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EGF abrogation-induced *fusilli*-form dysmorphogenesis of Meckel's cartilage during embryonic mouse mandibular morphogenesis in vitro

Lillian Shum[1,*], Yasunori Sakakura[1], Pablo Bringas, Jr.[1], Wen Luo[1], Malcolm L. Snead[1], Mark Mayo[1], Constant Crohin[1], Sharon Millar[1], Zena Werb[4], Sue Buckley[2], Fredrick L. Hall[3], David Warburton[2] and Harold C. Slavkin[1,†]

[1]The Center for Craniofacial Molecular Biology, School of Dentistry, University of Southern California, Los Angeles, CA, USA
[2]Developmental Lung Biology Research Center, Division of Neonatology and Pediatric Pulmonology, Department of Pediatrics, School of Medicine, University of Southern California, Los Angeles, USA
[3]Orthopedic Research Laboratory, Division of Pediatric Orthopedics, Childrens Hospital of Los Angeles, School of Medicine, University of Southern California, Los Angeles, CA,USA
[4]Laboratory of Radiobiology and Environmental Health, School of Medicine, University of California, San Francisco, CA 94143-0750, USA

*Present address: Department of Growth and Development, School of Dentistry, University of California, San Francisco, CA 94143-0640, USA
†Author for correspondence

SUMMARY

Mutations associated with genes of the EGF super-family are implicated in facial malformations arising from abnormal development of the first branchial arch. EGF and EGF receptor (EGFr) transcripts are expressed in the mouse embryonic first branchial arch and derivatives from E9 through E15. EGF transcripts are localized to ectomesenchymal cells associated with precartilage, cartilage, bone and tooth-forming cells. EGF and EGFr proteins co-localize to the same cells suggesting an autocrine regulation. To test whether EGF effects the timing and positional information required for Meckel's cartilage (MC) and tooth development, we cultured E10 mandibular explants in serumless, chemically defined medium with either antisense or sense EGF oligodeoxynucleotides. Antisense inhibition of EGF expression produces bilaterally symmetrical *Fusilli*-form dysmorphogenesis of MC and decreases tooth bud size; these effects are reversed by the addition of exogenous EGF to the culture medium. Tyrphostin RG 50864, which inhibits EGF receptor kinase activity, inhibits EGF stimulation of tyrosine phosphorylation in a concentration-dependent manner and severely retards mandibular development yet increases tooth size. These findings support the hypothesis that endogenous EGF and EGF-like proteins provide signalling to regulate the size and shape both of cartilage and tooth formation during craniofacial morphogenesis.

Key words: EGF, EGFr, Meckel's cartilage morphogenesis, tyrphostin, antisense, epithelial-mesenchymal interactions, mouse mandible

INTRODUCTION

Loss-of-function mutations that disrupt normal first branchial arch-derived mandibular development can define a set of genes that are required for morphogenesis (Bailey, 1985; Gruneberg and Truslove, 1960; Juriloff and Harris, 1991; Juriloff et al., 1992; Morrison-Graham et al., 1992). Mutations associated with the EGF super-family of regulatory molecules [e.g. transforming growth factor-alpha (TGF-alpha), amphiregulin, cripto and heparin-binding EGF-like growth factor (HB-EGF)] are implicated in first branchial arch malformations including ablation of tooth and cartilage formation as well as retarded mandibular development (Ardinger et al., 1989; Ferguson, 1991; Kronmiller et al., 1991a,b; Murray et al., 1992; Wilkinson and Poswillo, 1991).

EGF is a 53 amino acid polypeptide of 6×10^3 M_r (Carpenter, 1987; Carpenter and Cohen, 1990) derived from a precursor of 136×10^3 M_r, which contains not only EGF (amino acid residues 977-1029), but also eight EGF-like repeats and is encoded in a single gene localized to the mouse chromosome 3 (Scott et al., 1983; Zabel et al., 1985). EGF effects are mediated by EGF binding to specific cell surface receptors (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 EGFr by tyrphostins inhibits EGF-stimulated growth and cell proliferation (Lyall et al., 1989; Yaish et al., 1988).

Among mouse mutants, the first arch mutation, localized to chromosome 2 (Juriloff and Harris, 1991; Juriloff et al., 1992), and the Patch mutant, which is a deletion of the gene encoding the platelet-derived growth factor receptor alpha subunit (Morrison-Graham et al., 1992), demonstrate phenotypes of craniofacial defects that show features in common with inherited human mandibulofacial dysostosis and hemifacial microsomia syndromes (Gorlin et al., 1990). Several teratogens including glucocorticosteroids and retinoic acid (RA) produce mandibulofacial dysostosis and limb deformities in a number of mammalian species (Lammer et al., 1985; Shih et al., 1974; Sulik et al., 1988; Wilkinson and Poswillo, 1991). RA appears to up-regulate a number of Hox genes and growth factor genes, including EGF-associated genes, correlated with pattern formation associated with development of the first branchial arch (Abbott and Pratt, 1987a,b; 1988; Ferguson, 1991; Heath and Smith, 1989; Kessel and Gruss, 1991; Mackenzie et al., 1991; 1992; Maden et al., 1991; Mavilio et al., 1988; Simeone et al., 1990).

To elucidate the role of endogenous EGF signalling that controls cartilage and tooth size and shape, we have investigated the timing and position of EGF and EGFr expression during mandibular morphogenesis We report herein that antisense EGF inhibition induced a novel bilaterally symmetrical *Fusilli*-form dysmorphogenesis of Meckel's cartilage and decreased tooth size. Tyrphostins, which are inhibitors of the EGFr tyrosine kinase activity, inhibit the effect of EGF signalling on cartilage yet induced increased tooth size. These data support the hypothesis that activation of EGFr by members of the EGF super-family plays a key role in regulating the bilaterally symmetrical size and shape of Meckel's cartilage and tooth formation during embryonic craniofacial morphogenesis.

MATERIALS AND METHODS

Mandibular organ cultures

Mouse mandibles were cultured in serumless, chemically defined medium according to methods from our laboratory (Slavkin et. al., 1989; Mayo et al., 1992). Briefly, E10 Swiss Webster mouse embryos at Theiler stage 18 (Theiler, 1972) were isolated and the mandibular divisions of the first branchial arch were microdissected and explanted. Explants were supported by Millipore type AABP filters, 0.8 μm pore size and 6 mm diameter, on steel rafts and cultured in BGJb medium (GIBCO-BRL, Grand Island, New York). Cultures were maintained at 37°C and 5% carbon dioxide with medium change every two days. All experiments were done in triplicate.

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 hours culture was determined (Sigma, St. Louis, MO). With LD activity determined at no more than 200 units per ml of culture medium, cell death in this system was found to be negligible.

Reverse transcription-polymerase reaction (RT-PCR)

Total RNA was extracted from tissues according to Evans and Kandar (1990) and RT-PCR was performed according to Rappolee et al. (1988a,b). Briefly, the tissues were homogenized in 8 M guanidine hydrochloride: 3 M sodium acetate: 10% sodium sarcosyl (8:1:1, v/v/v) and sheared three times with a 25-gauge needle. Total RNA was precipitated with absolute ethanol at −20°C, re-dissolved in 8 M guanidine hydrochloride: 3 M sodium acetate (9:1, v/v), reprecipitated with absolute ethanol at −20°C, washed twice with 80% and absolute ethanol and stored in DEPC-treated water with 0.1 U/μl RNAse inhibitor (Promega, Madison, Wisconsin).

RT was performed in a 50 μl reaction volume. First, 6 μl of 0.1 M methyl mercury(II) hydroxide (Amoreaco, Ohio) was added to the extracted total RNA and allowed to stand for 7 minutes at room temperature. Then, 3.1 μl of 2-mercaptoethanol (Sigma, St. Louis) was added and allowed to stand for 5 minutes at room temperature. Subsequently, a master mix of 5× RT buffer (Gibco), 1 U M-MLV reverse transcriptase (Gibco), 25 mM dNTP (Pharmacia), RNAse inhibitor (Promega) and 1 μg random hexamer (Pharmacia) was added to each sample and incubated at 42°C for 1 hour. Thereafter, the samples were boiled for 5 minutes and immediately cooled on ice. RT was repeated 2 more times with the addition of 25 mM dNTP and M-MLV reverse transcriptase in each cycle.

PCR was performed in a 50 μl reaction volume. The sequences for the 3′ and 5′ amplimers were designed and synthesized based on the sequence for EGF precursor mRNA for the mouse submandibular gland (Scott et al., 1983). The oligonucleotides were synthesized unmodified using phosphoramidite chemistry (Caruthers et al., 1987) on a PCR Mate EP 391 DNA synthesizer (Applied Biosystems, Foster City, California). The sequences for EGF amplimers were as follows: 5′ AGA GCC AGT TCA GTA GAA ACT GGG and 3′ ACT TTG GTT TCT AAT GAT TTT TCT CC. The sequences for EGFr amplimers were as follows: 5′ AGA ACA ACA CCC TGG TCT GGA AGT and 3′ CCA GTC GCG ATG GAT GGG ATC TT (Petch et al., 1990). The PCR reaction mix was composed of DEPC-treated water, 1× PCR buffer containing 2.5 mM magnesium chloride, 10 mM dNTP, 2.5 U Taq polymerase, 20 pmoles each of the 3′ and 5′ amplimer and 2 μl of the RT product. This PCR mix was placed in a thermal cycler (Ericomp) programmed for 40 cycles of 94°C denaturation for 1 minute, 55°C annealing for 1 minute and 72°C elongation for 1 minute. Subsequently, the PCR products were visualized on a 4% agarose gel (Nusieve: BRL agarose; 3:1; w/w) and stained with 1 μg/ml ethidium bromide. The fragment size of the amplified EGF product was 255 base pair (bp) and the EGFr product was 146 bp. The authenticity of the amplified products for EGF and EGFr cDNA was confirmed by direct nucleotide sequencecing of the RT-PCR products taken from the gel. RT-PCR products taken from adult male submandibular gland were used as positive controls. Beta-actin was used as an internal control. RT-PCR products obtained from diethlypolycarbonate (DEPC)-treated water were used as negative controls (data not shown).

Histological preparation

Representative specimens were selected for histological preparation. Tissues were fixed in buffered formalin fixative for 3 hours at room temperature, dehydrated in graded series of ethanol, cleared in xylene, infiltrated and embedded in paraffin. The paraffin blocks were serial-sectioned at 5 μm thickness and stained with haematoxylin and eosin.

In situ hybridization

Serial sections of entire mandibles in vivo (E10-E14) and in culture (E10 mandibular explants cultured for 3, 6 and 9 days), were prepared for detection of EGF transcription using in situ hybridization. Tissue preparation, sense and antisense cRNA probe synthesis, hybridization and detection were essentially as previously described (Snead et al., 1989).

Immunohistochemistry

Representative specimens were selected for immunohistochemistry. These tissues were fixed in Carnoy's fixative (ethanol: chloroform: glacial acetic acid, 6/3/1) overnight, dehydrated in absolute ethanol, cleared in xylene, infiltrated and embedded in paraffin. These blocks were sectioned at 5 μm thickness and immunostained using the Zymed Kit (California). Polyclonal EGF precursor antibodies (a gift from Chiron, Emmeryville, California) were used at a dilution of 1:400, polyclonal EGF antibodies (UBI, Lake Placid, New York) were used at a dilution of 1:200, with a 6 hour incubation at room temperature. Monoclonal EGF receptor antibodies (ICN, Irvine, California) and polyclonal phosphotyrosine antibodies (UBI, Lake Placid, New York) were both used at a dilution of 1:50, with an overnight incubation at 37°C. The secondary antibodies were horse radish peroxidase conjugated for all three procedures. Positive reaction was localized as a red deposit in the Zymed detection system and for contrast, slides were briefly counterstained with haematoxylin.

Tyrphostin effects on mandibular development

Tyrphostin, [RG 50864 and RG 50862, gifts from Rorer Biotechnology, King of Prussia, Pennsylvannia], an inhibitor of EGFr tyrosine kinase activity, was added at concentrations of 20, 40, 80 and 160 μM in the presence or absence of exogenous EGF (Sigma, St. Louis, Missouri). Stock solutions of tyrphostin were made in dimethylsulfoxide (DMSO) and were diluted prior to use so that the final concentration of DMSO in the BGJb incubation medium was less than 0.1%. At 0.1% concentration DMSO had no effect on mandibular development. RG-50864 is among the most specific of the tyrphostins with an IC_{50} for the EGFr tyrosine kinase in the 1.8 μM range (Gazit et al., 1989; Posner et al., 1989). RG 50862 is the least active tyrphostin and was used as a control for no inhibitory effect upon mandibular development. Treated mandibles were compared with controls and representative specimens were evaluated morphologically and biochemically.

Assays for EGF stimulated EGFr tyrosine kinase activation or inhibition

E10 mandibular explants were incubated in the presence of 200 μCi [^{32}P]orthophosphate/ml BGJb culture medium for 4 hour to metabolically label the tissue phosphate pools to equilibrium. Explants were either controls (E10 plus 4 days in vitro), or were treated with either exogenous EGF (20-40 ng/ml) or 20-60 μM tyrphostin or both EGF and tyrphostin. Replicate mandibular explants were stimulated with EGF for 20 minutes following metabolic labeling with [^{32}P]orthophosphate and EGFr tyrosine kinase activation of tyrosine phosphorylation was then detected by immunoprecipitation of tyrosine phosphoproteins using PY-20 antibodies (ICN, Irvine, California) coupled to Sepharose-A beads. Immunoprecipitated tyrosine phosphoproteins were then separated by SDS-PAGE and autoradiographed as recently described (Warburton et al., 1992).

Determinations of DNA, RNA and protein

Representative specimens were used to determine total DNA, RNA and protein (Keleti and Lederer, 1973). The rate of DNA synthesis was determined by the incorporation of tritiated thymidine into mandibular tissues as previously described (Slavkin et al., 1989, 1989; Mayo et al., 1992). The mandibular cultures were pulse-labeled with 50 μCi/ml of [^{3}H]thymidine (1 mCi/ml stock, methyl-^{3}H, 77 Ci/mmole, New England Nuclear, Boston, Mass) for 4 hours and chased with 1 mg/ml unlabeled thymidine for an additional hour after sequential days in culture. The specimens were thoroughly rinsed 5 times with cold PBS and retrieved. These samples were solubilized in 100 μl of Solvable (New England Nuclear, Boston, Mass.) for 3 hours at 50°C and aliquots were taken for liquid scintillation counting.

EGF translation arrest by antisense oligonucleotide inhibition

EGF sense and antisense oligodeoxynucleotides 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. Theoretically, a 15-bp oligonucleotide provides uniqueness in 500 million bases of DNA. Oligonucleotides of both sense (5′ TCG GCC CCA GGG CAT 3′) and antisense (5′ ATG CCC TGG GGC CGA 3′) orientation were synthesized by using phosphoramidite chemistry (Caruthers et al., 1987) on the PCR Mate EP 391 DNA synthesizer (Applied Biosystems, Foster City, California) and purified using reverse-phase HPLC. The sense and antisense oligonucleotides were dissolved in double distilled water and quantified by optical density at OD_{260}. Medium was prepared fresh and changed every other day. In addition, oligonucleotides of both sense (5′ AGG TGG TAG GGG CAT 3′) and antisense (5′ ATG CCC CTA CCA CCT 3′) orientation were synthesized based on the sequence of mouse amelogenin (AMEL) (Snead et al., 1985) which was expressed at birth in the mouse mandibular first molar bell stage tooth organ (Snead et al., 1988). The designed oligodeoxynucleotides were cross-referenced with the current Genebank and IBI Sequence Analysis Program (International Biotechnologies, New Haven, Conn.) to ensure the specificity of the sense and antisense synthetic products. The sense and antisense pentadecaoligomers were used at 30 μM as additional controls (approximately 10^{-6} molecules). E10 mandibular explants were cultured with (1) control medium, (2) 30 μM sense, (3) 30 μM antisense, (4) exogenous EGF at a concentration of 20 ng/ml culture medium, (5) sense with exogenous EGF, or (6) antisense with exogenous EGF.

Whole-mount staining

At the termination of the experiments, specimens were processed to examine the three-dimensional architecture of Meckel's cartilage employing whole-mount staining with Alcian blue. Briefly, Alcian blue specifically stains the chondroitin-4- and chondroitin-6-sulphate components of cartilage. Explants were isolated and fixed overnight in 95% ethanol and 20% glacial acetic acid containing 10 mg/100 cc Alcian Blue (EM Science, Cherry Hill). Specimens were then hydrated through a graded series of ethanol (95%, 80%, 70%, 50% and 30%) twice; each step for 30 minutes. The specimens were hydrated in two changes of distilled deionized water, cleared in 0.1 potassium hydroxide for 1 hour and excessive Alcian Blue staining was then washed off and specimens were examined. The cartilage stained blue with all other soft tissues appearing transparent.

Morphometric assays from histological serial sections

E9-E14 mandibles and E10 mandibular explants cultured in vitro from experimental and control groups were processed through routine histology, serially sectioned at 5 μm, stained with hematoxylin and eosin and examined using light microscopy. Three explant samples from each group were used for volumetric determinations by the computer-assisted SigmaScan morphometric study program (Jandel, San Francisco, CA). Histologic sections, including in situ hybridization samples, 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. Mandible or tooth organ volumes were calculated by the integration of the area times the thickness of each histologic section (5 μm). Evaluation of areas was analyzed by computer-assisted three-dimensional image reconstruction of representative samples (PC3D, Jandel, San Francisco, CA). Images from all explants were

rotated to different degrees to allow maximum visibility of developing mandibular explants.

Statistical analyses

All studies were done in triplicate. Data were analyzed by using one-way analysis of variance and Student *t*-test, taking the confidence level at $P<0.05$ (Epistat statistics program).

RESULTS

E10 embryonic mouse first branchial arch undergoes mandibular morphogenesis in culture

The embryonic mouse mandibular processes consist of two bilateral halves including the medial tuberculum impar anlagen; ectoderm-derived epithelium surrounding cranial neural crest-derived ectomesenchymal cells, endothelial cells associated with a primitive vasculature and occipital somite-derived mesodermal cells (Fig. 1A, arrow). Under conditions where E10 mandibular processes are cultured in serumless, chemically defined medium, explants develop into a number of tissue phenotypes including tongue morphogenesis (Fig. 1B,C), formation of bilaterally symmetrical Meckel's cartilage (MC) (Fig. 1D,E), initial osteogenesis (Fig. 1E, arrow), as well as both incisor and molar tooth bud formation (Fig. 1F). E10 mandibular explants contained 0.5×10^6 cells, mandibles cultured for 9 days contained 1.2×10^6 cells.

EGF and EGFr transcripts are expressed during mouse embryonic mandibular morphogenesis

To identify, quantitate and correlate EGF and EGFr expression, we analyzed early embryonic mouse mandibular morphogenesis by mRNA phenotyping (Rappolee et al., 1988a,b). EGF and EGFr transcripts were expressed as early as E10 and the relative number of EGF and EGFr transcripts increased with subsequent development through E13 (Fig. 2). By E13 the mandible contained 8.1×10^6 cells. In culture, expression of EGF and EGFr are similar to the in vivo patterns (Fig. 3A-D). However, whereas EGF precursor mRNA levels increased 100-fold from E10 to E13 in vivo, EGF transcripts produced by E10 cultured explant per cell increased to 1000-fold higher levels by 9 days in vitro (Fig. 3C,D). Exogenous EGF significantly decreased the relative number of endogenous EGF transcripts expressd in vitro per mandibular cell (Table 1). In addition to EGF and EGFr, a number of other growth factor transcripts are also expressed during mandibular development in vivo (E10-E13) and in culture including TGF-alpha, IGF-I, IGF-II, bFGF and TGF-beta$_1$ (Slavkin et al., 1990).

EGF precursor and EGFr localize to the same epithelial and ectomesenchymal cells

To determine the timing and position within the forming mandibles of EGF transcription, the cellular localization and tissue distribution of EGF mRNAs were elucidated by in situ hybridization. Serial sections, representing E10 explants cultured for 3, 6 and 9 days, were hybridized to either sense or antisense radiolabeled cRNA probes corresponding to amino acids 1070-1081 of precursor mouse salivary gland EGF mRNA (Scott et al., 1983; Snead et al., 1989). EGF precursor mRNA was localized in cells within precartilage, perichondrium and cartilage associated with the perimeter and interior of Meckel's cartilage, as well as to sites of forming bone (Fig. 4A,B). EGF transcripts were also localized to cells within the incisor and molar tooth buds epithelium and ectomesenchyme.

To assess EGF and EGFr translation products, we used immunohistochemistry to localize epitopes contained within the EGF precursor (antibodies were directed against polypeptides containing amino acid residues 348-691), to epitopes within processed EGF, and to both EGFr and phosphotyrosine. EGF precursor antigen and EGFr immunostaining were localized with discrete immunostaining to the same cells as previous experiments identified EGF precursor mRNA expression (Fig. 5A,B), whereas post-translationally processed EGF immunostaining was a diffuse distribution within ectomesenchyme and epithelia throughout the developing mandible. EGF precursor antigen, EGFr and phosphotyrosine epitopes were all detected as immunostaining localized to precartilage, cartilage, adjacent osteoblasts and odontogenic cells. Phosphotyrosine immunostaining was localized to osteoblasts, perichondrium and dental papilla ectomesenchymal cells associated with tooth buds (Fig. 5C,D). EGFr tyrosine kinase mediates tyrosine phosphorylation when EGF, TGF-alpha or other members of the EGF super-family bind to the receptor. Taken together, these data clearly demonstrate that endogenous EGF signalling can function in both autocrine as well as paracrine controls for cartilage, bone and tooth formations.

Embryonic mandibular processes respond to exogenous EGF

To assess EGF receptor responsiveness to EGF signalling in E10 mandibular cultures under serumless conditions, we analyzed a dose-response for exogenous EGF supplemented to the culture medium. Under these experimental conditions mandibular explants were responsive in a dose-response manner to the administration of exogenous EGF. Four distinct effects were seen: (i) EGF mRNA levels was reduced; (ii) [^{3}H]thymidine incorporation into DNA increased as did the size of explants; (iii) Meckel's cartilage size increased; and (iv) increasing concentrations of EGF reduced tooth size in the mandibular explants. Addition of exogenous EGF (2 ng/ml) decreased steady-state levels for endogenous EGF mRNA transcription (Table 1). The effects of exogenous EGF on net DNA, RNA and protein accumulation and [^{3}H]thymidine incorporation into DNA are shown in Fig. 6. Exogenous EGF (10 ng/ml) produced a 50% increase in DNA synthesis, whereas 20 ng EGF/ml resulted in a 50% reduction in DNA synthesis without toxicity.

Antisense ablation of EGF expression induced fusilli-form Meckel's cartilage dysmorphogenesis

Meckel's cartilage (MC) is assumed to determine the size and shape of the developing mandible (Bhaskar, 1953; Bhaskar et al., 1953; Frommer and Margolies, 1971; Gaunt, 1964; Hall, 1991; Slavkin, 1978; Slavkin et al., 1989; Wilkinson and Poswillo, 1991). During embryonic mandibular morphogenesis, MC appears as a 'wish-bone' form consisting of three discrete regions: (i) a rostral medial segment which is triangularly shaped; (ii) two bilaterally symmetrical segments, which are straight, thin, rod-shaped

structures that connect the rostral medial segment to the posterior extensions; and (iii) two bilaterally symmetrical curving posterior segments, which are the rudiments for the malleus and incus of the ear (Fig. 7A).

If endogenous EGF precursor-derived growth factor signalling was exclusively rate-limiting to cartilage and tooth formation, inhibition of EGF precursor translation should ablate cartilage and tooth formation. However, mandibular explants treated with EGF antisense oligonucleotides resulted in *Fusilli*-form dysmorphogenesis of cartilage [a *Fusilli* pasta-like morphology, comparable to mandibular dysmorphogenesis in several first branchial arch syndromes such as oculo-auriculo-vertebral spectrum and mandibulo-facial dysostosis (Gorlin et al., 1990; Shih et al., 1974; Wilkinson and Poswillo, 1991)] as compared to untreated or sense-treated controls (Fig. 7A-D). The decreased

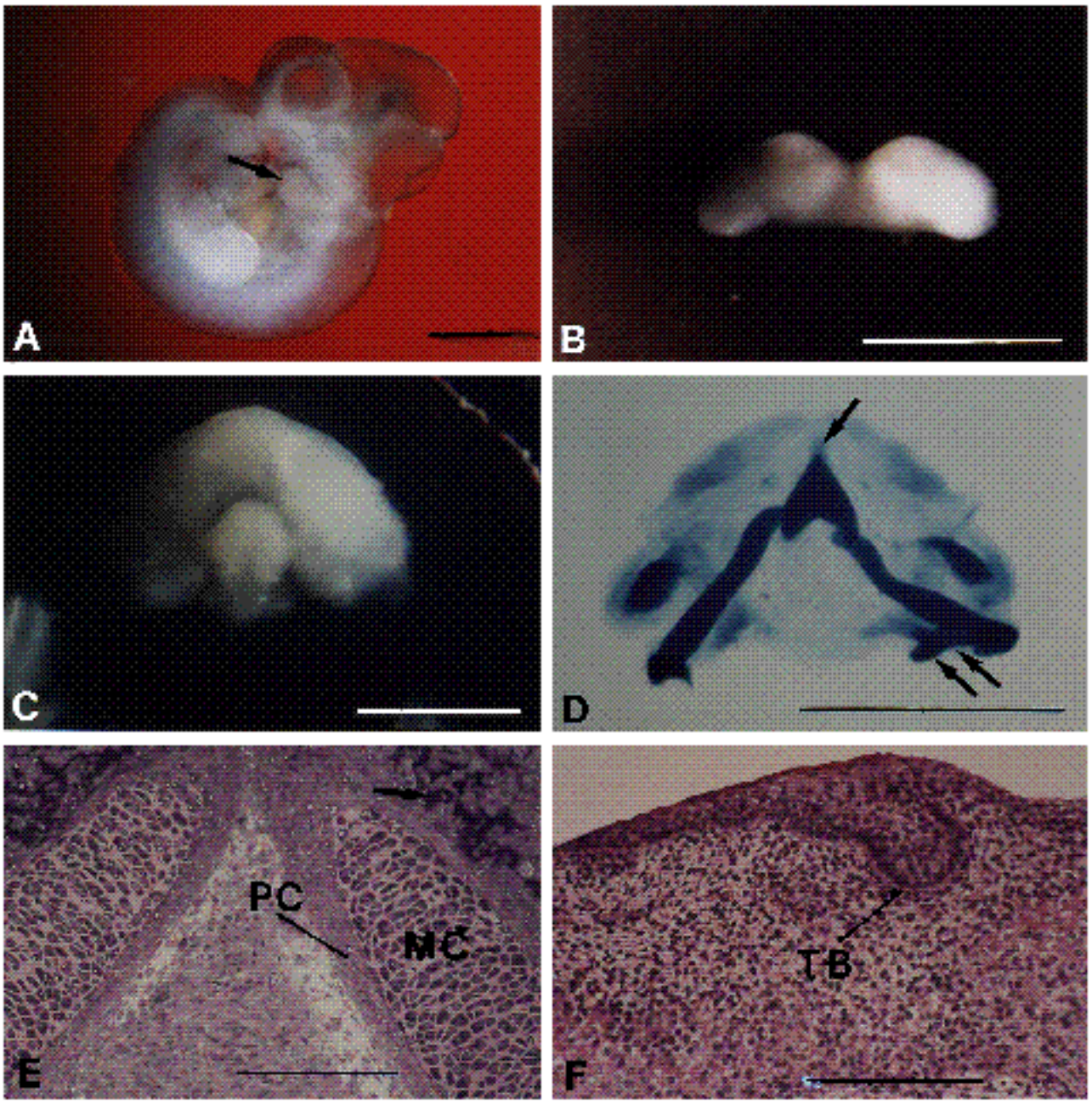

Fig. 1. Embryonic mouse first branchial arch-derived mandibular development in serumless, chemically defined medium. (A) E10 mouse embryo (42-44 somite pair, Theiler stage 18). Arrow indicates mandibular process. (B) E10 mandibular explant, including the tuberculum impar, placed on Millipore filter and cultured in serumless medium. (C) E10 mandibular explant cultured for 9 days demonstrated mandibular and tongue morphogenesis. (D) Whole-mount Alcian blue-stained Meckel's cartilage (MC) of mandibular explants cultured for 9 days in vitro. Single arrow indicates the rostral medial segment and double arrows indicate the extreme posterior segment of MC. [Note: A-D, bar line, 1.0 mm]. (E) Bilaterally symmetrical Meckel's cartilage (MC) with perichondrium (PC) and adjacent osteoid (arrow) converge in the rostral segment. Myotubes form within the forming tongue. (F) Molar tooth bud formation (TB). [Note: E-F, bar line, 100 μm].

expression of EGF induced mirror image helical malformations with periodicity, as well as an increased net production of cartilage under these experimental conditions (Fig. 7C). The malformation was particularly striking within the middle and posterior segments of MC; the rostral medial segment was less affected. Control explants and explants cultured with sense oligodexynucleotides expressed comparable patterns of chondrogenesis, osteogenesis and odontogenesis.

EGF antisense-treated cultures showed three overt alterations: (i) EGF abrogation induced *Fusilli*-form dysmorphogenesis of MC, (ii) cartilage was significantly increased compared to the non-treated, sense- and antisense amelogenin-treated controls (Fig. 7A-D) and (iii) tooth bud size and volume decreased compared with the controls. Antisense treatment induced a decrease in molar tooth bud volume, a reduction of 48%. Addition of 10 ng/ml exogenous EGF, a concentration that stimulates mandibular growth in

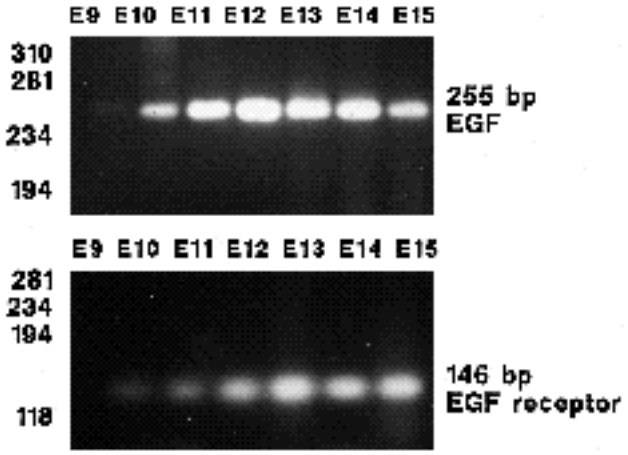

Fig. 2. Temporal expression of EGF precursor mRNA and EGFr mRNA by mouse embryonic processes in vivo from E9-E15 stages of development analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). 3 μl of the reverse transcriptase (RT) reaction was used in each polymerase chain reaction (PCR). The amplified sequences were resolved on 4% agarose gels and the bands viewed by ethidium bromide staining. A 0X174/*Hae*III (BRL, Bethesda, Maryland) ladder molecular weight markers was used to measure the size of the PCR fragments; 255 bp for EGF and 146 bp for EGFr. Adult mouse salivary gland EGF, EGFr and beta-actin RT-PCR products were used as positive controls, and RT-PCR amplification of DEPC water for EGF and E10 mandibles for insulin were used as negative controls (data not shown).

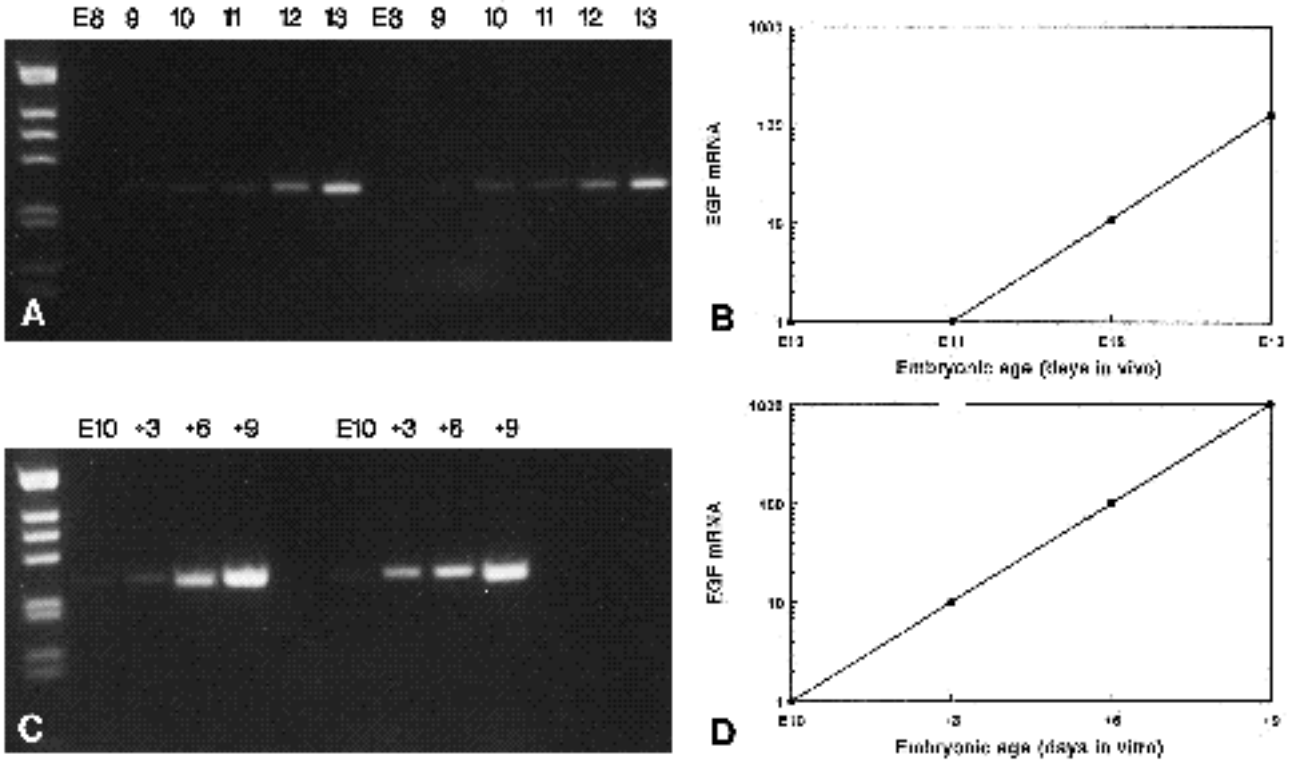

Fig. 3. Semi-quantitative RT-PCR comparison of EGF mRNA expressed in vivo versus within mandibular explants cultured in serumless medium for 3, 6 and 9 days in vitro. (A) duplicate analyses of E8-E13 mandibular expression of EGF transcripts. (B) A very weak PCR product for EGF mRNA at 255 bp was detected in E10 mandibles, which increased 100-fold by E13. (C) Duplicate analyses of E10 mandibular explants cultured for 3, 6 and 9 days in serumless medium in vitro. (D) A 1000-fold increase in EGF mRNA expression was detected in E10 explants cultured for 9 days in vitro. The relative level of EGF precursor mRNA per mandible cell (see Slavkin et al., 1989) is shown in B and D and is based on a comparison with internal EGF precursor mRNA standards obtained from adult male mouse submaxillary gland. Comparable data were also obtained using relative units EGF transcripts to beta-actin transcripts.

Table 1. Exogenous EGF effects on EGF precursor mRNA expression in E10 mandibular explants cultured for 9 days in vitro

Sample	Relative EGF mRNA
E10	1
E10 + 9 days	1,000
E10 + 9 days + EGF (2 ng/ml)	10
E13	100

3 μl of the reverse transcriptase (RT) reaction was used in each polyperase chain reaction (PCR). The amplified sequences were resolved on 4% agarose gels and the bands viewed by ethidium bromide staining. The approximate levels of EGF precursor mRNA per mandible cell is based on a comparison with purified EGF precursor mRNA from adult submaxillary gland (used as an internal standard for semi-quantitative RT-PCR). The E10 mandibles contained 0.5×10^6 cells, whereas E10 mandibles cultured for 9 days in serumless medium consisted of 1.1×10^6 cells. Addition of exogenous 2 ng/ml EGF did not change the cell number.

Table 2. Antisense EGF inhibition induces a significant decrease in tooth bud size which is recovered by exogenous EGF in vitro

Culture conditions	Volume (μm^3)
E10 + 9 days (Control)	3061±132
E10 + 9 days (Sense)	3275±606
E10 + 9 days (Antisense)	1713±287
E10 + 9 days (Antisense & EGF)	2405±197

The size and volume of tooth buds were calculated from computer-assisted, three-dimensional reconstructions of 5 um thick serial sections of mandibular explants (n=3). EGF abrogation reduced molar tooth bud volume by 48% (P<0.05, Student's t-test). Exogenous EGF (10 ng/ml) rescued tooth bud volume to 80% of the sense-treated control valves.

antisense oligonucleotide-treated explants, showed a substantial increase in tooth bud size and volume comparable to that of the normal phenotype; 20-40 ng/ml EGF decreased tooth size. Molar tooth volume recovered to 80% of the sense-treated or non-treated controls (Table 2). Amelogenin antisense or sense oligodeoxynucleotides had no effect on cartilage, bone or tooth development (data not shown).

Taken together these data clearly demonstrate that the primary effects of EGF abrogation on cartilage and tooth formation are mediated through inhibition of endogenous EGF growth factor availability for binding to and activating the EGFr.

Tyrphostin, which inhibits EGF signal transduction, inhibits mandibular morphogenesis and increased tooth size

If growth factor binding to the EGFr (as opposed to effects mediated directly due to the EGF precursor) accounts for the effects of EGF on first branchial arch-derived mandibular development, then abrogation of the function of the EGF receptor should produce the same clinical phenocopy as described for the EGF antisense experiment. If, on the other hand, other ligands are also acting through the EGF receptor, perhaps in spatially distinct sites and possibly through transmembrane bound ligands [e.g. TGF-alpha, *Notch*, *Motch*, *lin*-12, *glp*-1 (Brachmann et al., 1989; Del Amo et al., 1992; Greenwald. 1985; Taub et al., 1990; Twardzik, 1985; Wharton et al., 1985)], or substratum adhesion molecules containing multiple EGF-like repeats [e.g. cytotactin or tenascin, laminin, entactin, core protein of proteoglycans (Chiquet-Ehrismann et al., 1986; Durkin et al., 1988; Engel et al., 1987; Graf et al., 1987; Jones et al., 1988; Lawler and Hynes, 1986; Panayotou et al., 1989), then inhibition of the EGF receptor tyrosine kinase activity would only share some characteristics with the antisense EGF-induced mandibular and tooth malformations.

Untreated control explants demonstrated a very low amount of endogenous EGFr tyrosine kinase-mediated autophosphorylation, whereas 20 ng/ml exogenous EGF significantly increased EGF receptor phosphorylation (Fig. 8). Tyrphostin RG 50864 inhibited exogenous EGF stimulation of EGFr kinase-mediated phosphorylation of tyrosine residues (Fig. 8) and inhibited mandibular morphogenesis.

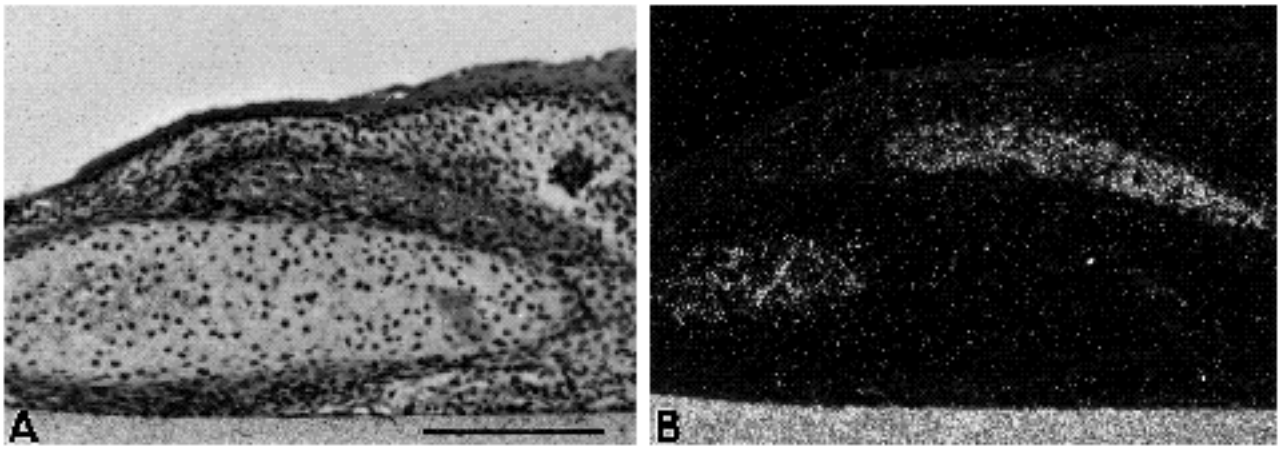

Fig. 4. In situ hybridization of EGF precursor mRNA in E10 explants cultured for 9 days in serumless, chemically defined medium. (A) Bright field of cross-section through Meckel's cartilage and adjacent bone formation in the posterior segment of the mandibular explant; (B) Dark field of the same area indicating intense hybridization signals associated with chondroblasts and adjacent osteoblasts forming osteoid. Bar line, 100 μm.

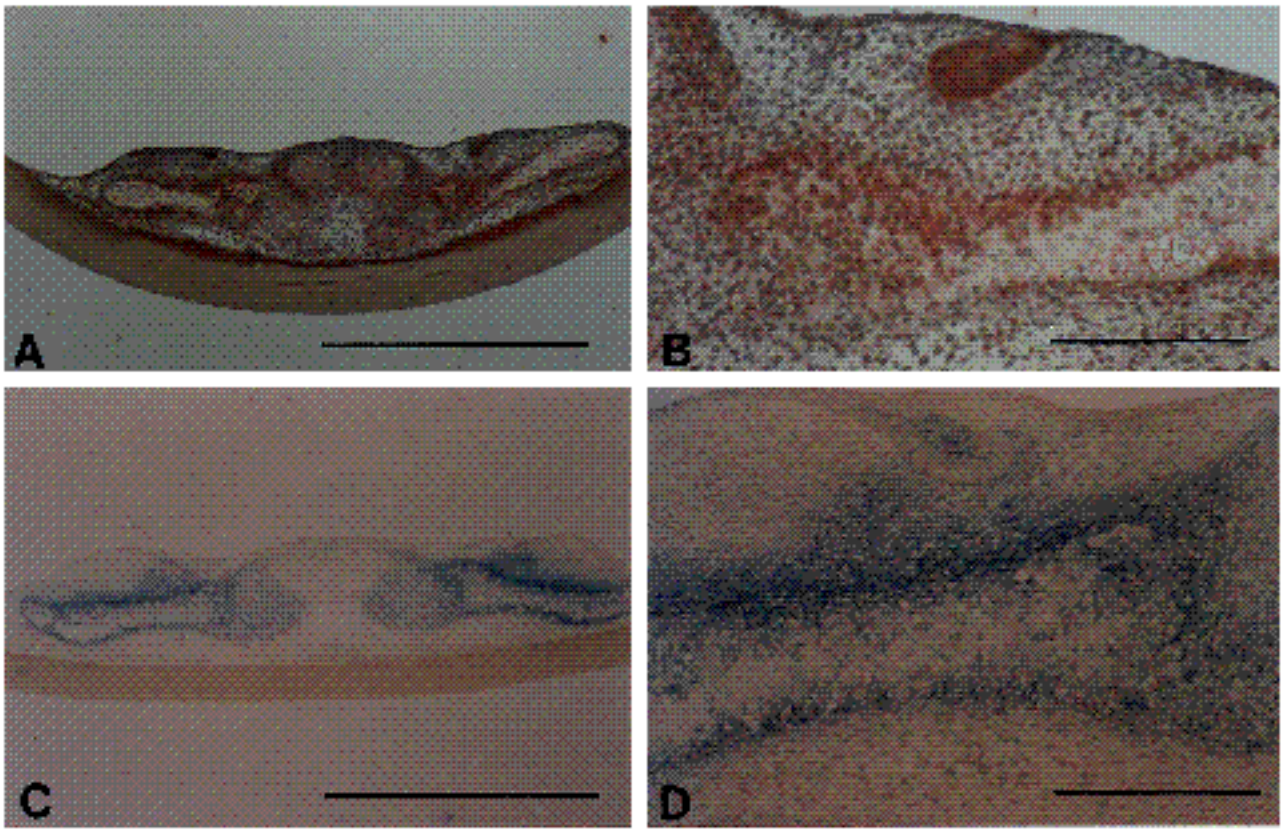

Fig. 5. Immunolocalization of EGFr, EGF precursor and phosphotyrosine antigens in cartilage, bone and tooth development when E10 mandibular explants were cultured for 9 days in vitro. (A) EGF precursor protein was localized to enamel organ epithelia, osteoblasts, perichondrium and chondroblasts (B) EGFr was localized to the same cell phenotypes as the EGF precursor. (C) Low magnification survey of phosphotyrosine immunostaining in cross-sections comparable to those used to localize EGF precursor and EGFr. (D) Intense phosphotyrosine immunostaining was localized to perichondrium, osteoblasts and dental papilla mesenchyme of tooth buds. [Note: A,C, bar line, 500 μm; B,D, bar line, 100 μm].

Total protein, RNA and DNA were significantly inhibited at 60, 80 and 160 μM tyrphostin after 4 days in culture (Fig. 9). Tyrphostin-treated explants were reduced in size, the cartilage phenotype was significantly diminished and tongue development was reduced (microglossia); thus mimicking the phenotype observed by EGF antisense inhibition (compare Figs 7 and 9). The 160 μM concentration was toxic based upon the LD (lactate dehydrogenase) activities of medium collected after 48 hours culture, whereas 20-80 μM tyrphostin did not induce cell death in the culture model. EGFr kinase inhibition with tyrphostins retarded both mandibular cell growth and morphogenesis. The inhibitory effect of tyrphostins on mandibular morphogenesis was irreversible, either by removal of tyrphostin from the culture medium following 2 days in vitro, or by supplementation with the addition of 10-20 ng/ml exogenous EGF in the presence of tyrphostin. The less active tyrphostin, RG 50862, has no inhibitory effect using this in vitro culture model (data not shown).

Additional studies were designed to compare the effects of exogenous EGF or tyrphostin treatment on tooth development within E10 mandibular explants cultured for 9 days in serumless, chemically defined medium. We predicted, based upon the antisense results, that exogenous EGF would increase tooth size, whereas tyrphostin would inhibit EGF-mediated signal transduction and thereby inhibit or ablate tooth formation. Curiously, the addition of 10-20 ng/ml EGF had no effect whereas addition of 40 ng/ml exogenous EGF reduced tooth size by 50% (Figs 10, 11). Tyrphostin treatment at 40 uM induced a statistically significant 20% increase in tooth size (Fig. 11). Tyrphostin treatment induced a precocious molar cap stage tooth development with no evidence of toxicity.

DISCUSSION

In this study, we have shown that activation of EGFr by endogenous EGF is directly required for the regulation of the size, shape and rates of development for embryonic mouse cartilage and tooth formation during mandibular morphogenesis. We used several approaches to support this demonstration. First, we analyzed the time and position for EGF and EGFr gene expression and determined that both ligand and receptor are expressed prior to and during cartilage and tooth development. We confirmed previous studies that indicated that EGF and EGFr are present in E10 mouse embryos (Adamson and Meek, 1984; Kronmiller et

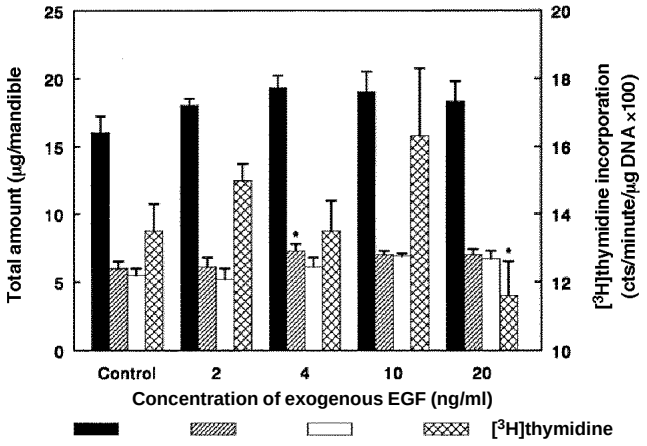

Fig. 6. Exogenous EGF effects on total protein, RNA, DNA and DNA synthesis in mandibular explants cultured for 9 days in vitro (see *Experimental Procedures*). Asterisk indicates statistical significance (P>0.05).

al., 1991a; Nexo et al., 1980). We demonstrated that EGF transcipts increased 1000-fold when E10 mandibular explants were cultured for 9 days in serumless, chemically defined medium (Fig. 3C,D; Table 1); addition of exogenous EGF to the serumless medium was found to reduce EGF transcripts (Table 1). Second, we determined the specificity of EGF signalling as a primary control for cartilage and tooth formations by either using EGF antisense inhibition to reduce endogenous EGF growth factor availability, or tyrphostin inhibition to block EGFr tyrosine kinase activity.

The present study demonstrates that EGF expression by embryonic mouse mandibular processes regulates Meckel's cartilage and tooth bud shape and form by signal transduction through binding with EGFr. Abrogation of EGF expression by antisense or of EGF signalling with tyrphostin, resulted in significant changes in the pattern and growth rates (Fig. 7 and 9). The novel bilaterally symmetrical *Fusilli*-form dysmorphogenesis of MC, [a *fusilli* pasta-like morphology, comparable to mandibular dysmorphogenesis in several first branchial arch syndromes such as oculo-auriculo-vertebral spectrum and mandibulofacial dysostosis (Gorlin et al., 1990; Wilkinson and Poswillo, 1991)], induced by EGF abrogation, produced helical transformations that were partially reversed by exogenous EGF (Fig. 7D). The partial recovery suggested that processed EGF per se was directly affected by the antisense strategy. However, tyrphostin inhibition of EGFr signalling produced decreased cell number, decreased extracellular matrix production and almost complete ablation of Meckel's cartilage,

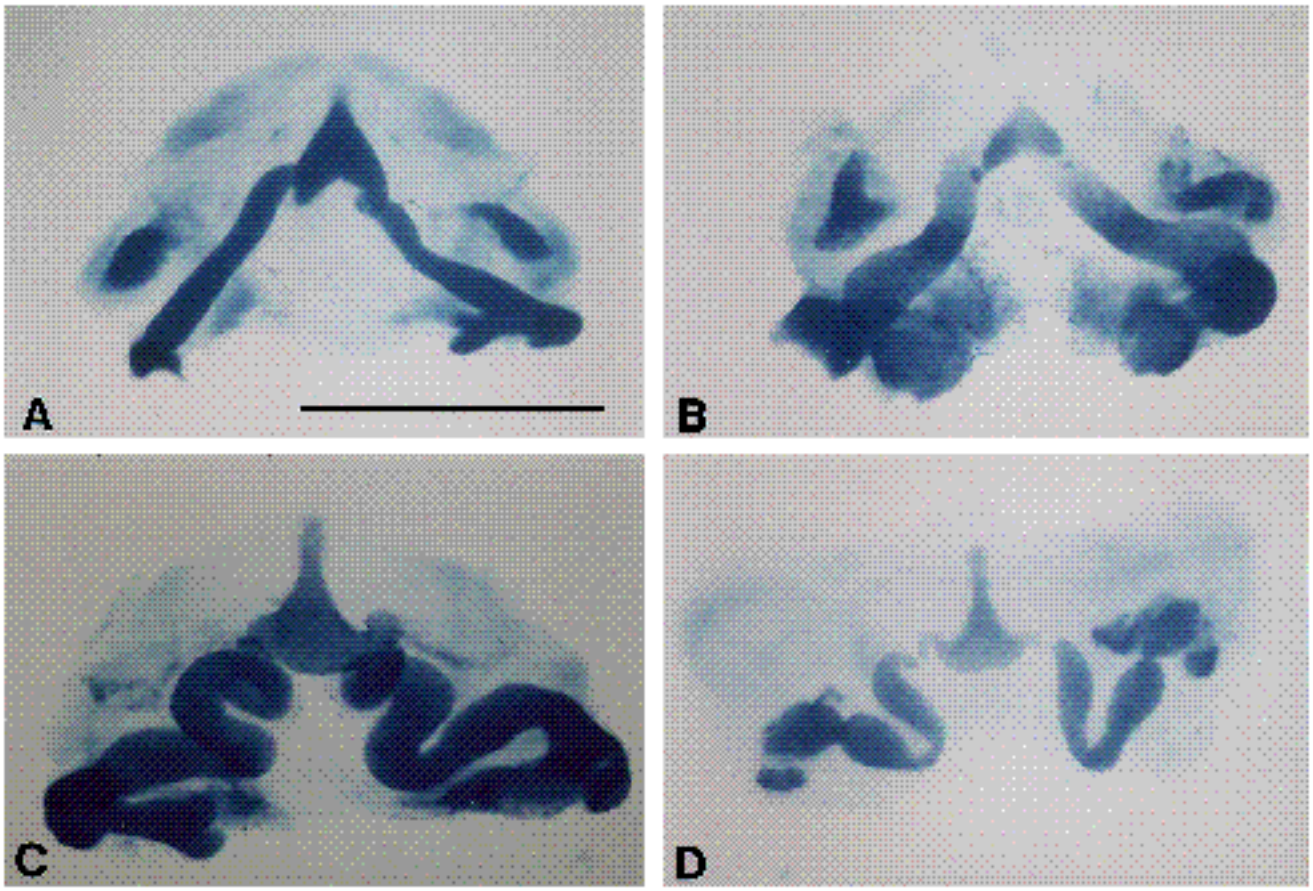

Fig. 7. EGF abrogation induced *Fusilli*-form dysmorphogenesis of Meckel's cartilage. Whole-mount Alcian blue staining of Meckel's cartilage after 9 days in culture. (A) Control; (B) sense-treated; (C) antisense-treated; and (D) antisense-treated plus 10 ng/ml exogenous EGF (see *Experimental Procedures*). Bar line, 1.0 mm.

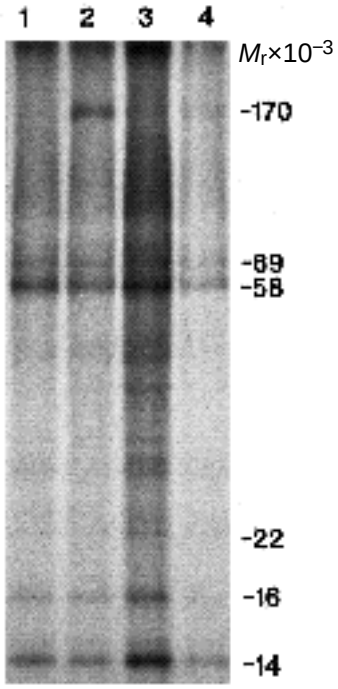

Fig. 8. Autoradiogram illustrating tyrosine phosphorylation in response to EGFr tyrosine kinase activation and inhibition during embryonic mouse mandible development. E10 mandibular explants cultured in vitro in serumless medium for 4 days were metabolically labeled for 4 hour with [^{32}P]P_i. Explants were either controls (lane 1), stimulated for 20 minutes with 10 ng/ml EGF (lane 2), stimulated for 20 minutes with 40 μM tyrphostin (TP) (lane 3), or pretreated with 40 μM tyrphostin (TP) followed by stimulation with 20 ng/ml EGF for 20 minutes (lane 4). Tyrosine phosphoproteins were immunoprecipitated, fractionated by SDS-PAGE and processed for autoradiography (see *Experimental Procedures*). EGF stimulation resulted in a prominent band at the correct molecular weight for the EGFr (170×10^3 M_r). Comparison of control with tyrphostin-treated cultures indicates that tyrphostin decreased basal tyrosine phosphorylation of the EGFr band. Tyrphostin inhibited subsequent stimulation of tyrosine phosphorylation by exogenous EGF.

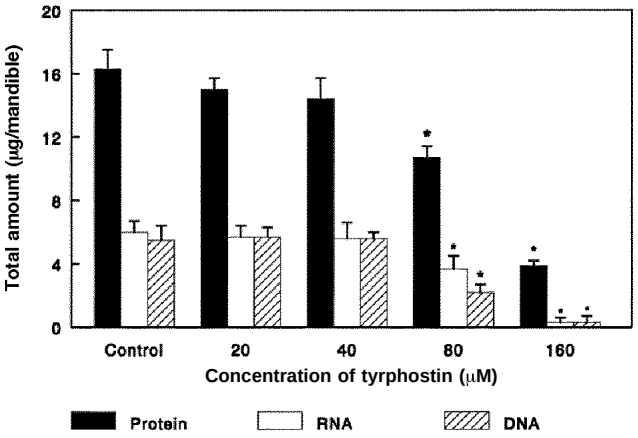

Fig. 9. Tyrphostin effects on total protein, RNA and DNA in mandibular explants cultured for 9 days in vitro. A concentration of 80 μM tyrphostin RG 50864 inhibited cartilage and tooth formation whereas tyrphostin RG 50862 had not effect (see *Experimental Procedures*). Asterisk indicates statistical significance (P>0.05).

which was not recovered by exogenous EGF. Curiously, 40 μM tyrphostin induced precocious molar tooth development with no evidence of toxicity (Figs 10, 11).

One explanation for this apparent discrepancy between EGF abrogation-induced mandibular dysmorphogenesis and tyrphostin inhibition producing micrognathia and reduced cartilage relates to a major problem in developmental biology, namely, biological redundancy. Preimplantation and postimplantation mouse embryos express several different types of growth factor receptors that contain tyrosine kinase activity (e.g. EGFr, insulin receptor, IGF-II/mannose-6-phosphate receptor, platelet-derived growth factor receptor) (Koch et al., 1991 and references therein; Rappolee et al., 1992; Werb, 1990). It is, therefore, quite feasible that different growth factors for which the cognate receptors are present within the embryonic mandibular process control the timing and positional information for cartilage and tooth development. If such is the case, and growth factors are involved in the controls for cartilage and tooth size and shape, a biological function of this redundancy would insure that first branchial arch-derivatives develop. In this regard, whereas EGF antisense inhibition could knock-out EGF availability to bind to the EGF receptor, other EGF-like factors such as TGF-alpha or EGF-like motifs found in substratum adhesion molecules as well as other growth factors (e.g. insulin-like growth factors) could serve as redundant regulatory controls for mandibular morphogenesis. In contrast, tyrphostin inhibition blocked the EGFr tyrosine kinase activity and prevented putative redundancy in the form of various growth factors from signalling cartilage but induced other growth factors to enhance precocious molar tooth morphogenesis (Figs 10, 11). The data presented in this report support the hypothesis that developmental redundancy in the form of EGF-like growth factors likely regulate cartilage using the same cognate receptor; however, tyrphostin treatment resulted in precocious tooth development.

The products of several developmentally important genes such as the *lin*-12 gene of *Caenorhabditis* and the *Notch* and *Delta* genes of *Drosophila* both contain multiple EGF-like repeats (Greenwald, 1985; Wharton et al., 1985). The *Motch* gene, which is expressed in mouse post-implantation embryos, and the *c-erb* gene, which encodes EGFr and TGF-alpha and is present in normal chicken erythrocytic progenitor cells, as well as EGF and TGF-alpha precursors, are apparently all transmembrane peptides (Del Amo et al., 1992; Greenwald et al., 1985; Kopczynski et al., 1988; Pain et al., 1991; Pfeffer and Ullrich, 1985; Rall et al., 1985; Teixido et al., 1987; Wharton et al., 1985). Transmembrane TGF-alpha precursors have been shown to activate EGFr on the same or adjacent cells (Brachmann et al., 1989; Pain et al., 1991; Wong et al., 1989), supporting our assumption that EGF-related proteins, [e.g. *Motch* expression during first branchial arch formation (Del Amo et al., 1992)], mediate cell-cell interactions associated with prechondrogenic and chondrogenic cells during Meckel's cartilage morphogenesis (Fig. 7C,D). Changes in the distribution of tenascin

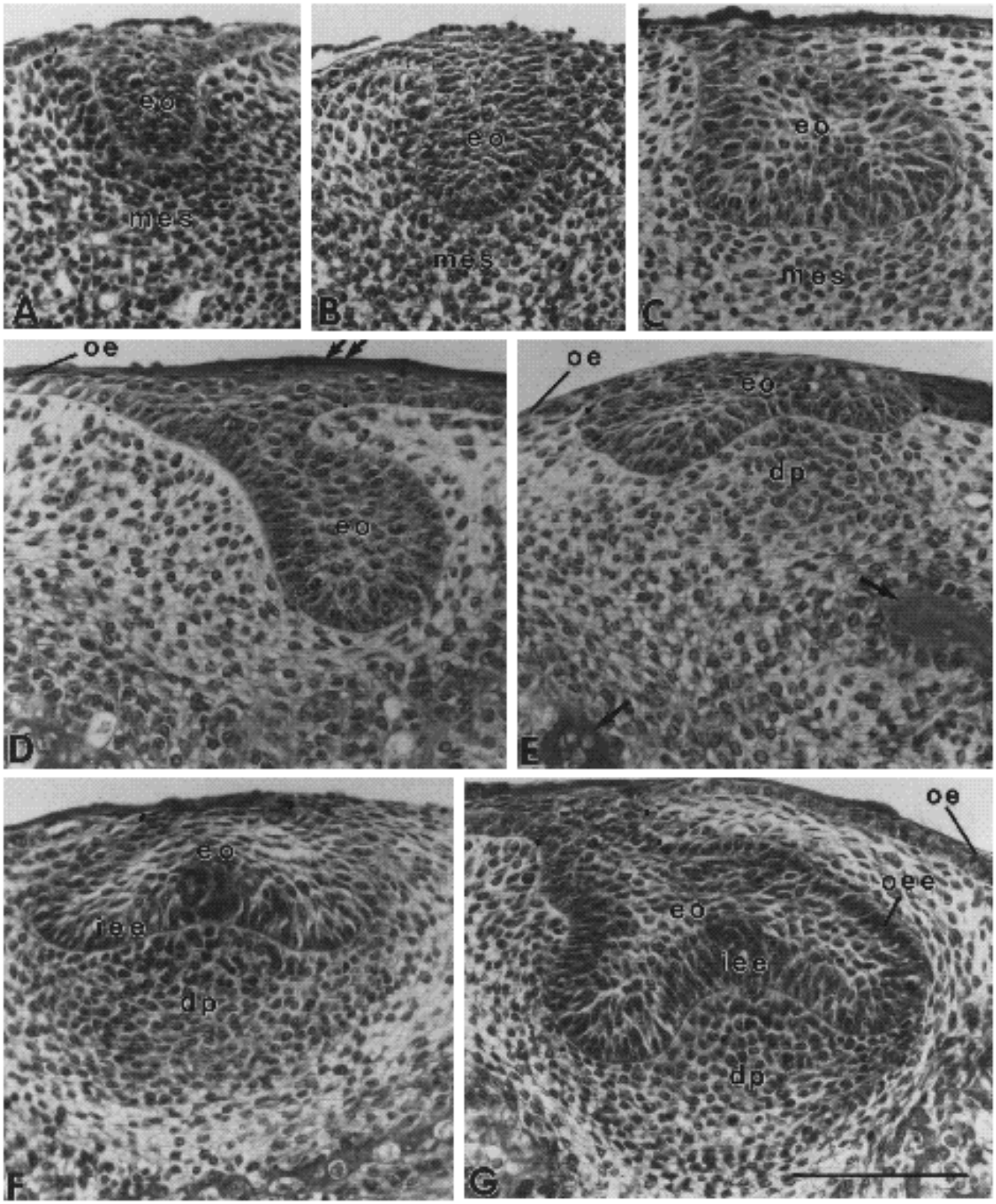

Fig. 10. Tyrphostin and exogenous EGF effects on molar tooth development. (A) E10 mandibular explant cultured for 3 days indicating for formation of the dental lamina. (B) 6 day cultures showed the formation of the tooth bud stage of development. (C) Explant cultured for 9 days indicated continued bud stage development. (D) Exogenous 20 ng/ml EGF added to 9 day cultures produced smaller tooth buds. Oral epidermis (oe) shows well-defined patterns of keratinization (double arrows). (E) Exogenous 40 ng/ml EGF significantly reduced tooth bud size. Arrows, adjacent bone formation. (F) Tyrphostin at 20 μM produced a cap stage tooth with reduced size, whereas (G) tyrphostin at 40 μM significantly increased tooth size and induced cap stage molar tooth formation. All figures at the same magnification (bar line, 100 μm). Abbreviations, eo, enamel organ; mes, cranial neural crest-derived extomesenchyme; dp, dental papilla mesenchyme; oe, oral epithelium; iee, inner enamel epithelium.

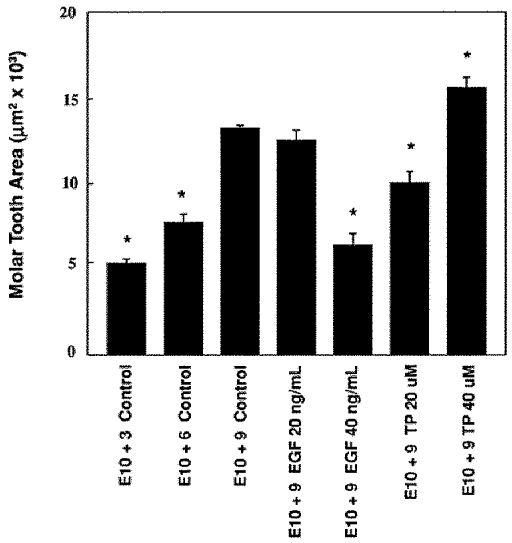

Fig. 11. Tyrphostin and exogenous EGF effects on the area of molar tooth development. Asterisk (*) designates statistical significance at a confidence level of $P>0.05$.

during embryonic mouse cartilage and tooth development in vivo (Mackie et al., 1987; Thesleff et al., 1987), are strikingly similar to our observations for EGF precursor mRNA expression, as well as EGFr and tyrosine phosphoprotein immunostaining during mandibular morphogenesis in vitro (Figs 4, 5).

Previous reports have demonstrated that exogenous EGF inhibits or delays mandibular cartilage (Coffin-Collins and Hall, 1989; Kronmiller et al., 1991b) and tooth development (Partanen et al., 1985; Huet et al., 1993). Whether the processing of TGF-alpha and/or EGF precursors to smaller peptides controls Meckel's cartilage morphogenesis in vivo is still unknown. EGF has been shown to disrupt the normal development of Meckel's cartilage in the avian and murine developing mandible (Coffin-Collins and Hall, 1989; Kronmiller et al., 1991b). EGF appears to up-regulate EGFr during chondrocyte differentiation and has been suggested to serve as an autocrine factor during early chondrogenesis (Kinoshita et al., 1992). EGF also has been found to stimulate cell proliferation in chondrocytes and decrease the synthesis of sulphated proteoglycans (Prins et al., 1982a,b). EGF stimulates mouse types I and III collagen, as well as glycosaminoglycan synthesis between E10 and E12 (Foreman et al., 1991), whereas EGF inhibits type I collagen in osteoblasts (Hata et al., 1984) and in tooth tissues (Hata et al., 1991). EGF has been shown to be preferentially incorporated into osteoprogenitor cells and stimulates cell proliferation and osteogenesis with bone nodule formation in vitro (Antoz et al., 1989). We suggest, therefore, that decreased endogenous EGF availability resulting from antisense inhibition induced malformations in mouse mandibular development significantly affected the control for the synthesis and/or degradation of extracellular matrix constituents associated with the prechondrogenic/chondrogenic tissue boundaries along the perimeter of forming Meckel's cartilage (Matrisian and Hogan, 1990; Partanen, 1990; and references therein). Cartilage ablation was not observed in the antisense studies, but was observed in the tyrphostin inhibition studies.

The specificity of the antisense and tyrphostin experiments is of major concern towards interpreting the results obtained in these investigations. The primary control for antisense inhibition of endogenous EGF production was the recovery strategy that used excess exogenous EGF in the presence of the antisense oligonucleotides thereby producing a partial recovery. Attempts to quantitate the relative number of EGF transcripts in control, sense, or antisense treated cultures have been equivocal; no difference in relative transcript number has been found (data not shown). We must emphasize that it is not known as to what extent the antisense oligomers used in our studies were specific for their intended target EGF mRNAs; clearly a large number of variables can compromise the specificity of oligonucleotides as antisense reagents (Woolf et al., 1992). Therefore, we have not yet been able to address the mechanism by which antisense oligomers specifically exert their Fusilli-like dysmorphogenesis of Meckel's cartilage during embryonic mouse mandibular morphogenesis in serumless medium.

Tyrophostins are a newly synthesized group of compounds designed to inhibit the action of protein tyrosine kinases. Since they are directed against the substrate-binding domain of the kinase and not to the ATP-binding domain, each tyrphostin has the specificity of the tyrosine kinase it was designed for, without cytotoxic effect. Compound 12 (RG60856) was potent in its ability to inhibit the tyrosine kinase activity of the EGFr specifically with an inhibitory constant of 0.85 μM in A131 cells (Lysall et al., 1989; Yaish et al., 1988). Tyrphostin specificity was demonstrated by its preference for the EGFr over the insulin receptor by over three orders of magnitude (Yaish et al., 1988). Most important, the inhibition of the kinase activity was not associated with interference to ligand binding, internalization, phospholipase C activation, phosphoinositides release, calcium release or receptor recycling activities. Therefore, we interpret our experimental results (e.g. tyrphostin inhibitory effects on cartilage morphogenesis and stimulatory effects of tooth development as seen in Figs 9-11) to suggest that tyrphostin acts preferentially but not exclusively on the EGFr and not only on this receptor.

In summary, the finding that EGF abrogation induced *Fusilli*-form malformations of Meckel's cartilage during mandibular development provides an explanation for several types of mandibulofacial dysostosis and oromandibular-limb hypogenesis syndromes (Gorlin et al., 1990). Mutations in the EGF super-family of peptide growth factors or EGF receptor possibly induce clinical first branchial arch syndromes (Ardinger et al., 1991; Kronmiller et al., 1991b). Our observations suggest that EGF or EGF-related peptide growth factors are pleiotropic and are associated with the timing and position both of Meckel's cartilage and molar tooth bud shape and form. This conclusion is further supported by the recent findings of Kronmiller and colleagues (1991b) showing that EGF antisense oligodeoxynucleotides ablated mouse molar tooth and cartilage formation when administered in E9 mouse embryonic mandibular cultures in vitro. The Rieger syndrome (RS) is an autosomal dominant disorder of morphogenesis characterized by craniofacial malformations including retarded tooth develop-

ment (Gorlin et al., 1990). Significant linkage of RS to 4q markers has been identified with tight linkage to EGF (Murray et al., 1992); EGF maps to chromosome 4q25. Therefore, EGF serves as a candidate gene for RS and other craniofacial malformations based upon the timing and tissue expression of EGF or EGF-related growth factors during craniofacial morphogenesis as described in the present study. Precisely how the EGF superfamily of peptide growth factors regulate the size and shape of first branchial arch-derived structures during craniofacial morphogenesis awaits further experimentation.

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