Int. J. Dev. Biol. 36: 000-000 (1992)

Original Article

Endogenous epidermal growth factor regulates the timing and pattern of embryonic mouse molar tooth morphogenesis

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ABSTRACT The tooth organ provides a model for discrete patterns of morphogenesis over short periods of developmental time. Studies were designed to test the hypothesis that endogenous epidermal growth factor (EGF) functions to regulate multiple cusp molar tooth morphogenesis during embryonic mouse development. The relative levels of endogenous EGF and EGF receptor (EGFR) transcripts were determined in both enamel organ epithelia and dental ectomesenchyme by reverse transcription-polymerase chain reaction (RT-PCR) assays. EGF and EGFR were localized by immunohistochemistry; both antigenic determinants were demonstrated on the same odontogenic cells in cultured tooth explants. To examine EGF-mediated signal transduction, cap stage mouse molar tooth organs (E16) were cultured in serumless, chemically-defined medium as either (i) controls, or supplemented with (ii) tyrphostin (an EGF receptor kinase inhibitor), (iii) tyrphostin plus exogenous EGF, and (iv) exogenous EGF. Antisense oligodeoxynucleotide (ODN) strategy was used to investigate the functions of endogenous EGF employing (i) non-treated control, (ii) sense ODN control, (iii) antisense ODN, (iv) exogenous EGF, (v) sense ODN with exogenous EGF, and (vi) antisense ODN with exogenous EGF. Tyrphostin inhibited DNA synthesis and produced a significant decrease in the volume of the explants. These effects were recovered by addition of exogenous EGF. Antisense ODN inhibition resulted in abnormal cusp formations, decreased DNA synthesis, total DNA, RNA and protein content, and decreased stellate reticulum and tooth explant volumes. The decreased tooth size was not uniform, the most pronounced effect was in the stellate reticulum. This pattern of changes was not seen when antisense ODN treatment was supplemented with exogenous EGF. These results suggest that during cap stage of odontogenesis endogenous EGF acts to stimulate DNA synthesis, which increases the cell number of specific phenotypes within the enamel organ epithelia, and thereby regulates molar tooth morphogenesis.

KEY WORDS: *EGF, tooth morphogenesis, tyrphostin, antisense, epithelial-mesenchymal interaction*

Introduction

Based upon embryological studies, instructive epithelial-mesenchymal interactions were demonstrated to mediate the timing, position and patterns of tooth morphogenesis (see reviews by Slavkin, 1974; Slavkin and Bringas, 1976; Thesleff, 1977, 1981; Kollar, 1983; Lumsden, 1984; Ruch, 1984). Whereas the specificity for initial tooth position appears to reside within embryonic oral epithelia (Lumsden and Buchanan, 1986), and subsequent tooth form appears to be controlled by cranial neural crest-derived ectomesenchyme (Lumsden, 1984, 1987), the molecular controls for these processes have remained evasive.

More recently, a number of growth factors have been identified, characterized and suggested to participate in dental development; the time and position of specific growth factor expression appears to implicate their role in odontogenic pattern formation (see reviews by Partanen, 1990a; Slavkin, 1990; Thesleff *et al.*, 1991, 1992). In particular, epidermal growth factor (EGF) effects on developing mouse tooth formation have been reported (Cohen, 1962; Partanen *et al.*, 1985; Rhodes *et al.*, 1987; Rihtniemi and Thesleff, 1987; Topham *et al.*, 1987; Hata *et al.*, 1990; Kronmiller *et al.*, 1991). For example, EGF when injected into newborn mice accelerated eyelid opening and incisor tooth eruption (Cohen, 1962). This EGF-induced precocious incisor eruption is associated with decreased tooth size (Rhodes *et al.*, 1987). EGF-induced cell proliferation of embryonic dental epithelium and non-dental mesenchyme in organ culture caused inhibition of morphogenesis (Partanen , 1987).

EGF is a 53 amino acid polypeptide with a molecular mass of 6

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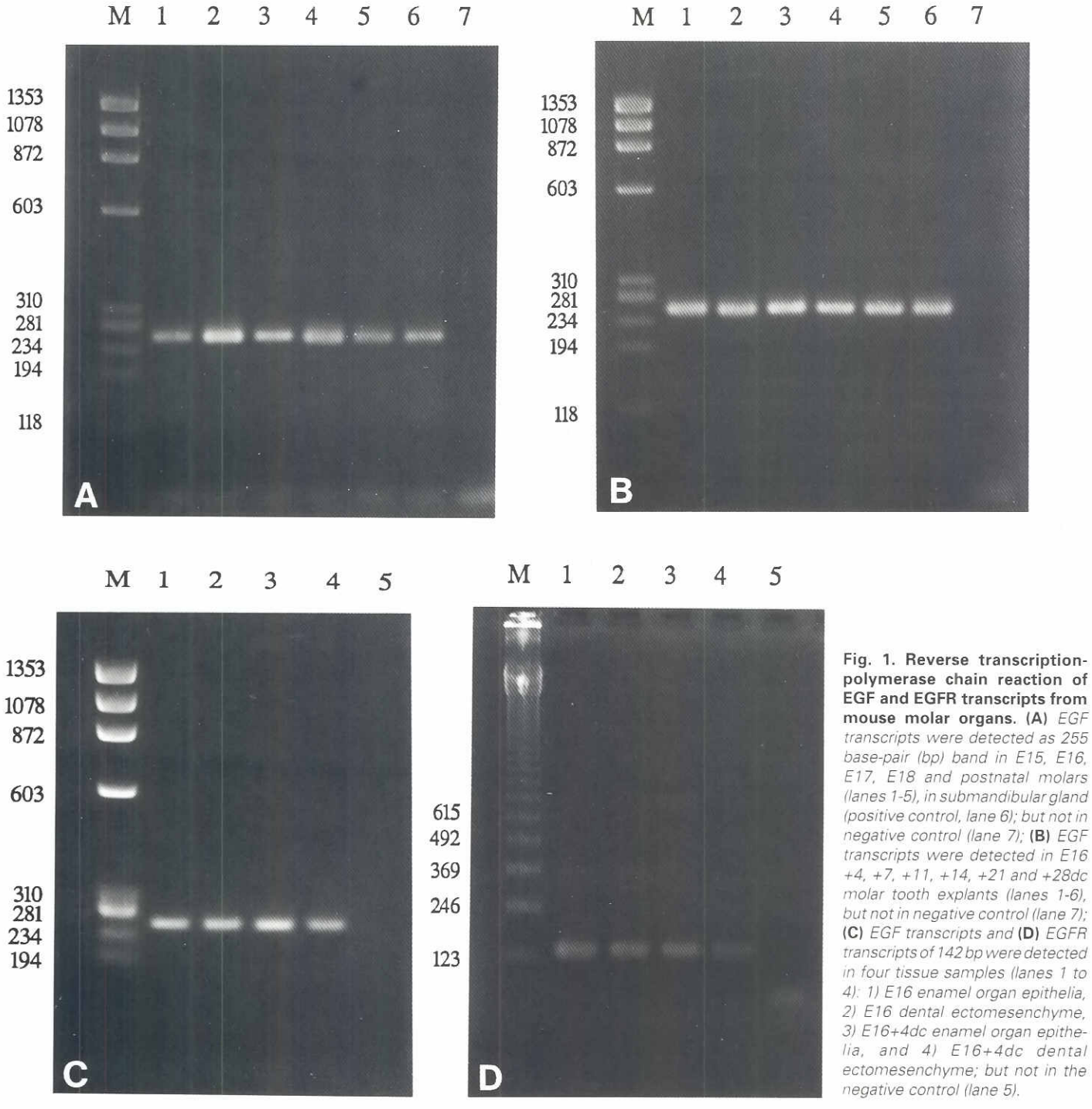


Fig. 1. Reverse transcription-polymerase chain reaction of EGF and EGFR transcripts from mouse molar organs. (A) *EGF transcripts were detected as 255 base-pair (bp) band in E15, E16, E17, E18 and postnatal molars (lanes 1-5), in submandibular gland (positive control, lane 6); but not in negative control (lane 7);* **(B)** *EGF transcripts were detected in E16 +4, +7, +11, +14, +21 and +28dc molar tooth explants (lanes 1-6), but not in negative control (lane 7);* **(C)** *EGF transcripts and* **(D)** *EGFR transcripts of 142 bp were detected in four tissue samples (lanes 1 to 4): 1) E16 enamel organ epithelia, 2) E16 dental ectomesenchyme, 3) E16+4dc enamel organ epithelia, and 4) E16+4dc dental ectomesenchyme; but not in the negative control (lane 5).*

kilodaltons (kDa) (Carpenter and Cohen, 1979), which is derived from a precursor molecule of 136 kDa, encoded by a structural gene localized to chromosome 4 in mice (Scott *et al.*, 1983). EGF effects are mediated by EGF binding specifically to its cell surface receptor (EGFR), a transmembrane glycoprotein composed of an extracellular EGF binding domain and an intracellular tyrosine kinase domain (Schlessinger *et al.*, 1983; Carpenter and Cohen, 1990). The activation of tyrosine kinase by EGF is an essential step to initiate the multiple intracellular responses that culminate in cell proliferation (Ushiro and Cohen 1980).

Inhibition of EGF can be achieved by disturbing ligand-induced autophosphorylation of the receptor, which results in the inhibition of phosphorylation of other intracellular protein substrates. A series of low molecular weight tyrosine kinase inhibitors, tyrphostins, have

been demonstrated to inhibit EGF-dependent autophosphorylation of EGFR without toxic effects (Yaish *et al.*, 1988). EGFR inhibition by tyrphostins correlates with the inhibition of EGF-stimulated growth and cell proliferation without affecting EGF binding or the internalization and degradation of both EGFR and EGF (Lyall *et al.*, 1989). A certain tyrphostin (compound RG 50864) is particularly specific, having a 2500-fold greater affinity for EGFR than to the insulin or PDGF receptors (Yaish *et al.*, 1988).

EGF and EGFR are both detected in mouse embryos at early stages of development (Nexo *et al.*, 1980; Adamson, 1990). The EGF distribution pattern during embryonic day 18 mouse molar development has been localized by *in situ* hybridization (Snead *et al.*, 1989). EGFR associated with odontogenic tissues were found to be competent to bind ^{125}I-EGF (Partanen and Thesleff, 1987). Investigations of EGF effects on tooth development in organ culture indicated that EGF inhibited morphogenesis and cell differentiation of cap-staged embryonic mouse molars (Partanen *et al.*, 1985); however, various cell lineages present in the developing tooth responded with different proliferation rates. EGF stimulates proliferation in the tooth, with dental mesenchyme being the primary target, which then secondarily regulates the proliferation of epithelial cells (see review Partanen, 1990a).

Although EGF is one of the most extensively studied growth factors, its function in embryonic tooth development remains obscure. The purpose of this study was to investigate the effects of endogenous EGF on the timing and patterns of tooth development using mouse E16 tooth organs cultured in a serumless, chemically-defined medium. This specific culture system supports morphogenesis and enables analyses of endogenous and/or exogenous growth factors. We report that EGF binding to EGFR invokes signal transduction processes which regulate tooth morphogenesis. Exogenous EGF enhances cell proliferation but alters cusp formation of embryonic mouse molars.

Results

EGF and EGFR expression during embryonic tooth morphogenesis

The PCR assay provides a method for sensitive detection and semi-quantitation of low abundance transcripts. We detected amplified EGF transcripts using as little as 1 ng of RNA from mouse molar tooth organs. The EGF and EGFR PCR products were detected on agarose gels as 255 and 142 bp bands respectively. Nonspecific bands were not seen in either assay. EGF transcripts were expressed as early as E15. The relative amount of EGF transcripts appeared to increase with subsequent developmental age. The E16 *in vitro* samples expressed a similar temporal pattern and suggested that two days *in vitro* development was equivalent to one day *in vivo* development based upon the relative patterns of RT-PCR amplification (Fig. 1A,B). A paracrine function of EGF on E18 mouse molars was previously suggested (Snead *et al.*, 1989). In order to determine the tissue source of EGF and EGFR, we dissociated individual tooth organs into either enamel epithelia or dental ectomesenchyme from E16 and E16+4dc tooth organs. Dispase-separated enamel epithelia and dental ectomesenchyme contained both EGF and EGFR transcripts (Fig. 1C,D). The dental ectomesenchyme from E16+4dc molars showed a reduction of EGFR transcripts (Fig. 1D, lane 4).

Endogenous EGF and EGFR localization to enamel organ epithelia and dental papilla ectomesenchyme tissues

The immunostaining positive control tissue, male mouse submandibular gland, clearly indicates a paracrine function of EGF since the EGFR is located on glandular stroma (Fig. 2C) and EGF is associated with acini cells (Fig. 2D).

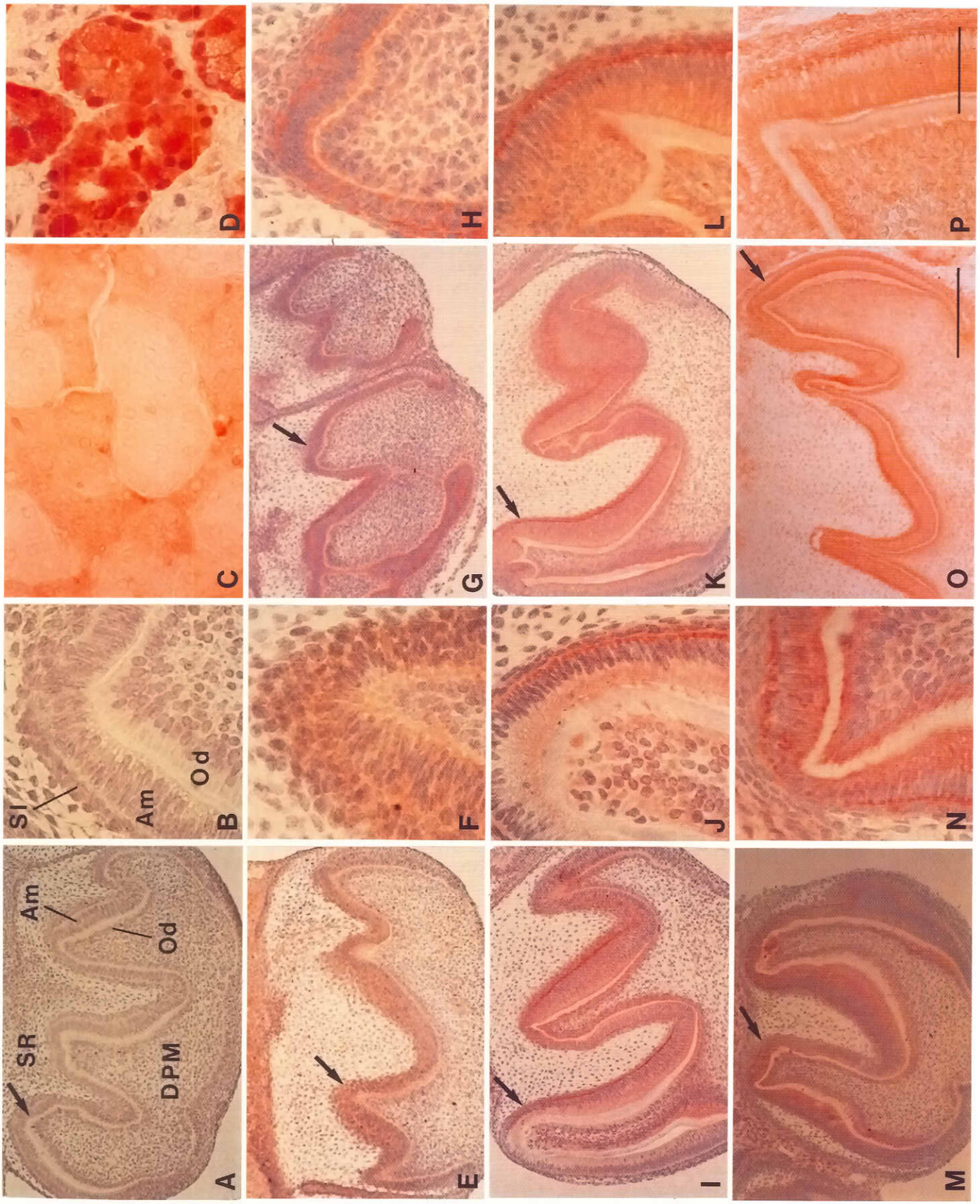
This study identified EGF and EGFR antigens during the critical transition from pre-ameloblasts to secretory ameloblasts. Positive immunostaining increased with culture time (Fig. 2E-P). EGF was found to concentrate on inner enamel epithelium (IEE) yet the EGFR was distributed in pericellular space of IEE. At E16+4dc there was positive immunostaining for EGF in the inner enamel epithelium (IEE), while the EGFR immunostaining was localized at the interface of IEE and the stratum intermedium. The most intensive staining was concentrated adjacent to the proliferative and pre-ameloblast cells within the IEE (Fig. 2E-H). By E16+7dc the pre-ameloblasts within the IEE were polarized. A gradient of EGF and EGFR immunostaining was observed, starting abruptly and most intensively at the ameloblast/stratum intermedium interface and radiating unidirectionally through the ameloblasts and into adjacent odontoblasts (Fig. 2I-L). By E16+12dc the staining was intense and diffuse in tandem with regions of tissue-specific mineralization (Fig. 2M-P).

EGF induces DNA synthesis

Exogenous EGF at 10 ng/ml was found to be the optimal dose to enhance DNA synthesis (Fig. 3). Exogenous EGF altered cusp morphogenesis in terms of size and shape at concentrations as low as 2 ng/ml. EGF-treated tooth organs contained an outgrowth of mesenchyme surrounding the cultured molar explants. The treated molar explants showed irregular cusp formation with increased mitotic figures in both enamel epithelia and dental ectomesenchyme (Fig. 4C, D, G, H). DNA synthesis was correlated with exogenous EGF concentration in a dose-dependent fashion with a high correlation ($r=0.92$). The increase in DNA synthesis was indicative of an increase in cell number as histological examination did not reveal the presence of multinucleated cells, although the cells appeared smaller and more densely packed. In tyrphostin-treated specimens, DNA synthesis was inhibited and the stellate reticulum area was significantly reduced in volume ($p<0.05$) (Figs. 4E, 5). At 80 μM tyrphostin (the most effective inhibitory dose), a 40% decrease of total DNA synthesis was observed (Fig. 5A). This inhibitory effect was reversed by adding exogenous EGF at a concentration of 10 ng/ml (Fig. 6A). An unexpected finding was that although the tooth organs treated with EGF or tyrphostin alone decreased in size (30% and 65% respectively), explants treated with EGF and tyrphostin together showed 30% decrease only (Fig. 6C). The reversal of the inhibitory effects of tyrphostin by the addition of exogenous EGF suggests that its actions are the result of a specific association with the EGFR.

EGF antisense oligodeoxynucleotide inhibits tooth morphogenesis

Tooth organs from experimental antisense ODN groups displayed dysmorphology of cusp formation as compared to non-treated or sense ODN controls (Fig. 7). Total DNA, RNA and protein content as well as tooth volume, stellate reticulum volume, and cell number all decreased after exposure to EGF antisense ODN ($p<0.05$) (Fig. 8). These effects were the consequence of a specific reduction in EGF expression since the inhibitory effects on DNA, RNA, protein and molar size using antisense ODN were reversible with exogenous EGF at 10 ng/ml supplementation (Fig. 8), although the dysmorphogenesis of molar cusps remained abnormal in the presence of exogenous EGF. Furthermore the addition of sense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ODN did not significantly change the explants relative to controls (Figs. 7, 8).

Discussion

The present study demonstrates that EGF is expressed during *in vivo* mouse molar tooth development from cap stage (E15) through crown formation (in the newborn) (Figs. 1, 2). A progressive increase in the amount of EGF mRNA was observed in molar tooth organs during this developmental period. This result is consistent with the findings of Nexø *et al.* (1980), who detected EGF in whole mouse embryo extracts by radioreceptor assay, as early as E11.5, and Slavkin *et al.* (1992) who reported that EGF is present *in vitro* from the early cap stage through crown completion and the beginning of root formation. We determined that EGFR is present in E16 to E16+12dc, Nexø *et al.* (1980) found EGFR in whole mouse embryo extracts before E14.5 with a substantial increase observed between E15.5-E17.5.

In addition to defining the presence or absence of EGF and EGFR transcripts in molar tooth organs, we also describe the temporal and spatial distributions of their expression. There is a distinct pattern of EGF and EGFR expression in the developing molar immediately before the differentiation of preameloblasts to ameloblasts. EGF is associated with the proliferative cells within the inner enamel epithelium (IEE), the post-mitotic polarized IEE and overtly differentiated ameloblasts (Figs. 1, 2). These results are consistent with recent studies by Cam *et al.* (1990) and Slavkin *et al.* (1992). The gradient of EGF and EGFR that is established is of unknown function. Although the transitory pattern preceded the initiation of mineralization, experimental evidence is not available to correlate EGF and/or EGFR localization with the amount of mineralization (as judged by amount of von Kossa staining). EGF inhibited by tyrphostin or antisense oligodeoxynucleotides was not correlated with dentine or enamel biomineralization in the present studies.

By separating the enamel epithelium and dental ectomesenchyme followed by RT-PCR it was discerned that *both* of these tissues were expressing EGF and EGFR. We found this result somewhat surprising. We expected to see one tissue expressing EGF while the other produced EGFR, which would have clearly defined the direction of an epithelial-mesenchymal interaction. With both tissues expressing both EGF and EGFR, it cannot be discerned whether the interaction is autocrine and/or paracrine in character.

The influence of endogenous EGF on tooth formation was tested by tyrphostin inhibiting EGFR and antisense strategy blocking EGF expression; the latter methodology has been reported as an effective experimental approach in blocking specific protein expression in pre-implantation mouse embryos (Rappolee *et al.*, 1992).

Previously, Gazit *et al.* (1989) demonstrated that the autophosphorylation of EGFR as well as of other growth factors is the first step in signal transduction and a prerequisite for the phosphorylation of exogenous substrates by the receptors. Through inhibiting EGFR tyrosine kinase activity, tyrphostin blocks the signal transduction pathway with attendant effects on cell proliferation and other EGF-mediated intracellular processes. Since the inhibition by tyrphostin is both competitive and reversible (Yaish *et al.*,

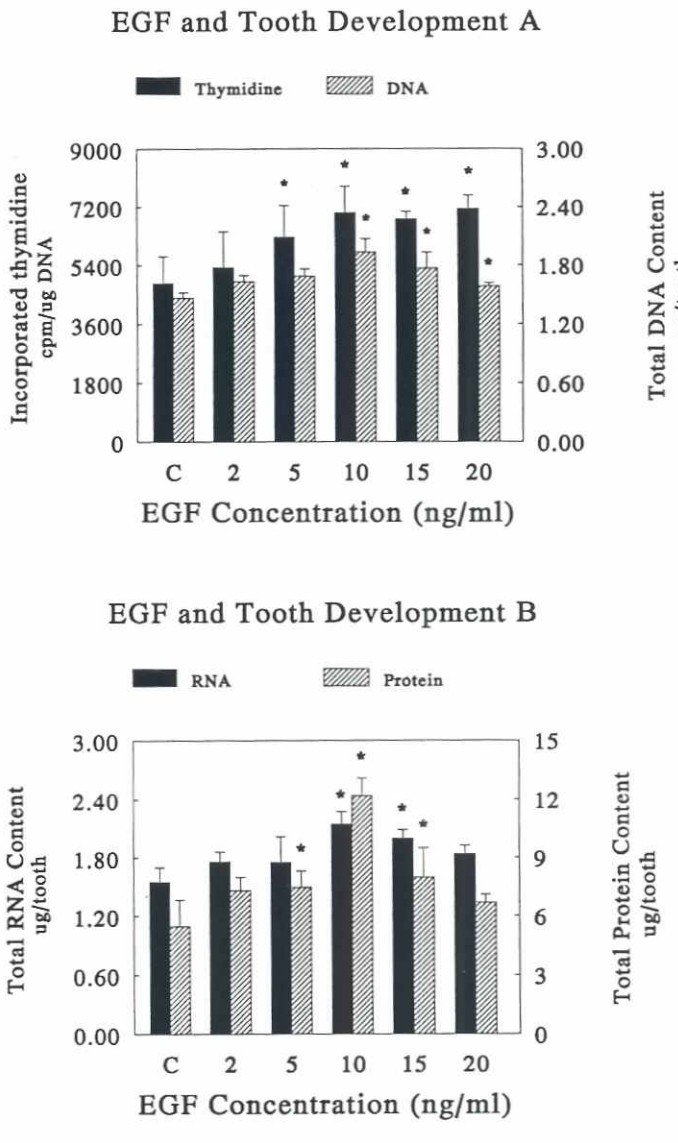

Fig. 3. Effects of EGF on E16+4dc cultured molars. *EGF was administered to cultured mouse molars at concentrations of 2-20 ng/ml. The effects on* **(A)** *the amount of DNA synthesis and total DNA content, and* **(B)** *total RNA and protein contents were measured. Exogenous EGF at concentrations of 10 and 15 ng/ml caused significant increases in all four measures of tooth morphogenesis. (*) Represents statistic significance ($p<0.05$).*

Fig. 2. Immunolocalization of EGF and EGFR on developing mouse molar organs. *Immunostained histologic sections of cultured molars, viewed at low and high magnifications. Red indicates positive immunostaining while the purple is hematoxylin counterstaining.* **(A,B)** *E16+7dc molar (negative control),* **(C)** *EGFR, and* **(D)** *EGF on submandibular gland (positive control). E16+4dc molars localized with* **(E,F)** *EGF and* **(G,H)** *EGFR. E16+7dc molars localized with* **(I,J)** *EGF and* **(K,L)** *EGFR. E16+12dc molars localized with* **(M,N)** *EGF and* **(O,P)** *EGFR without counter staining. Am, ameloblasts; Od, odontoblasts; SR, stellate reticulum; SI, stratum intermedium; DPM, dental papillae mesenchyme. Bars represent 200 μm in O and 50 μm in P.*

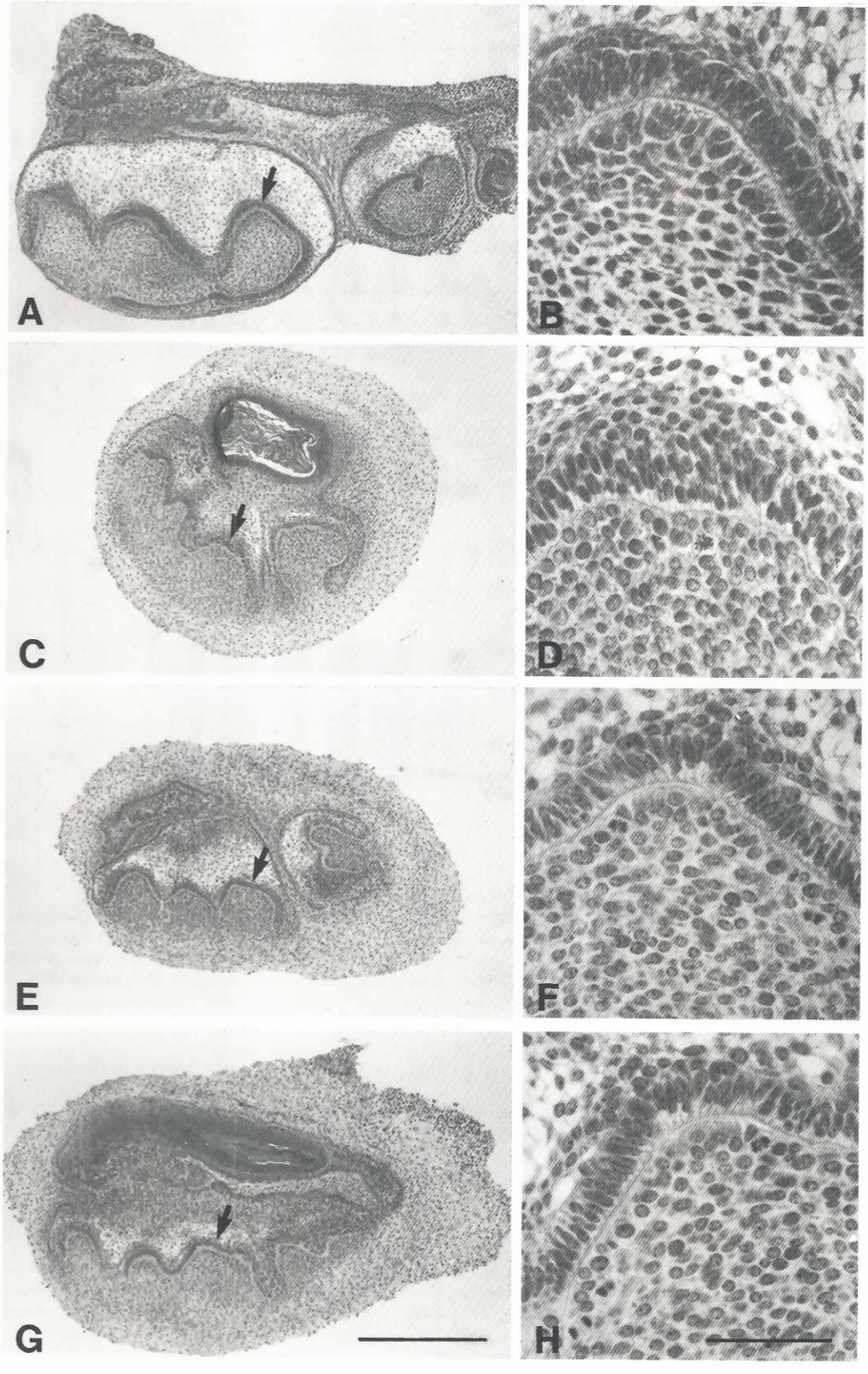

Fig. 4. Morphology of EGF- and tyrphostin-treated molars. *The histologic sections of E16+4dc cultured molars stained with H&E, viewed at low and high magnifications.* **(A,B)** *Control,* **(C,D)** *EGF 10 ng/ml,* **(E,F)** *tyrphostin 80 μM, and* **(G,H)** *EGF 10 ng/ml and tyrphostin 80 μM. All treatment groups show morphological disturbances in cusp formation. Arrow indicates the cusp in high magnification. Bars represent 200 μm in G and 50 μm in H.*

1988), the residual low tyrosine kinase activity may be insufficient for the signal to synthesize DNA but sufficient for the signal required for normal receptor trafficking, therefore certain pathways of signaling will not be influenced. With the information from Yaish *et al.* (1988), we did not expect an absolute block of EGFR activity. This explained why the decrease in cell number of the tooth organ induced by tyrphostin administration could be overcome by exogenous EGF supplementation.

By inhibiting EGF function through antisense ODN and tyrphostin we have demonstrated that EGF influences a number of important cellular activities during odontogenesis. When explants were exposed to exogenous EGF there was an increase in total DNA content of the explants as well as an increase in the amount of newly synthesized DNA. Because we did not observe multinucleated cells in the explants we infer that the exogenous EGF induced cell division and increased the total number of cells in the explants. Exogenous EGF also produced a net increase in total RNA and protein contents while decreasing the total volume of the explants. This suggests that the loss in volume may be due to a reduction in the secretion of carbohydrate-containing ECM. Exogenous EGF also effected a change in cusp morphogenesis.

When the expression of EGF in the molar explants was inhibited by tyrphostin or antisense ODNs we observed a decrease in both DNA synthesis and total explant volume. The volume of the stellate reticulum was reduced significantly more than the explants as a whole (-32% v. -23% in the antisense study). Cusp morphogenesis was irreversibly affected. It would seem inconsistent that inhibition of EGF and addition of exogenous EGF would both cause a reduction in explant volume (-65% and -30%, respectively). This anomaly would be understandable if cells could respond differently to high and low concentrations of EGF. Low concentrations of EGF could stimulate cell growth and/or the secretion of extracellular matrix and inhibit these activities at high concentrations. It is also possible that different cell types could respond to EGF with opposite effects. The observations could also be explained if most of the cap stage cells exposed to endogenous EGF were responding by producing more ECM while those not receiving endogenous EGF were programmed to respond by decreased production. When the latter group of cells experienced exogenous EGF stimulation they would reduce production of ECM and decrease explant volume. EGF appears to be pleiotropic. Whereas the mitogenic functions of EGF are well known (see review by Carpenter and Cohen, 1990), EGF clearly has several other functions. For example, Hata and colleagues demonstrated that EGF induces total protein synthesis during late cap stage tooth development, yet decreased type I collagen and enamel protein production (Hata *et al.*, 1990). More recently, EGF has been found to disrupt gap-junctional communication by inducing multiple phosphorylation of connexin 43 (gap junction protein) on serine residues (Lau *et al.*, 1992). Therefore, EGF binds to and activates the tyrosine kinase receptor (Ullrich and Schlessinger, 1990), thereby activating several second messenger systems which in turn activate several serine/threonine protein kinases (Sturgill and Wu, 1991).

During the cap stage of tooth formation the cells of the stellate reticulum secrete high levels of proteoglycans, primarily hyaluronate (Goldberg and Septier, 1987; Thesleff *et al.*, 1988; Mark *et al.*, 1990), which bind water and form a region against which molar cusps form. Knudson and Toole (1985) reported that cytodifferentiation of cartilage is accompanied by extensive intercellular matrix accumulation and that the structure of cartilage is dependent upon the aggregation of hyaluronate and proteoglycans. Similarly, the stellate reticulum of a tooth organ might function in controlling and supporting the size and shape of cusps through regulating its content of extracellular matrixes. Our results suggest that the SR is particularly sensitive to EGF levels and responds with a significant reduction in volume if EGF is either raised or lowered. This change in volume is more dramatic than the changes in cusp morphology. Therefore it seems unlikely that cusp morphology is molded by pressure from the SR or that the tooth surface is imprinted with its shape.

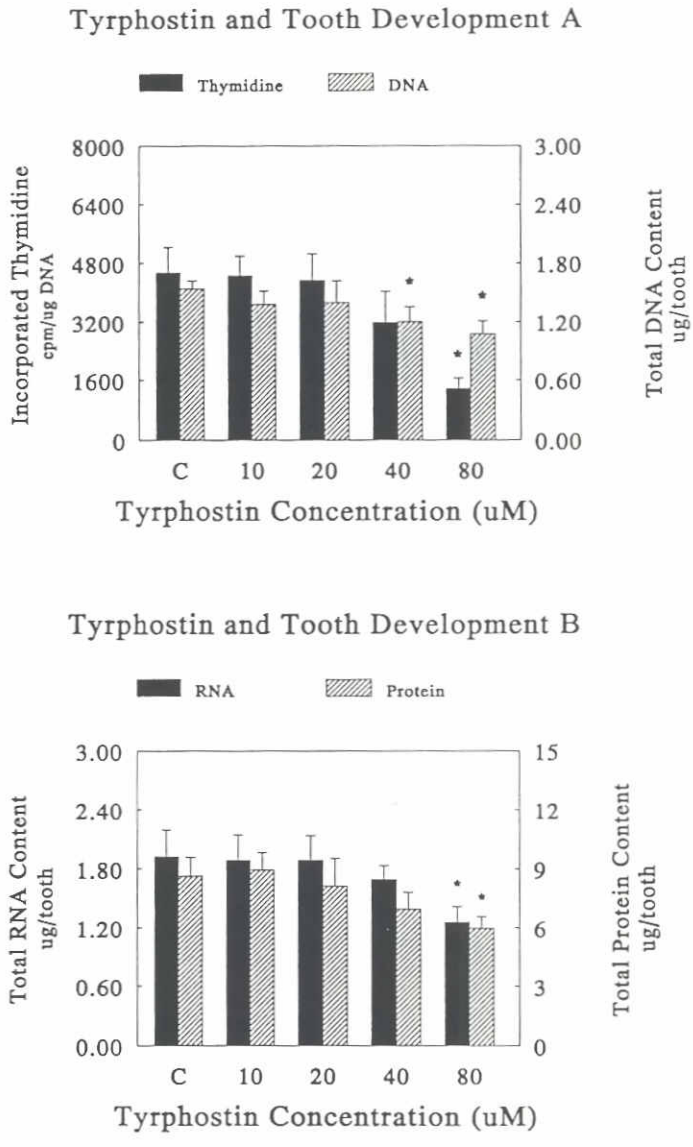

Fig. 5. Effects of tyrphostin on E16+4dc cultured molars. *Tyrphostin was administered to cultured mouse molars at concentrations of 10-80 μM. At a concentration of 80 μM tyrphostin inhibited development of the culture molars in all the assayed parameters* **(A)** *the amount of DNA synthesis and total DNA content and* **(B)** *total RNA and protein contents. (*) Represents statistic significance ($p<0.05$).*

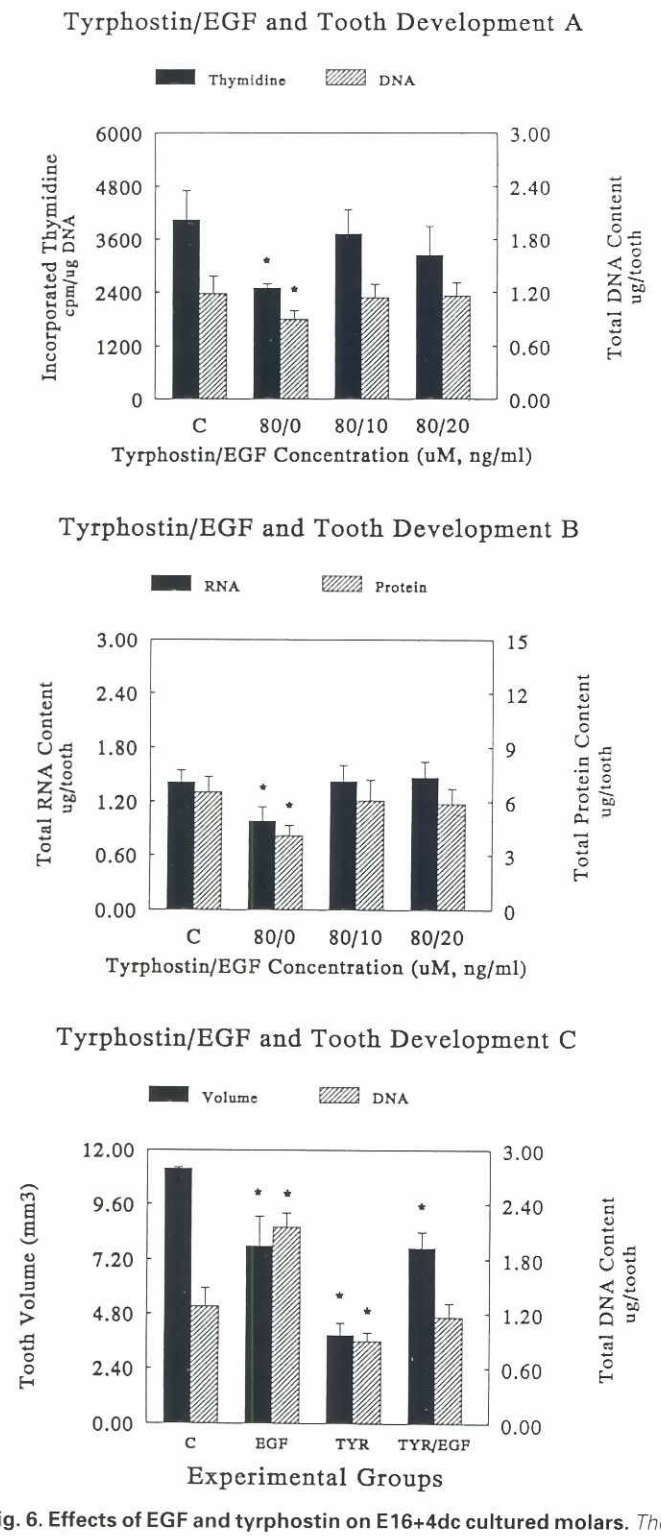

Fig. 6. Effects of EGF and tyrphostin on E16+4dc cultured molars. *The cultured mouse molars were studied under four conditions: 1) in chemically-defined medium, 2) medium + 10 ng/ml EGF, 3) medium + 80 μM tyrphostin, and 4) medium + 10 ng/ml EGF + 80 μM tyrphostin. Results presented as* **(A)** *the amount of DNA synthesis and total DNA content,* **(B)** *total RNA and protein contents, and* **(C)** *molar volume compared with total DNA content. Tooth organs from the EGF-treated group were found to have a 31% decrease in volume but a 32% increase in DNA content. (*) Represents statistic significance ($p<0.05$).*

The expression of EGF and EGFR is not uniform but follows a dynamic temporal and spatial pattern. A consistent effect of exposure to EGF was the induction of cell division. A mixed effect was the increase or decrease in the secretion of ECM depending upon the cell type and the timing and level of EGF exposure. An important finding in this study is that exogenous EGF as well as the inhibition of EGF irreversibly altered cusp morphogenesis. This suggests that more cells inside the developing tooth are expressing EGFR than are being stimulated at any one point in time. The effect of exogenous EGF is to stimulate *all* cells expressing EGFR. The selective *in vivo* EGF stimulation cannot be reconstituted by general supplementation. We conclude that the morphology of the mouse molar cusps is the aggregated effect of a complex pattern of EGF (as well as other growth factors) expression that induces selected cells to divide and alter their production of ECM. The sum total of these responses to the programmed spatial and temporal pattern of expression produces the final observed morphology.

Since EGF and TGF-alpha both bind to the same receptor (Pike *et al.*, 1982; Massague, 1983; Marquardt *et al.*, 1984; Adamson, 1990), and since exogenous TGF-alpha could mediate cell-cell interactions and transmit developmental information between neighboring cells in a paracrine manner similar to the action of EGF in the development of epithelial-mesenchymal organs (see review, Partanen, 1990a, b), we suggest that the family of EGF-related proteins mediate epithelial-mesenchymal interactions associated with tooth development via signal transduction. We predict that endogenous TGF-alpha would mimic the data sets as provided in this report. Studies are in progress to test this hypothesis.

Materials and Methods

Organ culture

Swiss Webster E16-day pregnant mice (plug day= day 0) were sacrificed by cervical dislocation. The uterus was dissected, embryos were removed, staged according to external characteristics (Theiler, 1972), and the mandibular first molar tooth organs were isolated. Selected intact tooth organs were cultured as previously described (Bringas *et al.*, 1987; Evans *et al.*, 1988). Briefly, cap stage explants were placed on type AA Millipore filter discs (6 mm in diameter with 0.8 μm pore size) supported by stainless steel rafts using BGJb medium (GIBCO, Gaithersburg, MD), pH 7.3-7.4, supplemented with 100 μg/ml ascorbic acid and 1% penicillin/streptomycin antibiotics. Cultures were incubated at 37°C in 95% air and 5% CO_2 for 4 to 28 days in culture (dc). Medium was changed every other day.

Cell death in the culture system was monitored by determining the activity of lactate dehydrogenase (LD) in the culture medium (Wroblewski and La Due, 1955). The LD activity of medium collected after 48 h culture was determined by using the LD reaction kit (Sigma, St. Louis, MO). With LD activity determined at no more than equals 200 units per ml of culture medium, cell death in this system was found to be negligible.

Reverse transcription-polymerase chain reaction (RT-PCR)

Tooth organs *in vivo* (E14 through E19) and E16 explants cultured from 4 to 28dc were collected, rinsed with phosphate buffered saline (PBS) and then frozen with liquid nitrogen and stored at -80°C. To Investigate EGF and EGFR transcripts in either enamel organ epithelia or dental papilla ectomesenchyme, E16 and E16+4dc were treated with Dispase (Boehringer Mannheim, Indianapolis, IN) for 1 h in Hank's medium (GIBCO, Gaithersburg, MD), separated, rinsed with PBS (pH 7.4), and stored at -80°C. Total RNA was extracted according to Evans and Kamdar (1990). RT-PCR was performed according to Rappolee *et al.* (1989). Briefly, seven tooth organs from each sample group were homogenized with sterile pestle in buffer containing 8M guanidine hydrochloride, 3M sodium acetate at pH 5.2, and 10% sodium sarcosyl (8:1:1 v/v/v) on ice. The total RNA was extracted and precipitated with absolute ethanol at -20°C to remove any contaminating DNA. The

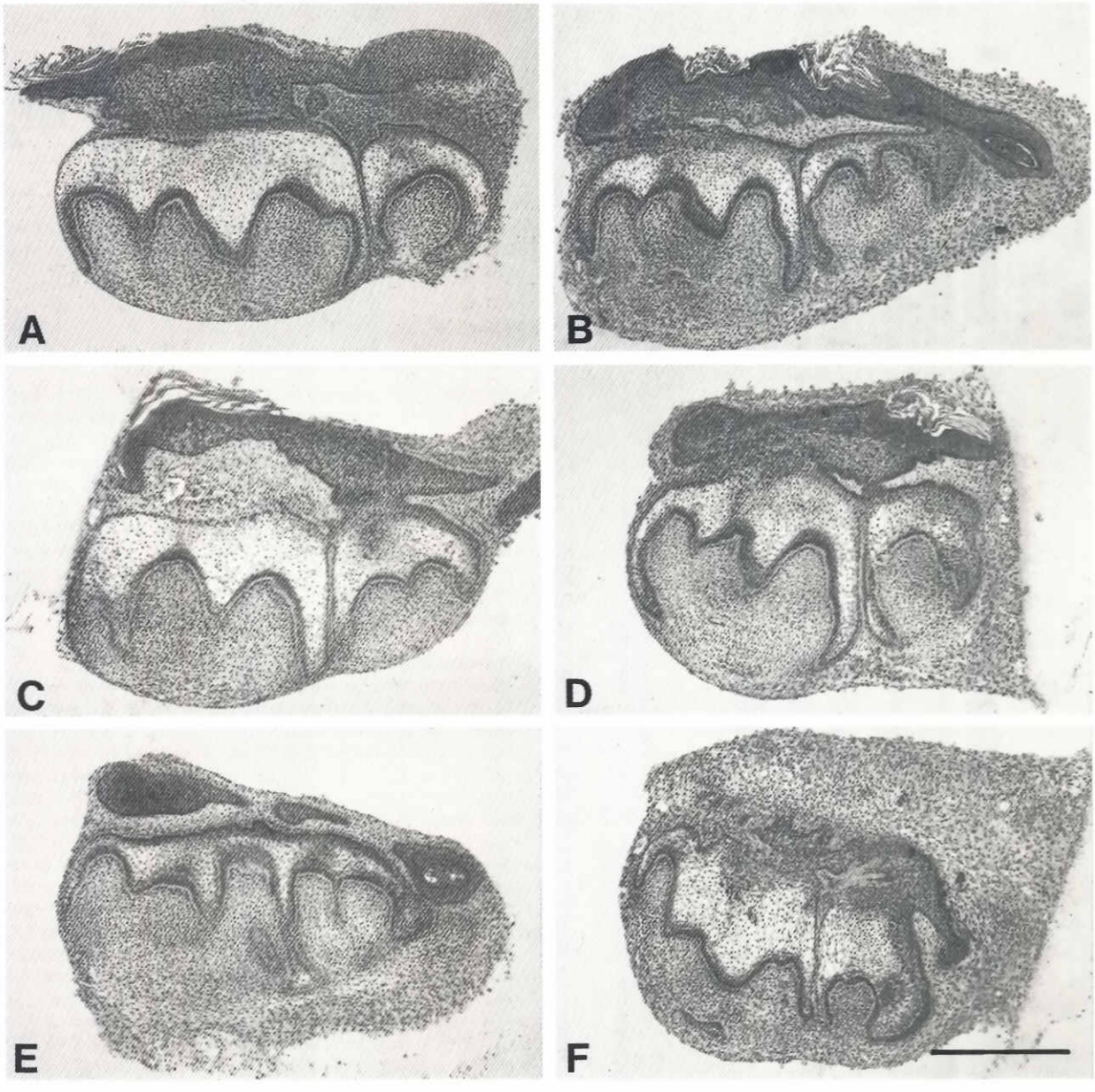

Fig. 7. Inhibition of cusp formation by EGF antisense ODN. *The histologic sections of E16+4dc cultured molars stained with H&E.* **(A)** *Control medium;* **(B)** *EGF 10 ng/ml;* **(C)** *sense ODN;* **(D)** *sense ODN+EGF;* **(E)** *antisense ODN and* **(F)** *antisense ODN+EGF. Arrow indicates the cusp in high magnification. Bars represent 50 μm in F.*

precipitated RNA pellet was then dissolved in diethylpyrocarbonate (DEPC) water containing RNase inhibitor (Promega, Madison, MI).

Reverse-transcription was started by adding 0.1 M methylmercury(II)hydroxide (Amresko, Solon, OH) to the sample for 7 min at room temperature, followed by 0.7M 2-mercaptoethanol (Sigma, St. Louis, MO) for 5 min. A mixture of random primers (Pharmacia, Alameda, CA), RNase inhibitor, M-MLV reverse transcriptase (GIBCO BRL, Gaithersburg, MD), dNTP (Pharmacia, Alameda, CA), and RT buffer (GIBCO BRL, Gaithersburg, MD) were added to the RNA extract. The samples were heated at 42°C for one hour, boiled at 95°C for 5 min, and immediately cooled on ice. The reaction was repeated three times.

PCR reaction solution was prepared by adding PCR buffer, 10 mM dNTP, *Thermus aquaticus (Taq)* polymerase (Perkin-Elmer Cetus, Norwalk, CT) in DEPC water. EGF primers were designed and synthesized based on the sequence of mouse submandibular gland (Scott *et al.*, 1983). The sequences of EGF primers used were as followings: 5' end AGA GCC AGT TCA GTA GAA ACT GGG, and 3' end ACT TTG GTT TCT AAT GAT TTT TCT CC. The EGFR primers were: 5' end AGA ACA ACA CCC TGG TCT GGA AGT, and 3' end CC AGT GGC GAT GGA TGG GAT CTT (Xu, *et al.*, 1984; Petch *et al.*, 1990). A mixture containing 43 μl PCR reaction solution, 2 μl of 3' primer, 2 μl of 5' primer, and 3 μl RT reaction product was then loaded in small Eppendorf tubes, sealed with mineral oil and placed in a thermal cycler machine to be

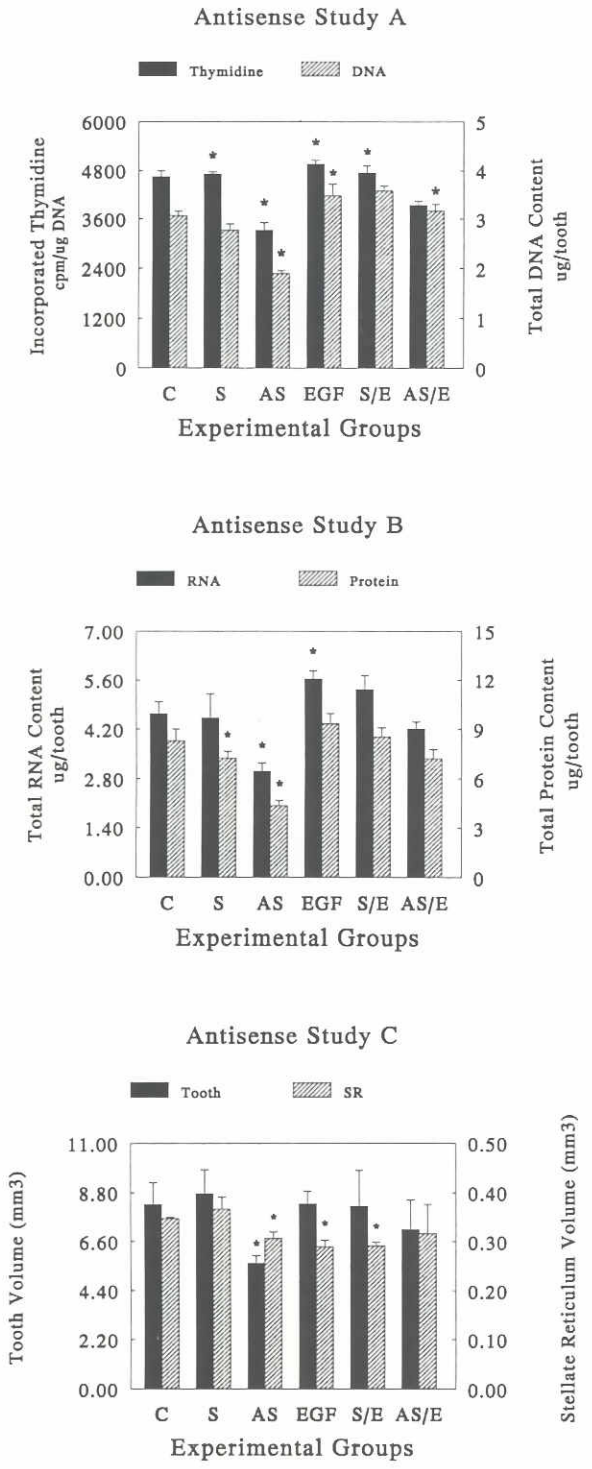

Fig. 8. EGF antisense ODN inhibits tooth development. *The E16+4dc cultured molars were studied under six conditions: 1) chemically-defined medium, 2) sense ODN, 3) antisense ODN, 4) EGF 10 ng/ml, 5) sense ODN + EGF, and 6) antisense ODN + EGF. Effects on* **(A)** *the amount of DNA synthesis and total DNA content and* **(B)** *total RNA and protein contents were measured. Volumes of (C) tooth organ and stellate reticulum area were quantified. The diminished DNA synthesis, tooth size and volume of stellate reticulum area caused by addition of antisense ODN was reversed by exogenous EGF supplementation. (*) Represents statistic significance ($p<0.05$).*

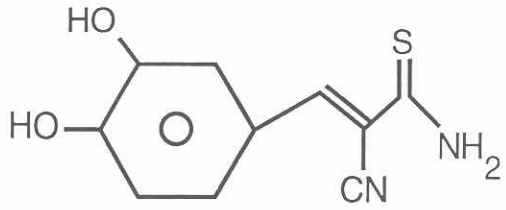

Fig. 9. The chemical structure of synthetic tyrphostin, compound RG 50864. *The specific EGFR tyrosine kinase inhibitor has a molecular weight of 220.247.*

processed at 95°C for 5 min then 94°C for 1 min, 55°C for 45 sec, and 72°C for 1 min in 60 cycles. Six microliters of the 50 µl PCR reaction product was fractionated by electrophoresis in a 3% Nusieve (FMC, Rockland, ME) and 1% agarose gel (GIBCO BRL, Gaithersburg, MD). Gels were run at 85 volt for 45-50 min, until the bromophenol blue dye front migrated 6 cm. Molecular weight standard used was a mixture of ficoll, trypan blue, and øx174/HaeIII (GIBCO BRL, Gaithersburg, MD). The gel was stained for 30 min in ethidium bromide, destained 10 min in water, viewed under UV light box, and photographed using Polaroid film. The positive control for endogenous EGF production was submaxillary gland extract from male Swiss Webster mice. Negative control used no tissue but all the regents.

Immunolocalization of EGF and EGFR molecules

Upon termination of cultures, specimens from 4-12dc were rinsed with PBS (pH7.4) and fixed with Carnoy's solution for 4 h and processed for paraffin embedding. Five micron serial sections were prepared from these tissue blocks. Immunohistochemistry was used to identify the cellular distribution of EGF and EGFR with the Zymed streptavidin biotin system (Zymed, San Francisco, CA). Sections were mounted on Histostik coated slides, deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by 30% hydrogen peroxide and absolute methanol (1:9, v/v), and slides were then washed in PBS. Slides were treated with serum blocking solution, primary antibody, biotinylated secondary antibody, enzyme conjugate, substrate chromogen mixture, and counter-stained with diluted hematoxylin according to the specifications of the Zymed peroxidase staining system.

Primary antibody raised against EGF (Sigma, St. Louis, MO) and monoclonal antibody raised against EGF receptor, specific to its extracellular domain (ICN, Costa Mesa, CA), were used for indirect immunostaining. The optimal dilution and incubation time for the EGF primary antibody was determined to be 1:200 with 2 h of incubation at room temperature. For the EGF receptor, a primary antibody concentration of 1:50 incubated at 37°C for 4 h was found to be optimal. Positive reaction was indicated by the presence of a local red precipitation. The positive control used was male submandibular gland, which has been shown to contain EGF at 1 µg/mg tissue wet weight (Byyny *et al.*, 1972). Specificity controls included pre-immune serum instead of the primary antibody, secondary antibody, and EGF antigen adsorption of the primary antibody.

EGF/EGFR inhibition

Explants were cultured with exogenous EGF at concentrations of 2-20 ng/ml. A replicate group was treated with tyrphostin, compound RG 50864 (Rorer research center, Horsham, PA) (Fig. 9) at concentrations of 20-80 µM from 0 to 4dc. A third group received a combined administration of exogenous EGF (10 ng/ml) and tyrphostin (80 µM). At the termination of the culture period, tooth organs in groups of 10 from control as well as experimental groups were incubated with a pulse of 100 µCi ^{3}H-thymidine (New England Nuclear, Boston, MA) for 4 h followed by a chase period of one additional hour in medium containing excessive non-labeled thymidine. Tooth organs were thoroughly rinsed in sterile PBS, collected and stored at -20°C. DNA, RNA and protein were determined by the method of Keleti (1974). DNA synthesis was determined by the incorporation of ^{3}H-thymidine

into DNA. Samples were also analyzed for protein content according to Bio-Rad Protein Assay (Bio-Rad, Richmond, CA).

Endogenous EGF inhibition by antisense oligodeoxynucleotide

EGF sense and antisense ODNs were designed according to the mouse submandibular gland EGF cDNA sequence (Scott *et al.*, 1983). We synthesized pentadecaoligomers (15 bases) targeted to the EGF mRNA beginning with the initiation codon. ODNs of both sense (5' ATG CCC TGG GGC CGA 3') and antisense (5' TCG GCC CCA GGG CAT 3') orientation were synthesized by using phosphoramidite chemistry on the PCR Mate EP 391 DNA synthesizer (Applied Biosystems, Foster City). Tooth explants were cultured with (1) control medium, (2) 30 μM sense ODN, (3) 30 μM antisense ODN, (4) EGF 10 ng/ml, (5) sense ODN with EGF, or (6) antisense ODN with EGF. Medium was prepared fresh and changed every other day. Explants were removed from culture on 5dc and were either (1) fixed with Carnoy's fixative for subsequent morphometric evaluations, or (2) used for pulse-chase ^{3}H-thymidine experiments, collected by immediate freezing, and subsequently assayed for either DNA, RNA or protein content or ^{3}H-thymidine incorporation into cold trichloroacetic acid (TCA) precipitates (Evans *et al.*, 1988).

Morphometric assays

Tooth organs from experimental and control groups were selected and processed through routine histology, serially sectioned at 5 μm, stained with hematoxylin and eosin, and examined using light microscopy. Three explant samples from each treatment group were used for volumetric determination by the computer-assisted SigmaScan morphometric study program (Jandel, San Francisco, CA). Histologic sections were projected onto a digitizing tablet connected to an IBM PC. The images were traced on the tablet and transferred to the computer for area calculations. Tooth organ and stellate reticulum volumes were calculated separately by the summation of the total area times the thickness of each histologic section (5 μm). Evaluation of the stellate reticulum area was analyzed by computer-assisted three-dimensional image reconstruction of representative samples (PC3D, Jandel, San Francisco, CA).

Statistical analyses

All studies were done in triplicate. Data were analyzed by using one-way analysis of variance and Student's *t* test, taking the confidence level at $p<0.05$ (Epistat statistic program).

Acknowledgments

We appreciate the generous gift of compound RG 50864 from Rhone-Poulenc Rorer Central Research (Horsham, PA) and the important technical assistance from Mrs. Sharon Groff and Mr. Valentino Santos. Thanks to Drs. Wen Luo and Yang Chai for advice in performing PC3D reconstruction. This work was supported by NIH grant DE-06425, USPHS.

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Accepted for publication: October 1992