places by short stretches of a thin epithelium reminiscent of reduced enamel epithelium. Only one specimen of follicular tissue was infiltrated by chronic inflammatory cells (predominantly plasma cells). Significantly fewer epithelial cells of follicles showed positive staining for pERK in comparison with radicular cysts ($18.4 \pm 7.3\%$ for follicle and $56.1 \pm 6.7\%$ for radicular cyst, $P < 0.05$) as demonstrated in Fig. 6.

The luminal lining epithelium of dentigerous cysts exhibited varied histomorphology. All of them contained simple cuboidal epithelium reminiscent of reduced enamel epithelium (a feature pathognomonic of dentigerous cysts), but in the presence of chronic inflammation, several of them demonstrated differentiated features such as those of a respiratory type or stratified squamous epithelium. The surface epithelia, as well as the larger epithelial clusters of the

cyst wall of the dentigerous cysts, as in the radicular cysts, expressed pERK intensely in both the nucleus and cytoplasm. ERK1 was expressed predominantly in the cytoplasm.

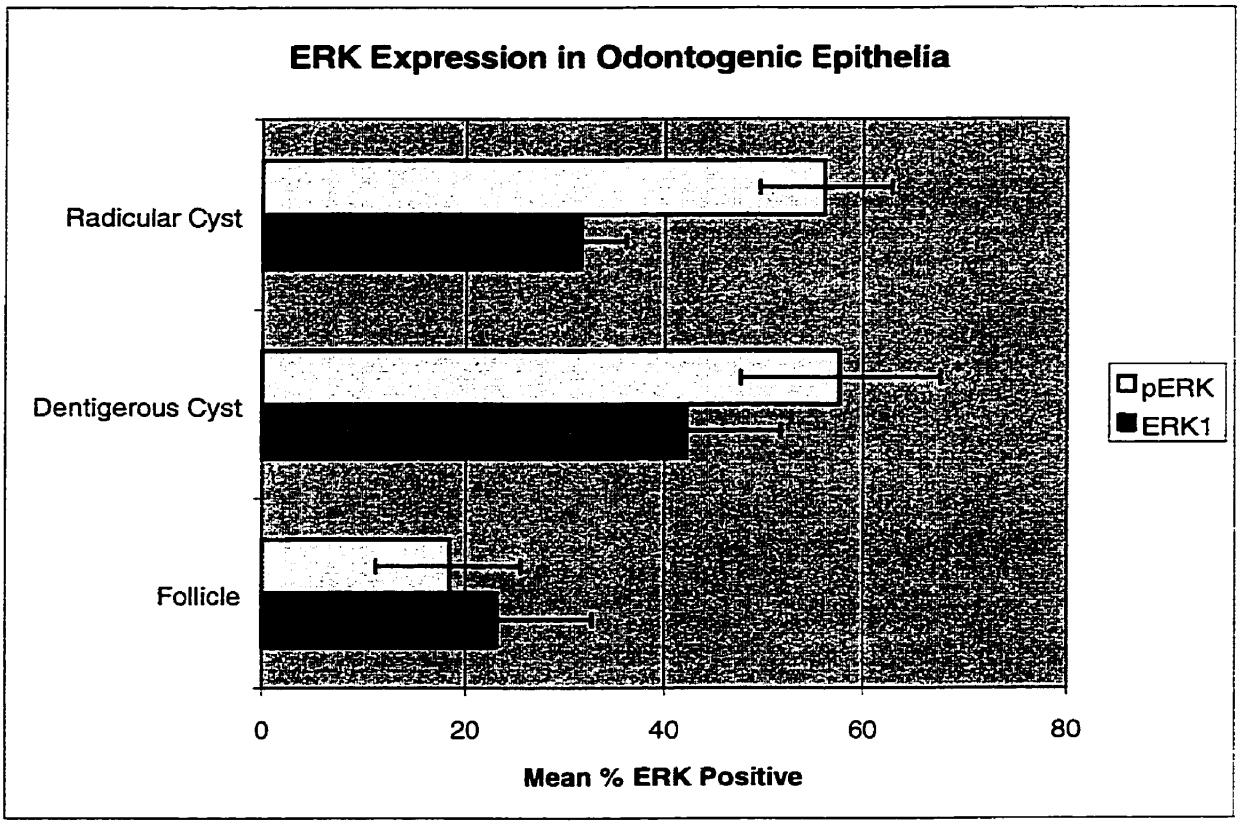

Fig. 6. ERK expression in odontogenic epithelia. The bars represent percentages (means ± standard errors) of ERK1/pERK positive epithelial cells per field (45,000 μm^2 as delineated by a graticule incorporated in the ocular lens at 40X objective) in the various odontogenic tissues examined. *The difference in pERK expression for follicles compared to radicular cysts and dentigerous cysts was significant ($P < 0.05$).

5.1 ERK Expression and Inflammation

As expected, the epithelial lining and the walls of the apical radicular cysts of our samples were heavily infiltrated with inflammatory cells and the lining showed the typical lace-like proliferation into the surrounding connective tissue. The walls of half of the dentigerous cysts examined also exhibited infiltration by chronic inflammatory cells (probably as a result of secondary inflammation of a developmental cyst). In this set of experiments, we have examined the difference in expression of ERK1 and pERK between epithelial cells surrounded by inflammatory infiltrate (chronic and/or acute cells) and epithelial cells in tissues devoid of inflammatory cells. While odontogenic epithelia of inflamed and uninflamed tissues showed no significant difference with regard to ERK1 expression, the phosphorylated form (pERK) is present in a significantly higher proportion of epithelial cells in inflamed tissue than in uninflamed tissue (56.7 ± 4.9% in inflamed tissue and 28.5 ± 9.2% in uninflamed tissue, $P < 0.05$)(Fig. 7).

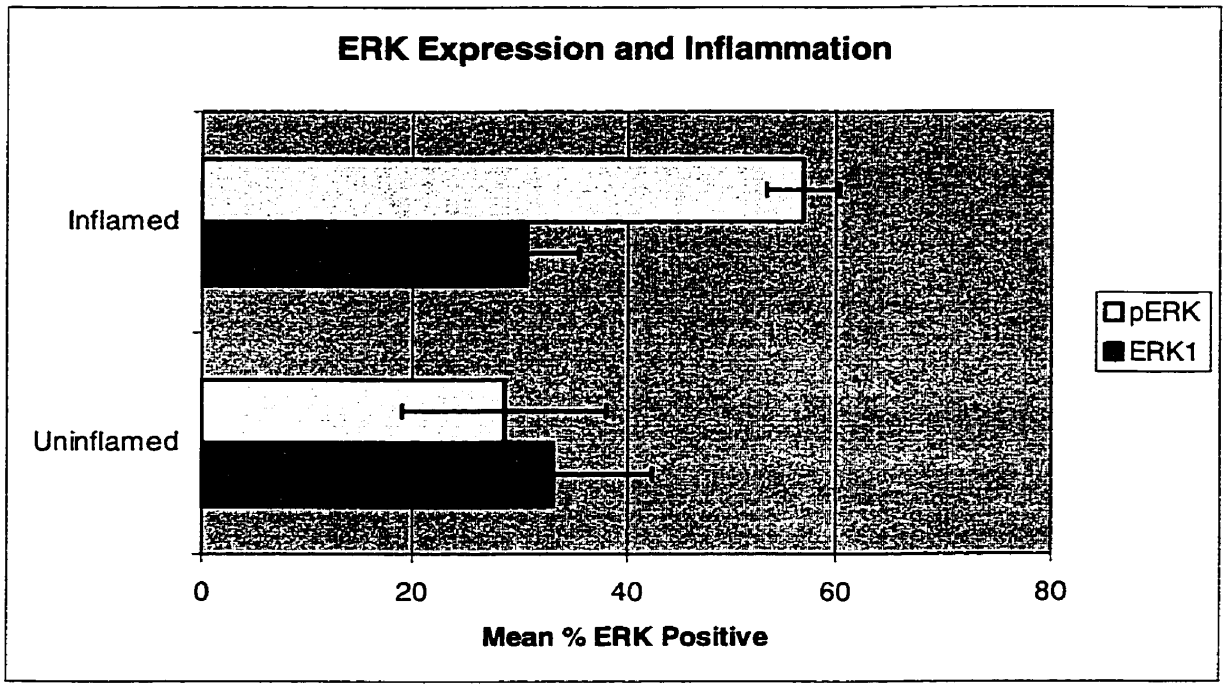

Fig. 7. ERK expression and inflammation. The bars represent percentages (means ± standard errors) of ERK1/pERK positive epithelial cells per field (45,000 μm^2 as delineated by a graticule incorporated in the ocular lens at 40X objective). The tissues are grouped according to the presence or absence of inflammation. *The difference in pERK expression between inflamed and uninflamed tissues was significant ($P < 0.05$). (Inflamed group: radicular cysts, follicles and developmental cysts infiltrated by chronic inflammatory cells with or without acute exacerbation; Uninflamed group: follicles and developmental cysts devoid of inflammatory cells)

5.2 ERK Expression and Proliferation

In this set of experiments, epithelial cell clusters were classified according to the proportion of cells undergoing proliferation (PCNA positive) as "none", "low", "moderate", and "high". ERK1 and pERK expression was determined and compared for each of these groups.

Expression of PCNA was generally limited to the nucleus in actively proliferating epithelial islands and vacuolated epithelial clusters; however, differentiated squamous epithelium consistently expressed PCNA in both the nucleus and cytoplasm of most cells. Actively proliferating epithelial cells (>25% PCNA positive) were more likely to express both ERK1 and pERK than relatively quiescent cells (<25% PCNA positive) as demonstrated in graph form in Fig. 8. Immunoperoxidase staining was more intense in the basal layers of stratified squamous epithelium and in the outer basal-like layer of epithelial islands.

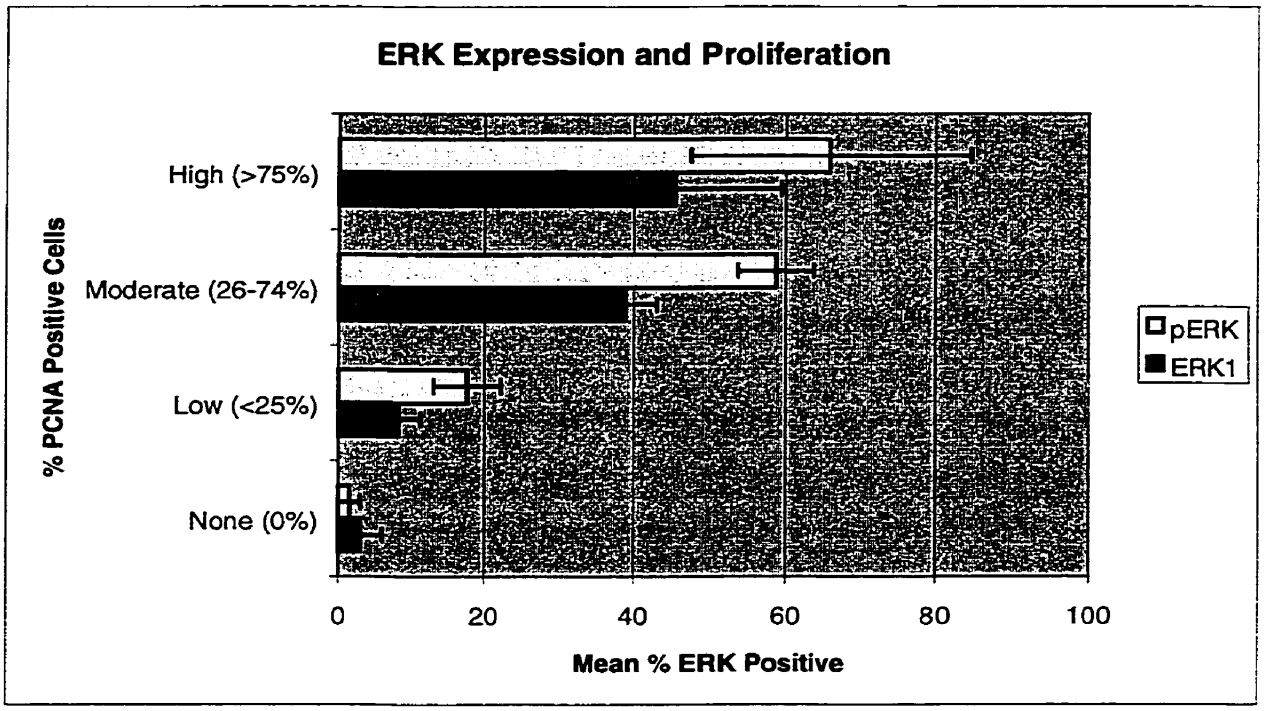

Fig. 8. ERK expression and epithelial cell proliferation. The bars represent percentages (means ± standard errors) of ERK1/pERK positive epithelial cells per field (45,000 μm^2 as delineated by a graticule incorporated in the ocular lens at 40X objective). The tissues are grouped according to the proportion of cells exhibiting PCNA binding. The asterisks indicate significant differences in pERK and ERK1 expression between *low and high and between the *low and moderate PCNA positive groups ($P < 0.05$).

5.3 ERK Expression and Epithelial Cell Differentiation

In this set of experiments, the epithelial cells were classified as either differentiated squamous, vacuolated, nondifferentiated epithelial cyst lining, or resting islands based on histomorphological characteristics and PAN cytokeratin expression. ERK1 and pERK expression was then compared in these epithelial classes.

With regards to both pERK and ERK1 expression, differentiated epithelial clusters showing both vacuolation and squamous differentiation contained a significantly higher proportion of positively-stained cells than the resting and nondifferentiated cyst lining epithelia (Table 2, comparison group I). This indicates that ERK1 expression followed by phosphorylation may take place (or be involved) in a higher level of differentiation. Those epithelial clusters containing either vacuolated or squamous epithelia did not differ significantly from each other with regards to pERK and ERK1 expression (Table 2, comparison group II). Similarly, ERK1 and pERK expression in resting epithelial islands did not differ significantly from nondifferentiated epithelial cyst lining (Table 2, comparison group III). While the means for these two groups appear to be quite different, there is statistically no difference between them. A likely reason for this is that the nondifferentiated epithelial cyst lining group was comprised of a low sample size ($n = 4$) with a high variance. Further studies with a larger sample size for this group would be necessary in order to draw definitive conclusions about this comparison.

Table 2. Epithelial Differentiation and ERK1/pERK expression

Comparison Group	Epithelial Differentiation	Mean % ERK1 (+/- SE)	P	Mean % pERK (+/- SE)	P
I	Resting Islands + Nondifferentiated Epithelial Cyst Lining	11.5 ± 3.9	p < 0.01	15.3 ± 4.4	p < 0.001
	Vs.				
	Vacuolated + Differentiated Squamous	40.7 ± 4.2		64.2 ± 4.3	
II	Vacuolated	45.8 ± 9.7	p > 0.05	55.0 ± 10.5	p > 0.05
	Vs.				
	Differentiated Squamous	38.2 ± 4.1		68.9 ± 3.7	
III	Resting Islands	6.3 ± 2.3	p > 0.05	9.0 ± 3.6	p > 0.05
	Vs.				
	Nondifferentiated Epithelial Cyst Lining	23.0 ± 10.1		29.3 ± 8.8	

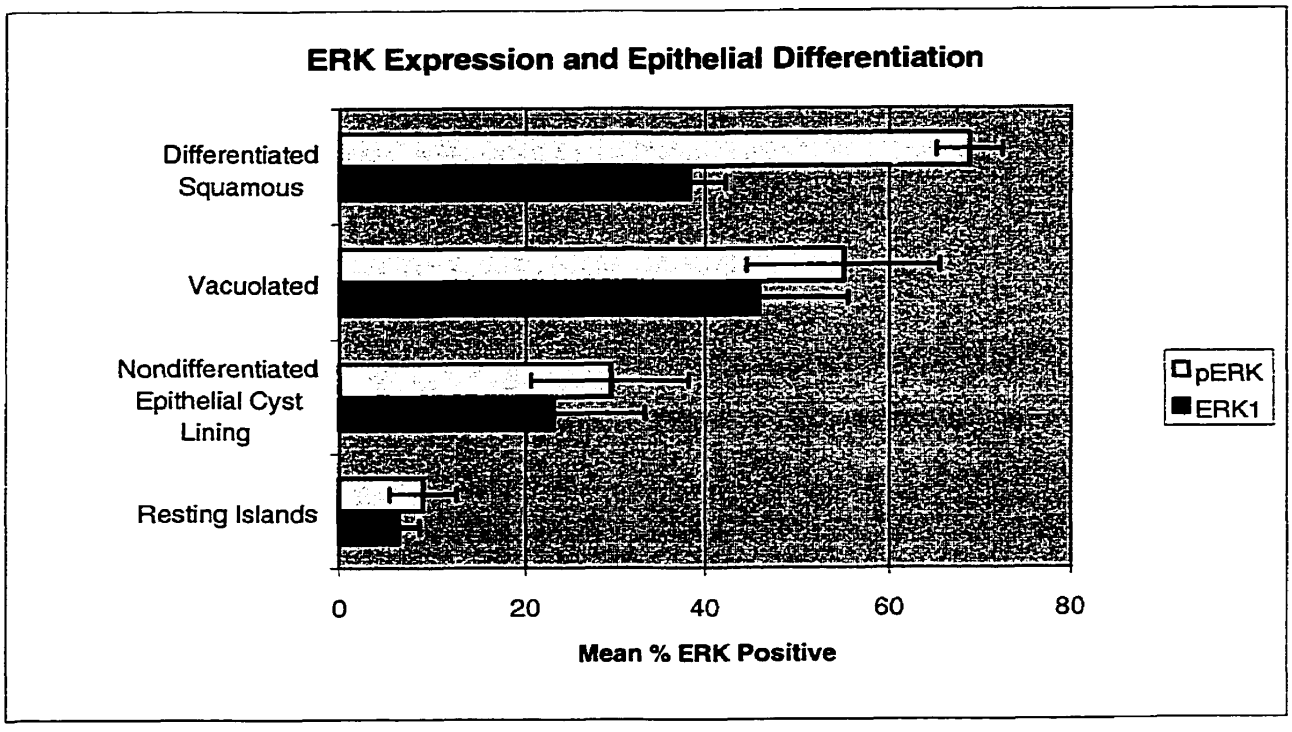

Fig. 9. ERK expression and epithelial cell differentiation. The bars represent percentages (means ± standard errors) of ERK1/pERK positive epithelial cells per field (45,000 μm^2 as delineated by a graticule incorporated in the ocular lens at 40X objective) in groups of tissue classified according to features of differentiation. The difference in ERK1 and pERK expression for resting islands and nondifferentiated epithelial cyst lining compared to vacuolated epithelia and differentiatied squamous epithelia was significant ($P < 0.01$).

In summary, the results of this study indicate that:

1. Both ERK1 and pERK, are expressed in epithelial tissues of odontogenic origin, particularly radicular cysts and dentigerous cysts.
2. ERK1 and pERK expression is associated with odontogenic epithelial cell proliferation.
3. pERK expression in odontogenic epithelium is associated with inflammation.
4. Differentiating epithelial cells (vacuolated or squamous) are associated with significantly greater ERK1 and pERK expression than resting islands or nondifferentiated cyst epithelium.

6. DISCUSSION

The data suggest that ERK activation, as monitored by expression of phosphorylated ERK, is involved in the activation of epithelial cells from "dormant" or "resting" epithelial remnants of the odontogenic apparatus, to actively proliferating and differentiating epithelial cells, during the histogenesis of odontogenic cysts. In this study, dual nuclear and cytoplasmic pERK expression, and predominantly cytoplasmic ERK1 distribution, is in keeping with the current general knowledge of MAP kinase activation in signal transduction: activation by phosphorylation at the cytoplasmic level and subsequent translocation to the nucleus to the site of transcription-factor-phosphorylation which results in mitosis and/or differentiation. For example, ERKs phosphorylate and activate transcription factors such as c-Myc and TCF/Elk1, both important regulators of the cell cycle (Marais *et al.* 1993). Further, transcription of cyclin D1 requires prolonged activation and nuclear retention of ERKs which implies a mechanism for ERK-mediated cell cycle entry (Lavoie *et al.* 1996, Cheng *et al.* 1998).

Although we can only speculate similar mechanisms may be operative in the activation of odontogenic epithelia, the following is a discussion of current knowledge of the role of MAP kinases in cellular signal transduction, as it may be relevant to the putative role of odontogenic epithelial cells in the histopathology of the periodontium. It is interesting to note that, with respect to pERK expression, observations similar to ours have been reported in other nonepithelial cell lines. For example, in human osteoblastic cells derived from the periodontal ligament mitogenic responses have been associated with selective phosphorylation and

activation of ERK 1/2 subsequent to EGF stimulation (Matsuda *et al.* 1998). **However, to the best of our knowledge this is the first report of ERK expression associated with the activation of odontogenic epithelium.**

6.0 The Role of MAP Kinases and Epithelial Differentiation

The data from this study clearly illustrate that both ERK1 and pERK are expressed more in epithelial cells undergoing differentiation. Epithelia exhibiting vacuolation and squamous differentiation exhibited a significantly higher proportion of positively-stained cells than resting and nondifferentiated cyst lining epithelium. No significant differences in ERK expression existed between vacuolated and differentiated squamous epithelia and between resting islands and nondifferentiated epithelial cyst lining. This supports the initial assumptions that both vacuolation and differentiation are histomorphological criteria for activation. Intracellular vacuolation, either in the presence or absence of squamous cells, appears to be a sign of differentiation. In addition, nondifferentiated cyst lining appears to be as quiescent as resting epithelial islands with respect to ERK expression.

A review of the literature by Lewis *et al.* (1998) shows that highly conserved MAPK cascades are important convergence points that transduce extracellular receptor signals to intracellular cues that coordinate responses (such as functional differentiation) appropriate for that specific MAPK. Three distinct signaling pathways characterize the MAPK family: ERKs, c-JUN amino-terminal kinases/stress-activated protein kinase (JNKs/SAPK) and p38/HOG genes (Cobb

& Goldsmith 1995, Groom *et al.* 1996, Hoffmeyer *et al.* 1999, Whitmarsh *et al.* 1998). While ERK and JNK/SAPK kinases are structurally related and activated by similar kinase cascades, they differ in extracellular stimuli and function. ERKs are activated by growth factors, whereas JNK/SAPK kinases are activated by various forms of environmental stress such as heat shock, UV, osmotic shock, cytokines, protein synthesis inhibitors, and antioxidants (Adachi et al. 2000, Adler *et al.* 1995, Sluss *et al.* 1994, Su *et al.* 1994).

While JNK/SAPK are not sensitive to growth factors and their activation contributes to apoptotic cell death (Cobb & Goldsmith 1995, Chen *et al.* 1996, Robinson & Cobb 1997), the association of ERK activation with cellular differentiation has been reviewed previously (Blenis 1993, Cobb & Goldsmith 1995, Lewis et al. 1998). Several developmental systems (*Drosophila* and *Xenopus* embryogenesis, *Caenorhabditis elegans* vulval development) exhibit ERK activation. One of the most studied differentiation systems is that in PC12 cells (neurite outgrowth) where both EGF and nerve growth factor (NGF) activate the MAP kinase cascade (Lewis et al. 1998, Seger & Krebs 1995). It is of interest that, while both result in ERK activation, EGF induces proliferation and NGF induces differentiation (Qui & Green 1992). It was observed that the difference was due to varied durations of ERK activity induced by each extracellular signal (Traverse et al. 1992). EGF-induced ERK activity is transient, whereas NGF-induced activity is more sustained. This conveniently explains how the same ERK cascade can affect different physiological responses. In this study, ERK also appears to be involved in both proliferation

and differentiation. Future *in vivo* studies are required to determine if different extracellular stimuli are responsible for activating the same ERK pathway in odontogenic epithelia and if different durations of ERK activation are responsible for the different physiological responses.

6.1 The Role of MAP Kinases in Cell Proliferation

Mammalian cell growth requires that the cell be continuously fired by signals during the first 6-8 hours that precede the onset of DNA replication (Chen *et al.* 1992, Wood *et al.* 1992, Marshall 1995). Cheng *et al.* (1998) have shown that sustained activation of ERK1/2 is required for cells to pass through G1 restriction point and enter S-phase. In addition, activation of ERK1/2 stimulates early gene transcription and reduces the growth factor requirement for DNA synthesis (Cowley *et al.* 1994, Brunet *et al.* 1994). Zi & Agarwal (1999) observed that a skin cancer preventative agent (silymarin) inhibits EGF-induced ERK1/2 activation which is further associated with growth inhibition of A431 cells with arrest in G1 and G2-M.

A minireview by Thomas (1992) summarized possible pathways involved in MAP kinase activation. Rapid activation of MAP kinases has been described in mammalian cells treated with mitogens. The basal rate of autophosphorylation appears much too slow to account for this, implying that other upstream mediators are required for MAP kinase activity. These factors could be effector molecules that accelerate autophosphorylation or kinases that independently phosphorylate and activate the enzyme. Such factors have been described in

EGF-stimulated Swiss 3T3 cells (Ahn *et al.* 1991) where they were able to raise basal MAP kinase activity or reactivate phosphatase-inactivated enzyme. In either case, activation or reactivation requires the presence of ATP.

Thomas (1992) noted it is odd that MAP kinases are activated during mitosis (or meiosis) and as well during the G0-G1 transition; however, this may simply suggest that these enzymes could be directing common metabolic events in both systems where exit from mitosis may have common features with reentry of quiescent cells into the cell cycle. A maturation promoting factor ($p34^{cdc2}$) has been detected at the G0-G1 boundary (Hall *et* al. 1991) and it appears to activate MAP kinases directly as it bypasses the growth factor receptor signaling pathway.

ERK1 and ERK 2 appear to be essential elements of mitogenic signaling since they are activated by mitogens in all cells (Seger & Krebs 1995). In the pathway from RTKs to ERK, several components including Ras, Raf and MEK may have oncogenic potential (Cowley *et al.* 1994, Mansour *et al.* 1994, Oka *et al.* 1995). ERKs themselves are active in various forms of cancer including skin cancer (Cowley *et al.* 1994; Mansour *et al.* 1994; Oka *et al.* 1995). Additional evidence that ERKs play a role in the carcinogenisis process comes from studies in which activation of ERK is associated with tumor promotion by oxidants and other tumor promoters (Guyton *et al.* 1996).

In this study, actively proliferating odontogenic epithelia expressed significantly more ERK and pERK than relatively quiescent epithelia, where proliferation was measured by immunoperoxidase staining of PCNA. PCNA

staining was confined almost entirely to the nucleus in most odontogenic epithelial cells. In proliferating islands of odontogenic epithelia, PCNA expression was generally limited to the outer basaloid epithelial cells while quiescent islands were mostly devoid of immunostaining for PCNA. PCNA expression in stratified epithelium was observed more in the proliferating basal layers and this correlated with the pattern of ERK and pERK staining. A consistent and unexpected finding was the presence of cytoplasmic and nuclear PCNA expression in differentiated stratified squamous epithelium and hyperplastic epithelial islands. According to Hall *et al.* (1990), cytoplasmic PCNA staining is often observed. However, the nature of this pattern of PCNA expression is unclear. It may represent cytoplasmic breakdown or synthesis of the protein. Mitotic cells will commonly show diffuse staining throughout the cell since the nuclear membrane is lost during mitosis. Accordingly, in this study all nuclear and cytoplasmic staining was considered insignificant.

The pattern of expression of PCNA in this study is consistent with other studies that indicate an association of PCNA with active cell proliferation. However, other studies have not shown a perfect correlation between PC10 immunoreactivity and other indices of proliferation (Yu *et al.* 1991, Rosa *et al.* 1992). One possible reason for this is the long half-life of PCNA (approximately 20 hours) which results in immunostaining of cells that have recently left the cell cycle (Scott *et al.* 1991). In addition, the association of PCNA with DNA repair processes (McCormick & Hall 1992) indicates that expression of PCNA may not necessarily be associated with the S-phase. Growth factors can also induce

expression of PCNA in cells which do not enter S-phase (Yu & Filipe 1993). The effects of different fixation conditions (i.e. duration of fixation, size of tissue block, and type of fixative) are of particular concern. Hall *et al.* (1990) showed that prolonged fixation can dramatically reduce PC10 immunoreactivity. Finally, poor correlations are observed between PCNA expression and cell proliferation in the context of some forms of neoplasia (Hall *et al.* 1990, Yu & Filipe 1993). Due to this complex pattern of PCNA expression, the use of PCNA antibodies as markers of cell proliferation should be interpreted with caution.

6.2 The Putative Role of MAP Kinases in Epithelial Proliferation of Odontogenic Cysts and Follicles

While odontogenic cysts are some of the most common locally destructive lesions of the human skeleton, the mechanisms responsible for their induction and growth remain unknown. All odontogenic cysts (inflammatory or developmental) arise from the epithelial residues of the tooth-forming organ. Seltzer *et al.* (1969) confirmed reports of previous investigators that ERM in the vicinity of the periapical foramina can proliferate in the presence of periapical inflammation, possibly forming a radicular cyst. Epithelial rests from the dental lamina give rise to the odontogenic keratocyst and the reduced enamel epithelium gives rise to follicular cysts (Harris & Toller 1975).

The main stimulus for radicular cyst formation is bacterial endotoxins which have been found in high concentration in the necrotic pulp (Meghji *et al.* 1996). Endotoxins can act as mitogens for epithelial cells (Meghji *et al.* 1992) and/or stimulate the production of cytokines from surrounding connective tissue and

inflammatory cells (Hanazawa *et al.* 1985). Meghji *et al.* (1996) proposed a scheme of cellular interactions leading to epithelial proliferation and bone destruction. For radicular cysts, it was proposed that bacterial endotoxins from the necrotic pulp stimulated ERM proliferation directly as well as indirectly by stimulation of cytokine synthesis from lymphocytes, monocytes, and fibroblasts. In follicular cysts, where no endotoxin is present, the unknown direct or indirect stimulus for ERM proliferation which may be represented by epithelial cell and fibroblast cytokine synthesis. Epithelial cells are known to produce cytokines IL-1α, -1β and –6; these may have an autocrine role in epithelial cell division, as well as a paracrine role on the fibroblast component of the cyst wall which, in turn, may produce EGF and osteolytic agents.

6.3 The Putative Role of Inflammatory Cytokines and MAP Kinases in Odontogenic Epithelial Activation

It is conceivable that the cellular interactions proposed by Meghji *et* al. (1996) are operative in odontogenic epithelial activation. The findings of our study allow us to speculate that cytokines released by inflammatory cells in the periapical tissues might play an important role in cellular signaling mechanisms of epithelial cell activation through the ERK/pERK pathway, during the histogenesis of odontogenics cysts, and perhaps during orthodontic tooth movement. The data from this study have clearly shown that expression of pERK in inflamed epithelial cells (i.e. radicular cyst) is significantly greater than uninflamed epithelia (i.e. follicle). It is conceivable that inflammatory cells release cytokines which act on local fibroblasts. Fibroblasts are capable of synthesizing a number of growth

factors apart from IL-1 and –6; these include keratinocyte growth factor (Rubin *et* al. 1989), EGF and fibroblast growth factor. EGF produced by fibroblasts then binds to the abundant EGF receptors on the surface of odontogenic epithelia (Thesleff 1987) and stimulates the ERK1/2 cascade. In this study, the main effect of inflammation on odontogenic epithelia appears to be phosphorylation of ERK1 to its active form. An elevation or upregulation of ERK1 was not evident; however, it cannot be ruled out that this does not occur.

A proposed scheme of cellular interactions leading to mitosis and differentiation in odontogenic epithelia cells is presented in Fig. 10. Although this scheme is plausible, as it is based on previous studies of periodontal cells (Thesleff 1987, Meghji *et* al. 1996, our observations in this study), further studies are needed to explore the interactions proposed.

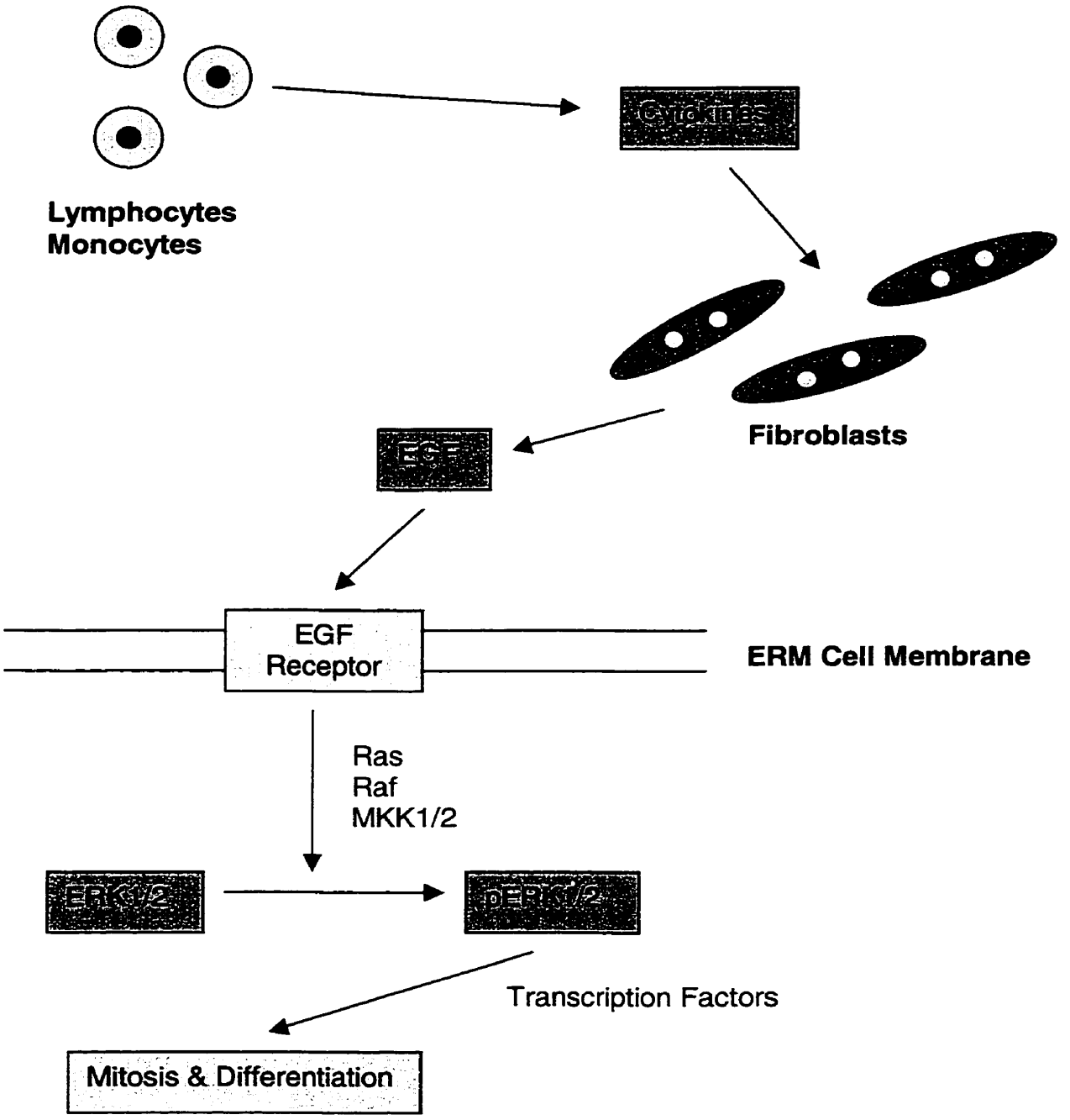

Fig. 10. A proposed scheme of cellular interaction leading to mitosis and differentiation of ERM. ERK1/2 activation/phosphorylation and proliferation is stimulated indirectly by fibroblast synthesis of EGF. Fibroblasts are stimulated to produce EGF by cytokines released from lymphocytes and monocytes (After Thesleff 1987, Meghji *et* al. 1996, and our observations).

7. CONCLUSIONS

The results of this study clearly indicate that:

1. Both ERK1 and its phosphorylated form, pERK, are expressed in epithelial tissues of odontogenic origin. In particular, pERK is well represented in odotogenic epithelial cells of follicles and odontogenic cysts.
2. In epithelial cells of dental follicles and odontogenic cysts, ERK1 and pERK expression is associated with an increased number of proliferating cells.
3. In individual islands of odontogenic epithelial cells and in cyst lining epithelium, cell differentiation is associated with ERK1 and pERK expression.
4. In odontogenic epithelium of dental follicles and odontogenic cysts, pERK expression is associated with inflammation.

These results allow speculation that cytokines released by inflammatory cells may indirectly stimulate the ERK1/2 cascade in odontogenic epithelia by stimulating fibroblasts to release EGF, which binds to the EGF surface receptor on epithelial cells.

8. FUTURE STUDIES

Future studies will be designed to prove the hypothesis that ERK1/2 activation is induced by EGF in cultured human or porcine ERM. Fresh, unfixed tissues (i.e. periodontal ligament obtained from surgical specimens of extracted teeth) will be minced under sterile conditions, explanted onto plastic dishes and incubated, in the presence or absence of EGF, under defined atmospheric conditions and in defined media (KBS) supplemented with bovine pituitary extract, reported recently to be indispensible for the differentiation and maintenance of human epithelia *in vitro*. The explant procedure will be similar to that employed for the culture of ERM from porcine PDL (Birek *et al.* 1983, Brunette *et al.* 1984). Cultures will be fixed at 3, 5, 7, 10 and 20 days after explantation, embedded in water permeable medium and processed further for light microscopic examination, immunohistochemical staining and Western blotting for EGF-receptors, ERK1/2 and pERK, with commercially available, highly specific monoclonal antibodies. Once the cell cultures are well established, repeat experiments will be performed in the presence of PD98059, a highly selective inhibitor of MEK1 activation and the MAP kinase cascade (Pang *et al.* 1995).

Further studies will be directed at demonstrating the role of MAP kinases and extracellular mitogenic signals in tooth movement.

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