EPITHELIUM IS REQUIRED FOR MAINTAINING FGFR-2 EXPRESSION LEVELS
IN FACIAL MESENCHYME OF THE DEVELOPING CHICK EMBRYO.

by

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ABSTRACT

In the developing chick embryo, FGFR-2 expression patterns correlate with outgrowth of facial prominences: frontonasal mass prominences which form the prenasal cartilage and upper beak express high levels of FGFR-2 receptor while maxillary prominences which form the flattened corners of the beak and palatal shelves express low FGFR-2 transcript levels. Facial epithelium is an abundant source of FGFs and is required to support outgrowth of mesenchymal tissue including cartilage rod formation. Since FGFR-2 is highly expressed in regions of facial outgrowth and epithelium is required for outgrowth of facial prominences, epithelium could be required to maintain FGFR-2 transcripts in facial mesenchyme. To test this hypothesis, we removed epithelium to inhibit outgrowth of regions of the embryonic face, grafted frontonasal mass and maxillary prominences into a host limb bud and then examined changes in FGFR-2 expression using in situ hybridization. We also hybridized adjacent sections with collagen II probe to identify regions undergoing chondrogenesis. Our results indicate that removal of epithelium from frontonasal mass lead to a decrease in FGFR-2 and collagen II expression 24 hours after grafting to host and that neither FGFR-2 nor collagen II expression increased to expected levels at 48 hours. These results suggest that there are signals in the epithelium required for increasing FGFR-2 and collagen II gene transcription and the expression of these genes are linked to outgrowth of facial prominences. We localized FGF8

transcripts in the developing chick face to determine whether FGF8 is a putative epithelial signal required for FGFR-2 expression. Our results indicate that FGF8 ectodermal expression does not overlap FGFR-2 expression in mesenchymal tissue and therefore is not a signal required for the expression of this receptor.

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INTRODUCTION

1

Factors involved in the development of an organism increase with time almost exponentially, hence, developmental biologists tend to focus on a specific aspect of development which can range from studying molecular determinants involved in specifying cell fate to how different tissues may regulate morphogenesis. My research seeks to link gene expression with morphogenesis by addressing how tissue interactions regulate gene transcription.

Generally, most vertebrates have a similar design where a midline splits the face in two symmetrical halves which results in the formation of two ears, two eyes, one nose and one mouth. This design is so similar among vertebrates that it is very difficult to distinguish an early chicken embryo from a mouse embryo from a human embryo. Hence, studying face development in one organism has relevance to understanding face development in many organisms.

Studying face development also has clinical relevance for humans. Craniofacial birth defects like cleft lip/palate affects 1:1000 individuals (Ross and Johnson, 1972) and craniosynostosis is inherited in 1:2500 individuals (Cohen, 1986). Craniofacial research can be applied to future treating of facial abnormalities like cleft lip, hemifacial microsomia and craniosynostosis (Winter and Baraitser, 1994). The genetic cause of some of these craniofacial defects has been determined. The main feature of Crouzon's syndrome is craniosynostosis and the genetic cause is mutations in genes that code for fibroblast growth factor receptors [FGFRs] (Yamaguchi and Rossant, 1995). This project aims to elucidate

development. Hence, by identifying proteins involved in normal face development we can apply this knowledge to understanding human abnormalities.

I have chosen to carry out my studies on the avian embryo because manipulations can be carried out in ovo, embryonic stages are described (Hamburger and Hamilton, 1951), easily monitored and the rate of development can be controlled by altering ambient temperature. It is also possible to study long term effects of early manipulations in the avian embryo and these effects can be related to human development.

THE EMBRYONIC CHICK FACE

In vertebrates, the majority of the embryonic face and head is comprised of tissue originating from cranial neural crest cells (Hall and Horstadius, 1988). The vertebrate neural crest is a population of cells which arise from the dorsal edges of the neural folds as they close to form the neural tube. Neural crest cells migrate extensively along well characterized pathways comprised of extracellular matrix molecules like fibronectin, tenascin and proteoglycans which guide these cells (Bronner-Fraser, 1996) to populate target sites and form sensory neurons, peripheral nervous system neurons, pigment cells, catecholamine secreting cells, and craniofacial skeletal tissue (Le Douarin, 1982). Using radioactive and lipophyllic dye labeling techniques, neural crest cells were shown to reach chick facial primordia at stage 14 when the face is first recognizable (Noden, 1975;

Lumsden et al, 1991). At stage 14 in the chick, the first branchial arch is present and will form the maxillary and mandibular prominences. By stage 15 the nasal placodes are bilateral epithelial thickenings on the edges of the presumptive frontonasal mass. The maxillary prominences are first apparent at stage 18 as buds of tissue bordering the sides of the presumptive mouth (Yee and Abbot, 1978). Non-neural crest derived tissue which contributes to face development include paraxial mesoderm adjacent to the developing brain which goes on to form craniofacial musculature (Noden, 1975; Couly and LeDouarin, 1989).

Initially, facial prominences surround the presumptive oral cavity as buds of mesenchyme covered with epithelium. The two halves of the face consist of paired lateral nasal, maxillary and mandibular prominences with the frontonasal mass prominence formed at the center to establish the midline of the face. Lateral nasal prominences form the conchae of the nose and tissues of the external nares (Romanoff, 1960). The maxillary prominences go on to form the palatal shelves, the pterygoid, quadratojugal, palatine, jugal and maxillary bones. In the center, the frontonasal mass forms the majority of the upper beak including prenasal cartilage, nasal septum and primary palate. The mandibular prominences form the entire lower beak supported by paired rods of Meckel's cartilage.

At stage 24 (4.5 days incubation) maxillary, mandibular and the frontonasal mass prominences are similar in volume but cell differentiation and cartilage formation are beginning to occur. At stage 26, the proximal dorsal part of the lateral nasal processes begin to fuse with the maxilla and this fusion is complete by

stage 30. By stage 30-31, distinct facial outgrowth of the frontonasal

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mass and mandible are visible. The present study seeks to elucidate signals involved in the outgrowth of facial prominences between stage 24 when all primordia are equal in volume and stage 28 where the frontonasal mass and mandible are beginning to differentiate and form cartilage rods.

EPITHELIAL-MESENCHYMAL INTERACTIONS

In the developing chick face, epithelial signals are required for mesenchymal outgrowth and differentiation of facial prominences (Wedden, 1987; Richman and Tickle, 1989; Sabera et al, 1989). Mesenchyme is programmed to specify the structure of facial prominences and can differentiate to form bone and cartilage in the absence of epithelium (Tyler, 1978; Hall, 1980; Richman and Tickle, 1989), however, without epithelium, mesenchymal outgrowth is inhibited (Wedden, 1987; Richman and Tickle, 1989). All facial epithelia are interchangeable and can support morphogenesis of beak structures; whereas, epithelium from areas outside the face such as the dorsal surface of the limb and the flank, cannot provide signals necessary for normal outgrowth of facial prominences (Richman and Tickle, 1989). These results suggest that facial epithelia contain specific signals to foster outgrowth of facial mesenchyme.

FGFs

Which epithelial signals are regulating mesenchymal outgrowth in developing facial prominences? Fibroblast growth factors (FGF) have been shown to regulate many aspects of growth and patterning during development (Yamaguchi and Rossant, 1995). FGF2 (Richman et al., 1997) is homogeneously expressed throughout facial prominences in both epithelium and mesenchymal tissue (summarized in fig.1B) and ectopic applications of this factor can increase the length of cartilage rods formed by frontonasal mass and mandibular mesenchyme (Richman et al., 1997). FGF4 is expressed in mandibular epithelium (Niswander and Martin, 1992; Barlow and Francis-West, 1997) and like FGF2, can also stimulate outgrowth of both mandibular and frontonasal mass mesenchyme (Richman et al., 1997). FGF8 is expressed in the epithelium lining the nasal pits, the corners of the frontonasal mass, the caudal surface of maxillary prominences and the cranial surface of mandibular prominences (Heikinheimo et al., 1994; Ohuchi et al., 1994; Crossley and Martin, 1995; Wall and Hogan, 1996; Helms et al., 1997) but its effects on facial mesenchyme are not known. FGF10 is expressed in developing limb mesenchyme and may activate receptors found in overlying epithelial cells (Ohuchi et al., 1997); its expression pattern in facial prominences has yet to be determined. Recently, four new members of the FGF family called FGF homologous factors (FHF) have been identified (Smallwood et al., 1996) and are expressed in the developing mouse embryo, however, it is unclear whether they

are involved in face development. In sum, FGF2, FGF4, and FGF8 are expressed in developing facial prominences and may provide epithelial signals required for outgrowth of mesenchyme.

FGFRs

Endogenous FGF signals may be mediated by high affinity fibroblast growth factor receptors (FGFRs). Four distinct FGFR genes have been identified (Miller and Rizzino, 1994; Yamaguchi and Rossant, 1995). The structure of FGFRs consists of 3 extracellular immunoglobulin domains, a transmembrane domain, and an intracellular tyrosine kinase domain which is split into 2 parts by a sequence without tyrosine kinase activity. Alternative splicing of the extracellular immunoglobulin domains (Chellaiah et al., 1994) and receptor heterodimerization (Shi et al., 1993) determine ligand binding specificity in vitro. In general, the expression of alternative spliced isoforms of receptors 1-3 are tissue specific (Orr-Urtreger et al., 1993; Shi et al., 1994). The b exon which codes for the carboxy portion of the third immunoglobulin domain is expressed in epithelial tissues while the c exon is found in mesenchymal and neural tissues (Orr-Urtreger et al., 1993; Shi et al., 1994).

FGFR-1,2 and 3 are expressed in facial prominences of the developing chick embryo (Richman and Leon Delgado, 1995; Wilke et al., 1997; summarized

in fig.1A). At stage 28, just prior to the onset of chondrogenesis, FGFR-1 is expressed at higher levels in the mesenchyme lateral to the nasal pits and in the lateral edges of the frontonasal mass and may play a role in stimulating fusion between the frontonasal mass, lateral nasal prominences and maxillary primordia. FGFR-2 is expressed at high levels in the center of the frontonasal mass and may be required for both establishing the midline of the frontonasal mass and for outgrowth of the prenasal cartilage. Moderate levels of FGFR-2 are found at the lateral edges of maxillary prominences and at the caudal third of mandibular prominences. Later on in development, FGFR-2 is found in precartilaginous cell aggregates (Szebenyi et al., 1995; Patstone et al., 1992; Peters et al., 1992; Orr-Urtreger et al., 1993). FGFR-3 transcripts are concentrated at the posterior edge of the frontonasal mass where these receptors may be transducing signals involved in the outgrowth of this primordium. In the mandible, FGFR-3 expression is confined to the lateral third of this prominence, an area that will later form the malleus and incus (Richman and Diewert, 1988; Richman unpublished data). In sum, the mesenchymal isoforms of FGFR-1, 2 and 3 have unique expression patterns in developing facial prominences which correlate with different phases of outgrowth and differentiation.

AIM OF STUDY

In the present study we wish to determine which mesenchymal signals are regulated by epithelium in the developing chick face. FGFs are expressed in the

epithelium of developing facial prominences and can stimulate outgrowth of facial mesenchyme. Moreover, FGFR expression patterns in mesenchymal tissue correlate with relative outgrowth of facial prominences (Richman and Leon Delgado, 1995; Wilke et al., 1997; fig.1A). Based on this data we hypothesize that the truncated outgrowth which occurs after removing facial epithelium may be due to a loss of signal required for maintaining mesenchymal FGFR expression. To test this hypothesis, we removed epithelium from developing facial prominences and then probed for changes in FGFR-2 expression. We chose to examine FGFR-2 because its expression pattern is more pronounced in regions of the face involved in outgrowth than the other FGFRs (fig.1A) and consequently changes in its expression pattern can be readily detected. Our data indicate that epithelium is required for the temporal up-regulation of mesenchymal FGFR-2 and collagen II expression in the frontonasal mass. Epithelium is also required for maintaining FGFR-2 and collagen II expression in maxillary prominences.

Another aim of this study is to map the location of transcripts for a putative ligand for FGFR-2. Hence we localized FGF8 mRNA transcripts in the developing chick face and found that although its expression pattern is exclusively ectodermal, it did not overlap endogenous mesenchymal FGFR-2 expression (fig.1).

Figure 1.

(A) Summary of the mesenchymal expression patterns of FGF receptors in the stage 24 chick embryo. Heavier stippling indicates higher levels of FGFR 1c around the nasal pits and in the mandibular prominence. Lighter stippling indicates lower levels of FGFR 1c. Heavy lines indicate the location of tissues dissected for grafting. FGFR, fibroblast growth factor receptor; FNM, frontonasal mass; LNP, lateral nasal prominence; Md, mandible; Mx, maxilla.

(B) Summary of the ectodermal expression patterns of FGF4 and FGF8 transcripts at stages 24 and 28. FGF2 protein is expressed in all facial ectoderm and is not depicted in this diagram.

A **B**

MATERIALS AND METHODS

Chicken embryos

Fertilized White Leghorn chick eggs were obtained from Coastline Chicks, Abbotsford, B.C. and incubated at 37.5°C with humidity. To stage the embryos, each egg was candled with a fiber optic light and gently rolled to release the embryo from the inner shell membrane. Eggs were windowed to view the embryo by cutting a hole through the eggshell with scissors. Embryos were viewed under a dissecting microscope and staged according to the criteria in Hamburger and Hamilton (1951) based on external characteristics. At stage 22, the limb bud is as long as it is wide and is the optimum stage for serving as a host site for the grafts (fig.2). Donor tissue was obtained from stage 24 embryos.

Grafting

Facial prominences in situ are not accessible for direct manipulation since embryos develop lying on one side. Also, techniques used to strip facial epithelium from mesenchymal tissue in situ (Yang and Niswander, 1995) result in rapid epithelial regeneration by surrounding tissue making it difficult to observe isolated mesenchyme behavior (Richman unpublished; McGonnal unpublished). Hence, we grafted stage 24 facial prominences into host limb buds to maintain an in vivo environment while at the same time allowing for observations at 24 and 48 hour increments (Wedden, 1987; Richman and Tickle, 1989).

concave dissection slide filled with dissecting media containing 1X Hanks buffered salt solution and 10% fetal calf serum (fig.2). The entire maxillary prominence and the central third of the frontonasal mass were dissected with irridectomy scissors. Epithelium was removed from mesenchyme by placing the tissue pieces in 2% crude trypsin in 1X Hanks buffered salt solution at 4°C for 30-60 minutes until epithelium easily peeled away from mesenchyme (fig.2). The tissue was transferred to a dish of cold serum containing media to stop the trypsin action and the epithelium was cleanly separated from each piece of mesenchyme with forceps while working on a frozen slab. Four types of grafts were made: frontonasal mass mesenchyme with epithelium and without epithelium as well as maxillary mesenchyme with and without epithelium. These grafts were positioned into a prepared site on the dorsal surface of a stage 22 chick wing bud (Wedden, 1987; Richman and Tickle, 1989). Host embryos containing grafts were incubated a further 24 and 48 hours, fixed in 4% paraformaldehyde/phosphate buffered saline overnight, and embedded in wax as described in Rowe et al. (1991).

Grafting Method

In situ hybridization

FGFR-2 antisense (gift from E. Pasquale), FGF8 antisense, collagen II antisense and sense RNA probes were used. The FGFR-2 sequence corresponds to nucleotides 60 to 1116, includes the three extracellular immunoglobulin domains and can therefore recognize both the IIIb and IIIc isoforms of the third immunoglobulin domain. A genomic clone for chick FGF 8 was the template for the FGF riboprobe (gift from J-C. Izpisua Belmonte). The collagen II cDNA transcribes as a 1kb RNA transcript and is discussed in Devlin et al. (1988).

Transverse sections were made through the trunks of embryos which included both wing buds. In situ hybridization was carried out as described in Rowe et al. (1991). Riboprobes were labeled with [³⁵S]UTP, partially hydrolyzed, mixed with hybridization buffer: 1X salts (0.5M NaCl, 0.1M Tris pH 7.5, 20mM NaPO₄ pH 6.8, 50 mM EDTA pH 7.5, 5X Denhardts), 50% formamide, 2% dextran sulphate, 50 mM DTT, 500µg/ml yeast total RNA, and placed on pretreated tissue sections. Sections were hybridized overnight at 55°C, treated with 40ug/ml of RNase, and washed up to 0.1 X SSC (0.015M NaCl, 0.015M sodium citrate; pH 7.0) for 20 minutes at 65°C. Slides were dipped in NTB-2 emulsion, exposed 1-2 weeks and counter-stained with malachite green. Photographs were taken with a compound microscope under darkfield illumination. Adjacent sections not subjected to in situ hybridization were stained with toluidine blue and photographed with brightfield illumination.

Facial prominences grafted to the dorsal limb bud surface attach to the underlying mesenchyme within a few hours. A vascular connection is established within 24 hours and the graft can be identified as a swelling on the dorsal surface of the wing bud (fig.2,4,6). By 48 hours, frontonasal mass and maxillary grafts with epithelium are larger than respective grafts without epithelium for example, compare fig.4D with fig.4J and fig.6D with 6J. At 48 hours growth the graft is located near the future elbow region (fig.2). Since it takes time for the graft to revascularize, there is some degree of developmental delay compared to *in vivo* growth. Donor tissue is taken from stage 24 embryos and we estimate that after 24 hours of growth on the host limb bud, the donor mesenchyme has reached stage 26. The 48 hour grafts would be equivalent to stage 28-29 when chondrogenesis in the frontonasal mass is underway. Ectoderm expresses abundant FGFR-2 isoform IIIb transcripts. This ectodermal signal is useful in determining when re-epithelialization occurs over isolated mesenchymal grafts. In general, mesenchymal grafts partly re-epithelialize at 24 hours growth on the limb bud and are fully covered with ectoderm by 48 hours. The FGFR-2 riboprobe recognizes various FGFR isoforms found in both epithelial and mesenchymal tissue, however, only changes in mesenchymal gene expression are scored in these experiments. FGFR-2 and collagen II expression levels were scored using the following system: 0 for background expression; + for low levels; ++ for moderate expression and +++ for high expression.

Temporal and spatial expression of FGFR-2 and collagen II in the chick face in vivo

At stage 24, FGFR-2 transcripts are expressed at moderate levels in the frontonasal mass center and mandibular prominences and at background levels in maxillary prominences (fig.3A). Stage 28 marks the beginning of chondrogenesis in the frontonasal mass and FGFR-2 mRNA is expressed at high levels in the center of this prominence and in developing Meckel's cartilage of the mandibular prominences (fig.3C). FGFR-2 is expressed at low levels in lateral maxillary mesenchyme (fig.3C).

At stage 24, prior to chondrogenesis, collagen II transcripts are expressed at background levels in the frontonasal mass and maxillary prominences and at high levels in the developing Meckel's cartilage of the mandibular prominence (fig.3B). At stage 28, collagen II expression patterns mirrored those of FGFR-2. Here, collagen II is highly expressed in the center of the frontonasal mass (fig.3D) and in mandibular prominences (data not shown) and at moderate levels in the medial mesenchyme of maxillary prominences that will give rise to the palatal shelves (data not shown). Sections hybridized to the sense mRNA probe for collagen II showed no signal above background (data not shown).

Epithelium is required for maintaining FGFR-2 and collagen II expression in frontonasal mass grafts

At 24 hours, half the frontonasal mass grafts with epithelium contain low FGFR-2 signal (table 1;fig.5A) while the other half express moderate to high FGFR-2 transcripts (table 1;fig.4B,5A). FGFR-2 expression levels increased after 48 hours growth where most grafts express high FGFR-2 levels (table 1;fig.4E,5A). This temporal increase in frontonasal mass FGFR-2 expression from 24 to 48 hours is consistent with in vivo expression patterns (fig.3A and C). In most cases transcripts were localized to the center of the graft.

At 24 hours, frontonasal mass grafts without epithelium express lower FGFR-2 levels (table 1;fig.4H,5A) than grafts with epithelium (table 1;fig.4B,5A). At 48 hours, mesenchymal grafts without epithelium express low to moderate FGFR-2 signal (table 1;fig.4K,5A) which is a slight increase in expression from levels found at 24 hours but the level of transcripts is much lower in isolated mesenchyme grafts at 48 hours than grafts with epithelium (table 1;fig.4E,5A).

At 24 hours, half the frontonasal mass grafts without epithelium expressed low collagen II levels (table 1; fig. 5B) while the other half expressed moderate to high levels (table1;fig.4I,5B). At 48 hours, collagen II signal is at background levels in almost all grafts without epithelium (table 1;fig.4L,5B) compared to grafts with epithelium where transcripts are high at 48 hours (table 1;fig.4F,5B). In

overlap areas expressing FGFR-2 (compare fig.4E with F).

FGFR-2 and collagen II transcript levels are strongly correlated in frontonasal mass grafts

At 24 hours growth, expression levels of FGFR-2 correlate with collagen II expression levels in most frontonasal mass with epithelium specimens (table1). For example, both FGFR-2 and collagen II expression are at background levels in specimen 2 and 5, low in specimens 1 and 8, moderate in specimens 4 and 6, and high in specimens 7 and 9 (table 1). At 48 hours growth, expression levels correlate in 6 out of 8 specimens (table 1). This correlation is not evident in frontonasal mass grafts without epithelium at the 48 hour time point (table 1).

Epithelium is required for maintaining FGFR-2 and collagen II expression in maxillary grafts

Maxillary grafts with epithelium express low to background FGFR-2 levels at 24 hours (fig.6B; fig.7A). However, at 48 hours, specimens display low to moderate FGFR-2 expression (fig.6E; fig.7A) confined to one edge of the graft which resembles the in vivo lateral expression pattern found in stage 28 prominences (fig.3C). Tissue orientation was not monitored during the grafting procedure hence, this peripheral expression pattern was not in the same location for each specimen with respect to the host limb bud. Maxillary grafts without epithelium express low to background FGFR-2 levels throughout the mesenchyme (fig.6H and K; fig.7A).

collagen II levels at 24 hours (fig.6C; fig.7B). At 48 hours, specimens display low to moderate collagen II transcripts concentrated at one side of the graft (fig.6F; fig.7B). However, this concentrated region of collagen II expression did not overlap areas expressing FGFR-2 transcripts (compare fig.6E with 6F). Like maxillary grafts with epithelium, grafts without epithelium express low to background collagen II levels after 24 hours growth (fig.6I; fig 7B). In contrast, maxillary grafts without epithelium had decreased levels of collagen II expression 48 hours after grafting (fig.6L) compared to respective grafts with epithelium (fig.6F; fig.7B).

Temporal and spatial expression of FGF8 in the chick face in vivo

In order to correlate the expression of a potential ligand for FGFR-2, we mapped the expression of FGF8. At stage 20, FGF8 transcripts are abundant in epithelium surrounding the nasal pits (fig.8A,B) and extend across the frontonasal mass (data not shown). At stage 24, FGF8 is highly expressed in epithelium surrounding the nasal pits which extends to the lateral thirds of the frontonasal mass but is down regulated in the central third. The caudal and medial surface of the maxillae, the proximal and cranial surface of the mandible and the caudal surface of the second branchial arch express high FGF8 transcript levels (fig.8C,D). At stage 28, high FGF8 expression continues in epithelium surrounding the nasal pits and in the lateral thirds of the frontonasal mass (fig.8E,F). The caudal surface of the maxillae continue to express high levels of FGF8 (fig.8E,F).

Figure 3.

Dark field (A,C) and bright field (B,D) views of stage 24 (A,B) and stage 28 (C,D) heads (frontal sections). Panels A and C were hybridized to antisense FGFR-2 riboprobe. Panels B and D were hybridized to collagen II cRNA probe.

Scale bar = 250 μ m

Key: (e)epithelium, (h)hours, (md) mandible, (mx)maxilla, (fnm) frontonasal mass, (mc) Meckel's cartilage, (np)nasal pit

A) Moderate FGFR-2 expression across the frontonasal mass and caudal mesenchyme of the mandible at stage 24. Epithelium contains abundant FGFR-2 transcripts.

B) High collagen II expression in condensing Meckel's cartilage and cranial mesenchyme of the frontonasal mass at stage 24.

C) High FGFR-2 expression in the frontonasal mass center (arrowheads) and in condensing Meckel's cartilage at stage 28. The lateral edges of maxillary prominences also express some FGFR-2 signal (arrows with tails). Epithelium contains abundant FGFR-2 transcripts.

D) High collagen II expression in the frontonasal mass center (arrowheads) and in developing Meckel's cartilage overlapping FGFR-2 expression (fig.1B) at stage 28. Collagen II is expressed at low levels in the medial portion of maxillary prominences (data not shown).

Figure 4.

Expression of FGFR-2 (B,E,H,K) and collagen II (C,F,I,L) mRNA in adjacent sections of grafts of frontonasal mass prominences. Panels A,D,G,J are sections stained with toluidine blue which clearly show graft position in the host limb bud.

Scale bar =250 μ m

Key: (u)ulna, (g)graft, (h)hours, (l)limb (FNM)frontonasal mass

B) FGFR-2 expression in frontonasal mass grafts with epithelium at 24 hours growth. FGFR-2 expression is abundant in epithelium (arrow with tails) while mesenchymal expression is moderate (arrowheads).

C) Collagen II expression in frontonasal mass grafts with epithelium at 24 hours growth. Transcripts are abundant in the centre of the graft (arrowheads).

E) FGFR-2 expression in frontonasal mass grafts with epithelium at 48 hours growth. FGFR-2 expression is high in epithelium (arrow with tails) and mesenchyme (arrowheads).

H) Frontonasal mass grafts without epithelium at 24 hours. FGFR-2 expression is low in mesenchymal tissue (arrowheads) and epithelium does not cover the entire graft (arrows with tails).

I) Collagen II expression in frontonasal mass grafts without epithelium at 24 hours growth. Collagen II is expressed at high levels at the edges of the graft mesenchyme (arrows with tails).

K) FGFR-2 expression in frontonasal mass grafts without epithelium at 48 hours growth. FGFR-2 is expressed at high levels in the epithelium which has regenerated over graft (arrowhead) and at background in the mesenchyme. This background level expression differs from the high expression levels found in grafts with epithelium at 48 hours (compare with panel E).

L) Collagen II expression in frontonasal mass grafts without epithelium at 48 hours growth. Collagen II expression is at background which differs from the high expression levels found in grafts with epithelium at 48 hours (compare with panel F).

Figure 5.

Gene expression levels in frontonasal mass grafts.

Key: (0) background expression, (+) low expression, (++) moderate expression, (+++) high expression, (Epi) epithelium, (Mes) mesenchyme, (H) hour

A) Summary of FGFR-2 expression levels in frontonasal mass grafts. At 24 hours, grafts without epithelium express lower FGFR-2 levels than grafts with epithelium. At 48 hours, most grafts with epithelium express high FGFR-2 levels while half the grafts without epithelium express low to background FGFR-2 levels.

B) Summary of collagen II expression levels in frontonasal mass grafts. At 48 hours, most grafts with epithelium express high collagen II levels while almost all the grafts without epithelium express background collagen II levels.

EXPRESSION IN FRONTONASAL MASS GRAFTS

Figure 6.

Expression of FGFR-2 (B,E,H,K) and collagen II (C,F,I,L) mRNA in adjacent sections of grafts of maxillary prominences. Panels A,D,G,J are sections stained with toluidine blue which clearly show graft position in the host limb bud.

Scale bar =250µm

Key: (e)epithelium, (u)ulna, (g)graft, (h)hours,(l) limb (MX)maxilla,

B) FGFR-2 expression in maxillary grafts with epithelium at 24 hours growth. FGFR-2 expression is high in epithelium (arrows with tail) and low in mesenchymal tissue -confined to one edge of the graft (arrowheads).

C) Collagen II expression in maxillary grafts with epithelium at 24 hours growth. Collagen II expression is at background levels.

E) FGFR-2 expression in maxillary grafts with epithelium after 48 hours growth. FGFR-2 expression is high in epithelium (arrow with tail) and low in mesenchymal tissue-confined to one edge of the graft (arrowheads).

F) Collagen II expression in maxillary grafts with epithelium at 48 hours growth. Collagen II expression is low and confined to one edge of the graft (arrowheads).

H) FGFR-2 expression in maxillary grafts without epithelium at 24hours growth. FGFR-2 expression is at background and epithelium does not cover the entire graft (arrowheads).

I) Collagen II expression in maxillary grafts without epithelium at 24 hours growth. Collagen II is expressed at background levels

K) FGFR-2 expression in maxillary grafts without epithelium at 48 hours growth. Epithelium has regenerated over the graft and FGFR-2 is expressed at high levels in the epithelium (arrow with tail) and lower levels around the periphery of the graft (arrowheads).

L) Collagen II expression in maxillary grafts without epithelium at 48 hours growth. Collagen II is expressed at background levels.

Figure 7.

Gene expression levels in maxillary grafts.

Key: (0) background expression, (+) low expression, (++) moderate expression, (+++) high expression, (Epi) epithelium, (Mes) mesenchyme, (H) hour

A) Summary of FGFR-2 expression levels in maxillary grafts. At 48 hours, grafts with epithelium display slightly more FGFR-2 expression than grafts without epithelium.

B) Summary of collagen II expression levels in maxillary grafts. At 48 hours, grafts with epithelium display low to moderate collagen II expression while grafts without epithelium are at background.

EXPRESSION IN MAXILLARY GRAFTS

Epithelium and Mesenchyme 24h Mesenchyme 24h

specimen no.	FGFR-2	Collagen II	specimen no.	FGFR-2	Collagen II
1	+	+	1	+	++
2	0	0	2	0	+
3	+	+++	3	+	0
4	++	++	4	++	+++
5	0	0	5	+	++
6	++	++	6	+	++
7	+++	+++	7	+	0
8	+	+			
9	+++	+++			

Epithelium and Mesenchyme 48h Mesenchyme 48h

specimen no.	FGFR-2	Collagen II	specimen no.	FGFR-2	Collagen II
1	++	++	1	0	0
2	+	+++	2	+	0
3	+++	+++	3	++	0
4	+++	+++	4	++	+++
5	+++	+++	5	++	0
6	+++	+++	6	+	0
7	+++	+++			
8	+++	++			

Table 1:

Expression scores of FGFR-2 and Collagen II in individual frontonasal mass specimens.

KEY: (0) background expression, (+) low expression, (++) moderate expression, (+++) high expression, (h) hours, (no.) number

Figure 8.

Dark field views of stage 20 (A,B), 24 (C,D) and stage 28 (E,F) heads hybridized to antisense FGF8 riboprobe. A,C,E are frontal sections and B, C, E are parasagittal sections.

Scale bar = 250 μ m

Key: (20)stage 20, (24) stage 24, (28) stage 28, (MD) mandible, (MX) maxilla, (fnm) frontonasal mass, (NP) nasal pit, (T) telencephalon, (2nd) second branchial arch, (E) eye

A) Stage 20 frontal section. High FGF8 expression in epithelium adjacent to the nasal pit, the lateral edges of the frontonasal mass, and at the second branchial arch.

B) Stage 20 parasagittal section. High FGF8 expression is found at the lateral edge of the frontonasal mass and in between the maxillary and mandibular prominence. Strong signal is also present at the isthmus of the mesencephalon and the rhombencephalon.

C) Stage 24 frontal section. Abundant transcripts are concentrated in nasal pit ectoderm and in epithelium encasing the maxillae.

D) Stage 24 parasagittal section. Expression pattern is similar to C.

E) Stage 28 frontal section. FGF8 is highly expressed in ectoderm covering the globular processes and the developing maxillae. FGF8 transcripts are high in lateral frontonasal mass epithelium but are not expressed in the centre of the prominence (arrowheads).

F) Stage 28 parasagittal section. Expression is restricted to the medial edge of the maxillae and in the cranial surface of the mandible.

DISCUSSION

Frontonasal mass and maxillary prominences behaved autonomously when grafted to the host limb bud

Previous morphological studies indicate that frontonasal mass grafts with epithelium grown for 7 days on a host embryo formed cartilage rods that were 80% of the average prenasal cartilage rod length in vivo (Richman and Tickle, 1989). These data show that the process of outgrowth and differentiation on the host limb bud is remarkably similar to in vivo growth. Our results indicate that after grafting facial prominences with epithelium, FGFR-2 and collagen II transcript levels are similar to levels found in the respective prominences in vivo. For example at 48 hours, maxillary prominences with epithelium express low FGFR-2 and collagen II levels as in vivo and control for possible effects that host limb tissue may be contributing to graft gene expression.

In addition, FGFR-2 and collagen II position-specific expression patterns in frontonasal mass and maxillary grafts were preserved in the ectopic location. The FGFR-2 and collagen II expression in the centre of frontonasal mass grafts at 24 hours growth occurs in pre-cartilage cell aggregates. Previous grafting studies have not detected cartilage formation until 48 hours after grafting in alcian green-stained wholemounts (Richman and Tickle, 1989). Position-specific pattern of expression is also preserved in maxillary grafts; here both FGFR-2 and collagen II transcripts maintained peripheral expression patterns concentrated to one edge of the graft. These

data are similar to in vivo expression patterns where FGFR-2 and collagen II transcripts are found at the lateral and medial edges respectively.

Why is collagen II expressed in the ventro-medial regions of maxillary prominences? There are no known cartilaginous tissues derived from the ventro-medial part of maxillary prominences (D. Noden, personal communication). Moreover, no cartilage forms when maxillary mesenchyme is grown in micromass culture (Wedden et al, 1987; Langille et al, 1989; Richman and Crosby, 1990). The expression of collagen II in the maxillae may be acting as a signalling molecule which controls when and where cyto-differentiative events take place (Thorogood et al., 1986) or the expression is occurring in non-chondrogenic tissues as it does in the basement membrane of epithelia, dorsal and lateral surface ectoderm, lateral and ventral gut endoderm and other tissues (Kosher and Solursh, 1989; Wood et al., 1991; Cheah et al., 1991) as a transient expression not indicative of cartilage formation (Cheah et al., 1991). FGFR-2 expression in dorso-lateral regions of the maxilla is also not correlated with any chondrogenic activity but may contribute to patterning bones derived from the maxillary prominence.

**Epithelium is required to maintain mesenchymal FGFR-2 levels in
the developing frontonasal mass**

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Following 24 hours of growth on a host limb bud, grafts of frontonasal mass mesenchyme have not been re-epithelialized and the mesenchyme exhibits reduced levels of FGFR-2 expression when compared to respective grafts with epithelium. This down-regulation of FGFR-2 expression suggests that frontonasal mass mesenchyme requires signals provided by facial epithelium in order to maintain expression. Rapid down-regulation of gene expression following ectodermal removal has been demonstrated in the limb bud. Within hours of removing the FGF-rich apical ectodermal ridge down-regulation of AP-2, Cck-8 and Msx-1 is observed (Shen et al., 1997; Patel et al., 1996; Ros et al., 1992). While we have not done a detailed time course for FGFR-2 down-regulation in grafts of frontonasal mass mesenchyme, it is likely that decrease in signal would be seen at earlier time points.

After 48 hours of growth, frontonasal mass mesenchyme is covered with limb epithelium and gene expression has increased to moderate levels in 50% of the grafts. In contrast, intact frontonasal mass grafts with epithelium had high levels of FGFR-2 expression, similar to the high levels found in vivo. There are two possible explanations for the modest up-regulation observed in frontonasal mesenchyme grafts at 48 hours. The first possibility is that the limb epithelium inhibited increased expression of FGFR-2. The second possibility is that the initial lack of facial epithelium over the grafts resulted in loss of signals necessary for the temporal up-regulation of FGFR-2 transcription. Our data do not distinguish between these two

possibilities. Both immediate and delayed contact of frontonasal mass mesenchyme with limb ectoderm have been shown to inhibit outgrowth of the mesenchyme (Richman and Tickle, 1989; Richman and Tickle 1992). It is clear from these data that dorsal limb ectoderm cannot completely replace facial ectoderm and one of the reasons why outgrowth is inhibited might be due to an inhibitory effect of the limb ectoderm on FGFR-2 expression in the mesenchyme. The alternative possibility, that re-growth of facial epithelium would allow normal gene expression, is not supported by recent data from Imai et al. (1997). Grafts of first arch mesenchyme into other regions of the face do not form cartilage unless they are simultaneously grafted with facial ectoderm. Thus the delay in re-epithelialization in grafts of facial mesenchyme placed within the face may be enough to prevent the cascade of molecular events that normally precede chondrogenesis (including FGFR-2 expression).

FGFR-2 and collagen II expression are differentially affected by the removal of epithelium

Both FGFR-2 transcript levels and expression domains in frontonasal mass grafts with epithelium closely overlap collagen II expression levels and domains suggesting that there may be a relationship between FGFR-2 expression and chondrogenesis in this prominence. However, in frontonasal mass grafts without epithelium there is a slight increase of FGFR-2 expression while collagen II transcripts are down-regulated to background levels. These results indicate that the

expression patterns of FGFR-2 and collagen II are no longer as strongly

correlated in isolated mesenchyme as with the presence of facial epithelia. The differential regulatory effects on FGFR-2 and collagen II that are caused by removing epithelium may reflect the hierarchy of signaling involved in cyto-differentiation.

FGFR-2 activity may be further upstream from the collagen II signaling which occurs just prior to cartilage formation. FGFR-2 is specifically expressed across the mid-line of the frontonasal mass in stage 24 embryos and is one of the earliest markers for the future chondrogenic region. In the limb bud, FGFR-2 is also the earliest of the three FGFRs examined to be localized to cartilage condensations (Szebenyi et al., 1995) and precedes the expression of type II collagen (Devlin et al., 1988). We know that cartilage is formed in nearly all grafts of isolated frontonasal mass mesenchyme if they continue developing in the host limb bud (Richman and Tickle, 1989; 1992) which means type II collagen mRNA will be ultimately be up-regulated. This delay in collagen II expression may be linked to the formation of truncated cartilage rods.

Endogenous FGFs may be acting on facial mesenchyme

FGF2, 4 and 8 are ectodermal signals which may activate mesenchymally expressed FGFR-2 in the developing face. FGF2 is homogeneously expressed throughout facial prominences (Richman et al., 1997) and has mitogenic activity when bound to the mesenchymally expressed isoform of FGFR-2 (Ornitz et al., 1996). FGFR-2 is present in the frontonasal mass and mandibular prominences and ectopic

mesenchyme (Richman et al., 1997) which makes FGF2 a good candidate for activating FGFR-2 *in vivo*. Dorsal wing ectoderm also contains abundant levels of FGF2 protein (Savage et al., 1993) yet the presence of dorsal wing ectoderm cannot support outgrowth of facial mesenchyme (Richman and Tickle, 1989). Hence, other factors in addition to FGF2 are required for outgrowth of facial mesenchyme.

FGF4 can also promote outgrowth of frontonasal mass and mandibular mesenchyme (Richman et al., 1997) and has high mitogenic activity when bound to the mesenchymal isoform of FGFR-2 (Ornitz et al., 1996), however, the *in vivo* distribution of FGF4 transcripts in the developing chick face has only been detected at the medial-rostral surface of mandibular ectoderm (Barlow and Francis-West, 1997; Francis-West personal communication) where it may have paracrine regulatory effects on neighbouring mesenchymal FGFR-2 expression. More work is needed to determine whether FGF4 is expressed in the frontonasal mass.

In the stage 24 face, the expression pattern of FGF-8 is concentrated around the nasal pits (Helms et al., 1997; Richman et al., 1997) adjacent but not overlapping FGFR-2 expression in the frontonasal mass. Moreover FGF-8 has no mitogenic effects when bound to FGFR-2 (IIIc isoform, Ornitz et al., 1996). Other putative FGFR-2 activators include FGF-6 (de Lapeyriere et al., 1993; Coulier et al., 1994; Han and Martin, 1993), FGF-9 (Hecht et al., 1995; Tagashira et al., 1995), FGF-10 (Ohuchi et al., 1997) and FHF1-4 (Smallwood et al., 1996) all of which have not been described in the face.

eliminated a ligand required to activate FGFR-2 signalling and dismantled the positive feedback required for FGFR-2 up-regulation. A feedback loop was demonstrated in developing chick skin (Song et al., 1996). The application of exogenous FGF2 lead to up-regulation of FGFR-1 expression. Experiments are underway to test for the presence of a similar feedback loop involving FGFR-2 in facial mesenchyme.

Relevance to Crouzon's syndrome

Mutations in fibroblast growth factor receptors (FGFR) have been identified in humans with craniofacial disorders including Crouzon, Jackson Weiss, Pfeiffer and Aperts syndromes (Reardon et al., 1994; Jabs et al., 1994; Rutland et al., 1995; Wilkie et al., 1995; Oldridge et al., 1995; Galvin et al., 1996). The phenotype of Crouzon's syndrome includes shallow orbits, parrot nose, mandibular prognathism and maxillary hypoplasia (Cohen, 1986). In the developing chick, abundant FGFR-2 transcripts are found in regions of the head affected in Crouzon's syndrome (Wilke et al., 1997). We found that removing epithelia from facial mesenchyme results in diminished FGFR-2 expression which may cause the truncated outgrowth of cartilage rods that occurs later on in development (Richman and Tickle, 1989). Hence, our results in the chicken embryo may parallel the defects found in Crouzon's syndrome.

In addition to the FGFs, several other proteins have recently been shown to play very important roles in patterning various parts of the embryo. Proteins such as sonic hedgehog (Shh) and bone morphogenetic proteins (Bmp) are present at sites where epithelial-mesenchymal interactions occur and may mediate in vivo FGF functions.

A vertebrate homolog of the *Drosophila* Hedgehog gene (Ingham, 1995), Shh, is expressed in many epithelial tissues, frequently at sites where inductive interactions between epithelial and mesenchymal cells occur (Roelink, 1996). Throughout embryogenesis, Shh is found in organizing centers like Hensen's node, the notochord, the floor plate of the neural tube, and the posterior part of the limb bud. In developing limb buds, Shh mediates polarizing activity (Riddle et al, 1993), and its expression can be induced and maintained by FGFs (Crossley et al, 1996). In addition, Shh can induce FGF and Bmp-2 expression in the limb (Laufer et al, 1994).

Since Shh is involved in patterning, interacts with FGFs and Bmps -- molecules with distinctly localized transcripts in the developing face, it is necessary to investigate possible Shh functions in facial morphogenesis. Throughout craniofacial development, Shh is exclusively expressed in epithelium. During earlier stages (st.16), Shh mRNA is expressed in the presumptive oral cavity and in the posterior margin of the 2nd branchial arch which goes on to form facial muscles bones and nerves (Wall and Hogan, 1995). At stage 25, Shh is expressed in

epithelium at the inferior borders between the frontonasal mass, lateral nasal prominences, and maxillary prominences where it may mediate fusion of these tissues (Helms et al., 1997). At stage 29, Shh expression is highly restricted to the caudal edge of frontonasal mass ectoderm, maxillae and in the midline of the stomodeum (Helms et al., 1997). In sum, early Shh expression in the presumptive oral cavity may participate in putative signalling pathways delegating overall facial symmetry while Shh expression which occurs in already formed facial prominences may be involved in outgrowth and patterning. Interestingly, Shh mouse knockouts display a cyclops phenotype (Chiang et al., 1996) which already indicates that this protein is involved in establishing the mid-line of the face. Since patched, the proposed receptor for Shh (Stone et al., 1996; Marigo et al., 1996), is almost exclusively expressed in epithelium, it is unlikely that the ligand-receptor model postulated for FGF signalling where epithelium provides the ligand required for the activation of a mesenchymally expressed receptor applies to Shh mechanism of action. How epithelial expression of Shh interacts with underlying mesenchymal tissue is an intriguing question which has yet to be addressed.

Bmps are also expressed at inductive sites of epithelial-mesenchymal interactions. Bmp-2 and Bmp-4 are members of the TGF β super family which are growth factors involved in various aspects of development (Hogan, 1996). In the developing chick face, Bmp-2 and Bmp-4 mRNA expression is complex, shifting from epithelium to mesenchyme in regions involved in outgrowth (Francis-West et al., 1994). Recently, ectopic applications of Bmp-2 and 4 have been shown to

participate in a signaling cascade involving FGF4 that controls outgrowth and patterning of the facial primordia (Barlow and Francis-West, 1997) and it has been postulated that Bmp-2 and FGF4 may interact to regulate development of the mandible (Barlow and Francis-West, 1997) as they do during limb development (Laufer et al., 1994).

Signaling cascades found at various sites of epithelial-mesenchymal interactions may also be present in face development. In *Drosophila* imaginal discs, the Bmp related factor 'decapentaplegic' (Dpp) is an important downstream target of hedgehog (HH), a homologue of Shh, action. Here, Dpp appears to be involved in several organizing effects of HH (Roelink, 1996). In the chick, Shh induces ectopic mesenchymal Bmp expression in the developing hindgut (Roberts et al., 1995), and in the developing wing bud (Laufer et al., 1994). Since Shh can regulate Bmp expression at various sites of epithelial-mesenchymal interactions, perhaps Bmp is a downstream target of Shh function in the developing face. Moreover, since FGFs can induce Shh expression in developing limbs, Shh may be a downstream target for FGF functions in the face. Therefore, the relationship that exists between these proteins in other regions of the developing embryo may also be present in the developing face.

SUMMARY

In sum, my thesis addresses the role of overlying epithelium in the regulation of mesenchymal FGFR-2 expression during face development. My results suggest that there are signals in overlying epithelium required for the maintenance of FGFR-2 expression in the developing chick face. We believe that the epithelium may provide a ligand which activates FGFR-2, triggering a positive feedback mechanism that is dismantled when mesenchyme is stripped of epithelium and consequently, FGFR-2 receptor expression is not up-regulated to levels found in mesenchyme with endogenous epithelium. We postulate a putative mechanism for how epithelial and mesenchymal interactions occur; epithelium may provide the ligand necessary for activating receptors present in underlying mesenchyme. We localized FGF8 mRNA in the developing face to determine whether this protein co-localizes with FGFR-2 and found that it does not. Hence, more work is needed to determine the endogenous ligand for this receptor which may be expressed in ectoderm as well as to elucidate other proteins which may function upstream or downstream from FGF signalling to gain a better understanding of how FGFs contribute to face development.

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