THE RELATIONSHIP OF BASIC FIBROBLAST GROWTH FACTOR (bFGF) TO EMBRYONIC FACIAL GROWTH

by

JOANNE M. WALIN

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

MASTER OF SCIENCE

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ABSTRACT

Characteristic face shape is the result of differential growth of mesenchyme cells within the facial primordia. My hypothesis is that facial mesenchyme from different primordia will respond similarly to exogenous growth factors applied in a culture system provided the cells are derived from the same level of neural crest. The upper beak primordia (the lateral nasal process, frontonasal mass, and upper maxilla) derive from a different level of neural crest than the lower maxilla and mandible.

Micromass cultures of stage 24 embryonic chick facial mesenchyme were grown in defined media, fetal calf serum-containing media, and defined media with basic fibroblast growth factor (bFGF), and cell numbers were counted at 48 and 96 hours. Other cultures were stained with Alcian blue, which stains cartilage matrix. Medium containing bFGF (100 ng/ml) preferentially stimulated upper beak mesenchyme. The cell number data and the chondrogenesis patterns were similar in lateral nasal process and frontonasal mass cultures. Upper maxilla cultures also had cell numbers similar to lateral nasal process. Lower maxilla had cell numbers equivalent to mandibular cultures. Behaviour of facial cells in fetal calf serum-containing medium and defined medium was not as clearly linked to neural crest origins of the mesenchyme.

The role of endogenous bFGF *in vivo* was investigated. bFGF protein was mapped using immunocytochemistry. The antigenicity of bFGF was best preserved in non-fixed, frozen tissues. bFGF was evenly distributed at all stages (stage 15 to 28) and in all facial primordia. Epithelium appeared to express more bFGF than the mesenchyme. This could mean bFGF is involved in epithelial-mesenchymal interactions in the face.

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CHAPTER III

This thesis is dedicated to

Christopher William Hall

We have such wonderful times together,
especially in the woods and mountains
that we love.

Thanks for your encouragement and perseverance
during the course of this program, the writing
of this thesis, and the hours of labour that you
have spent manipulating the unruly computer.
Heli-skiing passes loom large in your future!

Many thanks to my husband,
my best friend.

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CHAPTER I

GENERAL INTRODUCTION

As a student of orthodontics, facial growth is an area of developmental biology which I find particularly interesting. An understanding of normal development, at its most basic biologic level, could lead to the possibility of manipulating abnormal growth patterns to follow more characteristic patterns of development. The study of development can elicit important clues to the complex processes involved in differential growth which determines face shape.

The intricacies of biochemical interactions between molecules, cells and tissues during development, are far from resolved. Therefore, a number of animal models have been used for the study of development processes. Studies of the face have concentrated on avian and mammalian systems. This has been primarily due to the accessability of chicks, mice and rats for research purposes, but the apparent similarities between these animals and humans during early embryonic development makes them good choices as research models.

The extensive body of knowledge regarding chick development makes the chick a suitable model for further investigation. The chick face looks remarkably like a human embryo in the earliest stages of development, although the final outcome of development produces a strikingly different facial pattern. A drawback of using the chick is that it has features not found in the human face. The secondary palate remains open in the bird, rather than the palatal shelves elevating and fusing to form the roof of the mouth. Also,

the bird has no teeth so the signals required for tooth germs found in the upper and lower jaws of humans are absent in the developing chick.

I. Stages of development in the chick embryo

The chick embryo has been used for embryological studies since the time of Aristotle, when chronological age was the basis for classification. The time of incubation can be used only as a rough guide in structural development, as there are wide variations in development due to biological differences and incubation techniques. The classification scheme described by Hamburger and Hamilton (1951), based on external morphological characteristics rather than chronologic age, was used to assess the stage of development of each embryo. Different characteristics become prominent during each phase of development. From stage 17 onward, the development of the limbs provide the most convenient criteria for determining the stage. The staging criteria are illustrated in Figure 1.1.

II. Normal development of the chick face

The embryonic face is composed of buds of mesenchyme covered by epithelium which surrounds the primitive oral cavity, or stomodeum. These swellings of tissue are called primordia. The facial primordia consist of the frontonasal mass, the paired lateral nasal processes, the paired maxillae, and the paired mandibular primordia (Fig. 1.2).

The face of the chick embryo is first recognized at stage 14 (Hamburger and Hamilton,1951) with the development of the first branchial arch which will form the lower beak, or mandible and part of the upper maxilla. At stage 15 the nasal placodes develop as epithelial thickenings bilaterally at the edges of the frontonasal mass. The

maxillae are first apparent at stage 18 as buds of tissue bordering the sides of the presumptive mouth (Yee and Abbot, 1978). Between stages 20 and 28 the primitive oral cavity (stomodeum) is surrounded by developed, but separate facial primordia. Mesenchymal proliferation around the nasal placodes produces the lateral nasal processes leaving the nasal placodes in depressions which later form the nasal pits (Yee and Abbot, 1978).

These nearly equally sized facial primordia, grow differentially to produce the characteristic facial form of the bird. Cell differentiation and cartilage formation begins as early as stage 24. It is the frontonasal mass which forms most of the upper beak, including the prenasal cartilage, nasal septum and primary palate. The lateral nasal processes form the conchae of the nose and tissues of the external nares (Romanoff, 1960). The maxillae form the corners of the beak, tissues under the eye, and the palatal shelves. The mandibular primordia fuse and form the entire lower beak and contain paired rods of Meckel's cartilage. At stage 26, the proximal dorsal part of the lateral nasal processes starts to fuse with the maxilla. By stage 30 the maxilla and lateral nasal process are completely united.

III. Origin of facial tissue

The facial mesenchyme develops from neural-epithelial cells, known as the neural crest. The neural crest cells arise from the edges of the neural folds as the neural tube closes, and migrate extensively within the developing embryo (Noden, 1984; Le Douarin, 1982). While still attached to the neural tube, the neural crest cells are typical epithelial cells but as they migrate, the cells transform from epithelial to mesenchymal cells and

begin to secrete a pericellular matrix (Hall, 1991). Molecules, such as fibronectin, laminin, heparan sulfate proteoglycan and tenascin, act as signals to guide cell migration to their target organs (Hall, 1991).

After migrating, cranial crest cells differentiate into a wide range of cell types such as cartilage, bone, glial cells, sensory and autonomic ganglia, and pigment cells (Le Lièvre, 1978; Le Douarin, 1982; Noden, 1988). Neural crest cells are formed along the entire length of the neural tube from head to tail but only the cranial crest cells make skeletal tissue.

The cranial region of the neural tube develops swellings that later on give rise to the brain and hindbrain. The most cranial of these compartments is the prosencephalon. The mesencephalon forms just caudal to the prosencephalon. By the 9 somite stage, the rhombencephalon has formed caudal to the mesencephalon consisting of two parts, the metencephalon and the myencephalon and the prosencephalon has subdivided into the telencephalon and diencephalon.

In order to determine which level of crest gives rise to facial mesenchyme, crest cells were labelled and their migration mapped. The migration of neural crest cells was demonstrated initially by ^{3}H-thymidine-labelling studies in the chick embryo (Johnston, 1966; Noden, 1975). The neural crest cells first reach the chick facial primordia at stage 14 (Johnston, 1966; Noden,1975; Lumsden *et al.*, 1991). However, progressive isotope dilution as tissues developed prevented a detailed analysis of fully differentiated structures. Subsequently, mapping of migration was done with permanent nuclear markers using quail-chick chimeras (Le Douarin, 1973; Le Lièvre, 1978; Noden, 1978a).

Quail nuclei can be distinguished from chick based on their chromatin pattern and the marker is not diluted with development. Lumsden *et al.* (1991) further refined the chick neural crest fate map by labelling small regions of the premigratory cranial crest *in situ*. The fluorescent vital dye DiI was injected into premigratory crest cells and labelled cells could be followed up to stage 15. These findings are of limited use in mapping lineage of the face, as the most cranial injections were in the midbrain area and did not include prosencephalic neural crest cells.

Facial primordia have contributions from neural crest cells originating from different locations along the neural tube. The frontonasal mass and the lateral nasal process mesenchyme cells are derived from neural crest located on either side of the prosencephalic-mesencephalic junction. The two primordia therefore have similar neural crest lineages (Le Lièvre, 1978; Le Douarin, 1982). The maxillary primordia is also composed of cells from both prosencephalic and mesencephalic neural crest although there appears to be regional separation of the two cell populations. The upper maxilla is formed primarily by prosencephalic neural crest cells while cells from the mesencephalic crest are thought to migrate into the lower maxilla (Le Lièvre, 1978). The mandibular mesenchyme is derived primarily from mesencephalic crest, although there may also be a small contribution from metencephalic neural crest (Noden, 1978a).

Although most facial mesenchyme is derived from neural crest, cells derived from mesoderm are also found within the face. Muscle cells are derived from the paraxial mesoderm (Noden, 1984; Ralphs *et al.*, 1989). The paraxial mesoderm, alongside the notochord, divides into a series of segmental blocks, or somites, which are prominent

during embryonic development. Each somite differentiates into 3 parts: the dermatome, the sclerotome, and the myotome. The myotome differentiates into the muscles of the trunk and limbs (Sperber, 1976), and all of the voluntary muscles of the head (Noden, 1984).

IV. Molecular markers identify subpopulations in the embryo

In recent years, previously undetected boundaries within the embryo have been discovered. It is now accepted that from its earliest stages of development, the embryo is comprised of subpopulations of cells. Molecular markers can be used to distinguish these subpopulations within the developing embryo even though the cells all look identical in tissue sections (Noden, 1992).

An example of a molecule which has uneven distribution within the face is retinoic acid receptor-beta (RAR-β). Retinoic acid, a derivative of vitamin A, is required for normal development of the embryo, but an excess of retinoic acid acts as a teratogen, causing facial defects (Richman, 1992). In chicks treated with retinoic acid, no upper beaks formed, while the lower beak was unaffected (Tamarin *et al.*, 1984). The expression of nuclear receptors for retinoic acid has been shown to be unevenly distributed within the facial primordia of chicks (Rowe *et al.*, 1991, 1992). RAR-β transcripts had higher levels of expression in the lateral nasal process, specific areas within the frontonasal mass, and the anterior part of the maxilla. Stage 18 to 24 embryos displayed a sharp boundary of expression between the upper and lower maxilla. This curious expression of RAR-β could be related to the neural crest origins of the maxillary mesenchyme. The RAR-β rich anterior cells seem to be derived from prosencephalic

crest whereas the posterior cells are principally derived from mesencephalic crest (Le Lièvre, 1978; Rowe *et al., 1992).* The expression of this molecule could be related to subpopulations of cells within the face, and these subpopulations could be related to neural crest lineage.

V. Growth factors

Growth factors are a group of molecules that may be useful in identifying subpopulations within the developing embryo. Growth factors are proteins isolated from tissues or tumours that promote growth. Initially, growth factors could not be accurately identified, but nucleotide sequencing technology has made it possible to compare the polypeptide sequences of different growth factors and to classify those with homologous domains into super families (Mercola and Stiles, 1988). The main families of growth factors are transforming growth factors, insulin-like growth factors, epidermal growth factors, platelet-derived growth factors, and heparin-binding growth factors (HBGF). Members of all growth factor families can induce proliferation in some cell types and differentiation in others.

The HBGF family contains two closely related growth factors, acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF), and at least five other structurally related genes products identified by recombinant DNA techniques which have 30-55% amino acid sequence homology (Thomas, 1987; Benharroch and Birnbaum, 1990). Most of the variation in amino acid sequence occurs at the amino end of the protein. In this work I have focused on one member of the HBGF family, basic fibroblast growth factor (bFGF). The related genes in the HBGF family are known as int-2, hst-1,

FGF-5, FGF-6, and KGF. Historically, aFGF and bFGF were characterized by their ability to bind to heparin, and to stimulate fibroblast proliferation. Other effects on differentiation have also been reported (Baird *et al*, 1986; Gospodarowicz *et al*, 1986,1987; Rifkin and Moscatelli, 1989). Recently, a change in terminology was proposed (Baird and Klagsbrun, 1991). Fibroblast growth factors are now classified according to their structural similarity and are named in a series such that aFGF and bFGF will be called FGF-1 and FGF-2 respectively (Baird and Klagsbrun, 1991). However, I will continue to use bFGF in this thesis.

A. Role of FGF in development

i) Effects of exogenous bFGF on development

Fibroblast growth factors have been shown to stimulate proliferation and/or differentiation in mesoderm-derived cells in culture, such as muscle cells (Seed and Hauschka, 1988), and limb bud cells (Aono and Ide, 1988). In addition, bFGF stimulates neuroectoderm-derived cells such as facial mesenchyme (Richman and Crosby, 1990).

Gospodarowicz and Moran (1976) demonstrated that a growth factor, such as bFGF, could act as both a mitogen and a differentiation factor, properties thought to be mutually exclusive. bFGF stimulated proliferation of endothelial cells and the concomitant synthesis of extracellular matrix components, including collagen, fibronectin, and laminin.

Mesoderm induction occurs during development of vertebrates (Smith, 1989; Green and Smith, 1991; de Pablo and Roth, 1990; Cooke and Wong, 1991). The role of bFGF in the induction of mesoderm in amphibian embryos has been investigated in most

detail (Slack *et al.*, 1987). In *Xenopus* blastulae there are only two types of cells, prospective ectoderm in the animal pole, and prospective endoderm in the vegetal pole. One of the key events in the development of amphibian embryos is the differentiation of mesodermal cells from animal pole cells, induced by signals from the vegetal pole. If the vegetal pole is removed, the animal pole forms only epidermal structures. Slack *et al.* (1987) demonstrated that mesoderm was induced when the vegetable pole was removed an the animal pole was exposed to exogenous bFGF. When heparin and bFGF were added simultaneously, mesoderm induction was blocked. A variety of mesodermal cell types differentiate after induction of mesoderm, the most abundant being muscle cells. Cartilage was also formed in these experiments.

ii) The role of endogenous bFGF in development

Descriptive evidence that fibroblast growth factors are involved with embryonic development comes from two major types of studies. The first type involves biochemical extraction of the protein or RNA from tissues or cells. The second type of study localizes bFGF protein or RNA in embryos, tissues, or cells.

bFGF protein has been extracted from embryos of all stages of development from pregastrula stages (Kimelman and Kirshner, 1987; Slack and Issacs, 1989; Mitrani *et al.*, 1990) to stages when the definitive body pattern has been established (Parlow *et al.*, 1991; Seed *et al.*, 1988). bFGF has also been extracted from the yolk and white of unfertilized chick eggs (Seed *et al.*, 1988). The face was rarely included in these studies, with one exception. bFGF RNA was found in the mouse face after fusion of the facial primordia (Hébert *et al.*, 1990).

bFGF protein has been localized *in vivo* with immunocytochemical techniques in the myocardium of early chick embryos (Parlow *et al.*, 1991; Kardami and Fandrich, 1989), and in the striated muscle of the tongue (Joseph-Silverstein *et al.*, 1989). In older rat fetuses, bFGF is integrated within the basement membrane (Gonzales *et al.*, 1990).

Another piece of evidence supporting a role for endogenous bFGF is that many receptors for this growth factor have been discovered (Lee *et al.*, 1989; Jaye *et al.*, *1992)* Receptors for bFGF have been identified on a variety of cultured cells, such as early chick mesenchyme (Parlow *et al.*, 1991), and with *in situ* analysis in the facial primordia of rat and chick embryos (Wanaka *et al.*, 1991; Heuer *et al.*, 1990).

B. Mechanisms of action of bFGF

Consideration of the biological activities of exogenous bFGF must be tempered by the fact that the mechanism for secretion of the native protein is not understood. All translational products of bFGF mRNA lack a signal sequence which would direct their release by secretion (Logan, 1990). There has been no defined mechanism for release, except cell death and rupture of the cell membrane. Despite not being secreted from cells, FGF is present outside of cells in the embryo and is localized in the basement membrane (Gonzalez *et al.*, 1990). bFGF seems to be incorporated into the extracellular matrix.

There are two ways in which extracellular bFGF can interact with cells (Klagsbrun and Baird, 1991). The mechanisms for the FGF activity appears to be related to its ability to bind to extracellular matrix components such as heparan sulfate proteoglycans (HSPG). HSPG's are related chemically to heparin and are found within

cells, at the cell surface, and within the extracellular matrix of cartilage basement membranes (Moscatelli *et al.*, 1991). When bound to HSPG, bFGF is protected from heat denaturing and proteolytic degradation (Gospdarowicz, 1990). FGF may be stored in a complex with heparan sulfate on the cell surface and in the extracellular matrix and then slowly released to potentiate the interaction with high-affinity cell surface receptors (Moscatelli *et al.*, 1991; Vlodavsky *et al.*, 1991). One proposed mechanism for release of FGF from extracellular matrix involves heparinase (de Iongh and McAvoy, 1992) or other agents which disrupt the heparin bond. Thus, the reserve of extracellular bFGF can be mobilized when required by tissue proteases.

A functional role for the cell surface receptor has been confirmed. Amaya *et al.* (1991) used a dominant negative mutant of the FGF receptor to block receptor function in frog embryos. Explants from embryos, which expressed this dominant negative mutant, failed to induce mesoderm in response to endogenous FGF. Embryos had normal head development with extreme trunk deficiencies. A role for FGF in head development was not excluded based on these findings. Thus, high affinity cell surface receptors are required for normal development of embryos.

Intracellular bFGF protein is found both in the cytosol and in the nucleus (Brigstock *et al.*, 1991) and may interact directly with DNA during cell division (Baldin *et al.*, 1990). In a cell-free system, bFGF has been shown to directly affect gene transcription (Nakanishi *et al.*, 1992). Shiurba *et al.* (1991) showed an *in vivo* change in distribution of bFGF protein from intracellular to nuclear, at the time when transcription and mesoderm induction begins. Therefore, a major mechanism of action

of bFGF may be to act directly on gene transcription and affect the synthesis of other proteins (Logan, 1990).

C. Role of bFGF in the face

bFGF preferentially stimulates an increase in cell number and proliferation in the frontonasal mass mesenchyme in culture (Richman and Crosby, 1990). These results suggest that bFGF may be involved in differential growth of the facial primordia in the embryo. This may be due to unequal distribution of FGF, variable receptor numbers in each primordia, or differences in the efficiency of signal transduction in each population of facial cells. The *in vivo* localization of bFGF and its receptors in the face would determine which of the aforementioned scenarios is true.

VI. AIMS

Subpopulations of cells in the developing face could contribute to the differential growth pattern that produces characteristic facial form. The objective of this study are two fold: to search for similarities or differences in facial mesenchyme cells, based on their response to growth factors in micromass culture; and to determine if basic fibroblast growth factor is present in the chick face at various stages of development and whether it is localized to particular regions of the face.

The first objective will be met by culturing the lateral nasal process, upper and lower maxilla, frontonasal mass, and mandible in three different culture media, defined medium, fetal calf serum-containing medium, and bFGF-containing medium. The increase in cell number and the pattern of chondrogenesis will be assayed. The prediction is that the lateral nasal process will behave like frontonasal mass cultures because it is

derived from the same neural crest cell lineage, and that upper and lower maxilla will behave differently from each other reflecting differences in neural crest origin.

The second objective will be addressed by using immunocytochemical techniques to determine the spatial and temporal location of endogenous bFGF protein in the face at stages prior to and during fusion of facial primordia.

Figure 1.1 **Diagrams of chick embryos at different stages of development.**

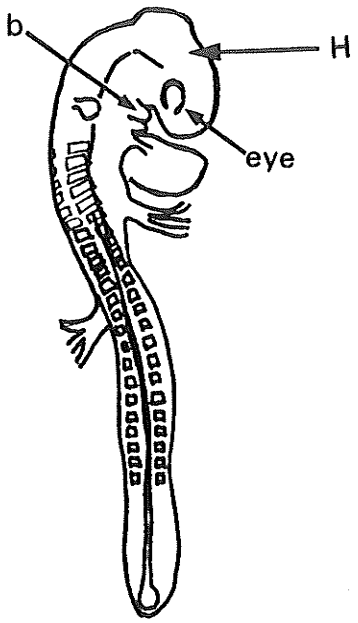
Scale bar represents 1 mm for all figures. Key: b = first branchial arch, h = head, l = leg bud, md = mandible, mx = maxilla, w = wing bud.

A. Stage 15 chick embryo (2.5 days incubation). The embryo is straight, the head well developed, and the prospective limb areas flat.

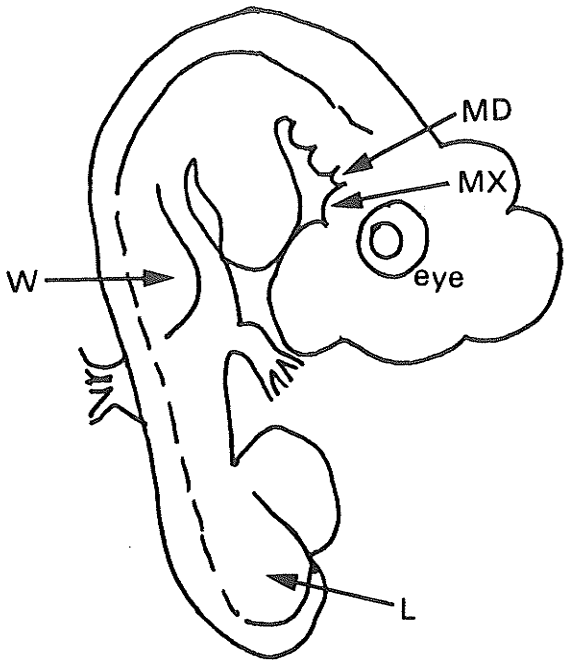
B. Stage 20 chick embryo (3.5 days incubation). The wing bud is a discrete swelling, leg buds are larger than wing buds, the body contour mid-trunk is straight, the neck region is very curved, and faint grey eye pigment is visible.

C. Stage 24 chick embryo (4.5 days incubation). The wing bud is longer than it is wide, distinct eye pigmentation, and the body is curved from head to tail.

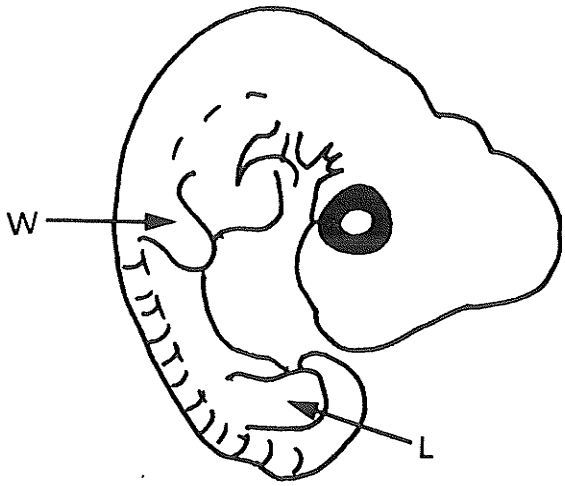
D. Stage 28 chick embryo (5.5 days incubation). The wing has a distinct elbow formation and the eye is large and prominent.



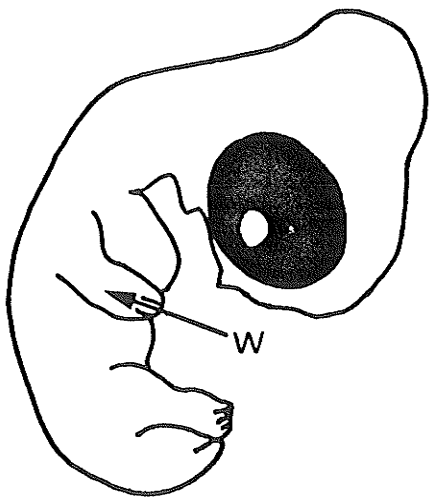
A ____ mm



B ____ mm



C ____ mm



D ____ mm

Figure 1.2 **Diagram of a stage 24 chick face.**

The frontal view of the embryo indicates how the facial primordia were dissected. The nasal pit was cut to separate the frontonasal mass and the lateral nasal process. The maxilla was either dissected as one entire piece, or divided in half to yield the upper maxilla and the lower maxilla.

Key: LNP = lateral nasal process, FNM = frontonasal mass, MX = maxilla, UMX = upper maxilla, LMX = lower maxilla, MD = mandible

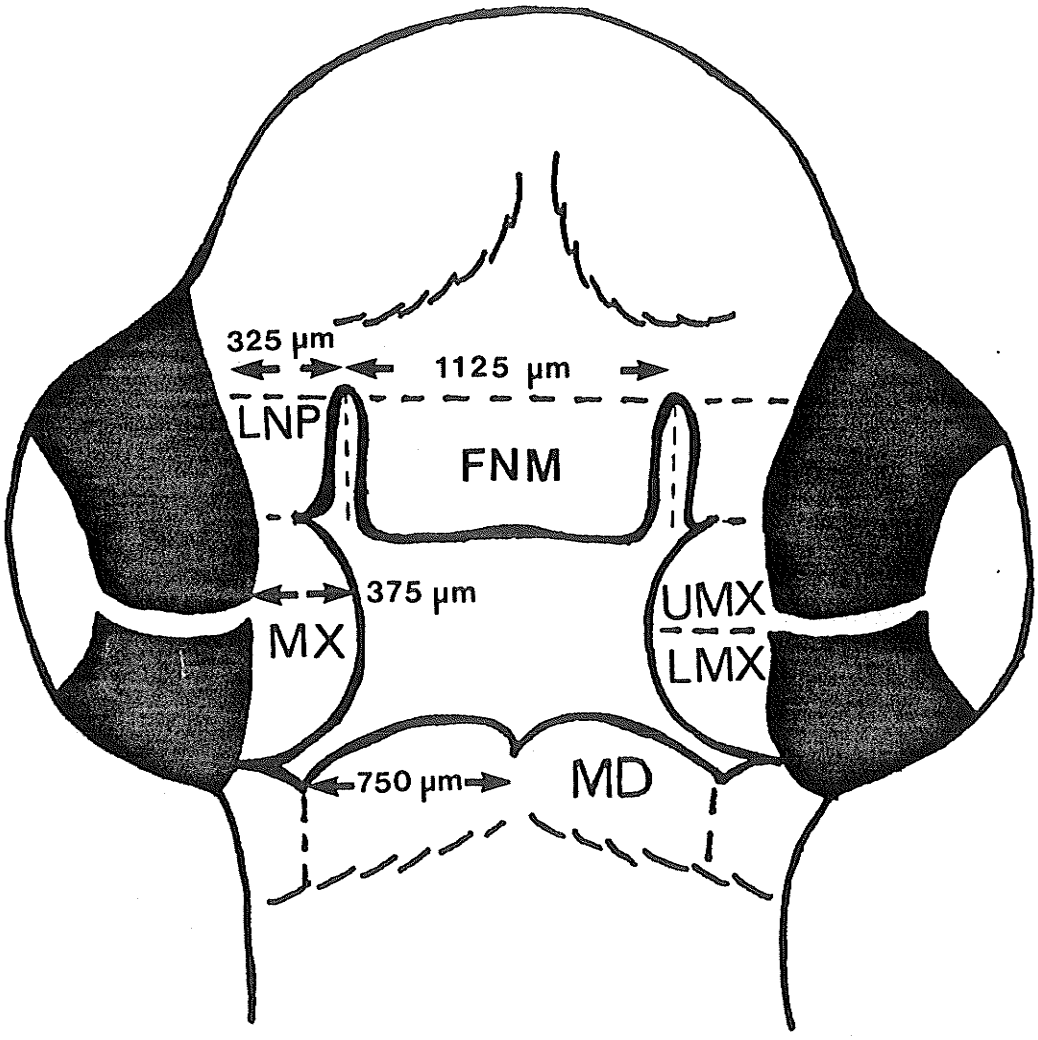

325 µm
1125 µm
LNP
FNM
375 µm
MX
UMX
LMX
750 µm
MD

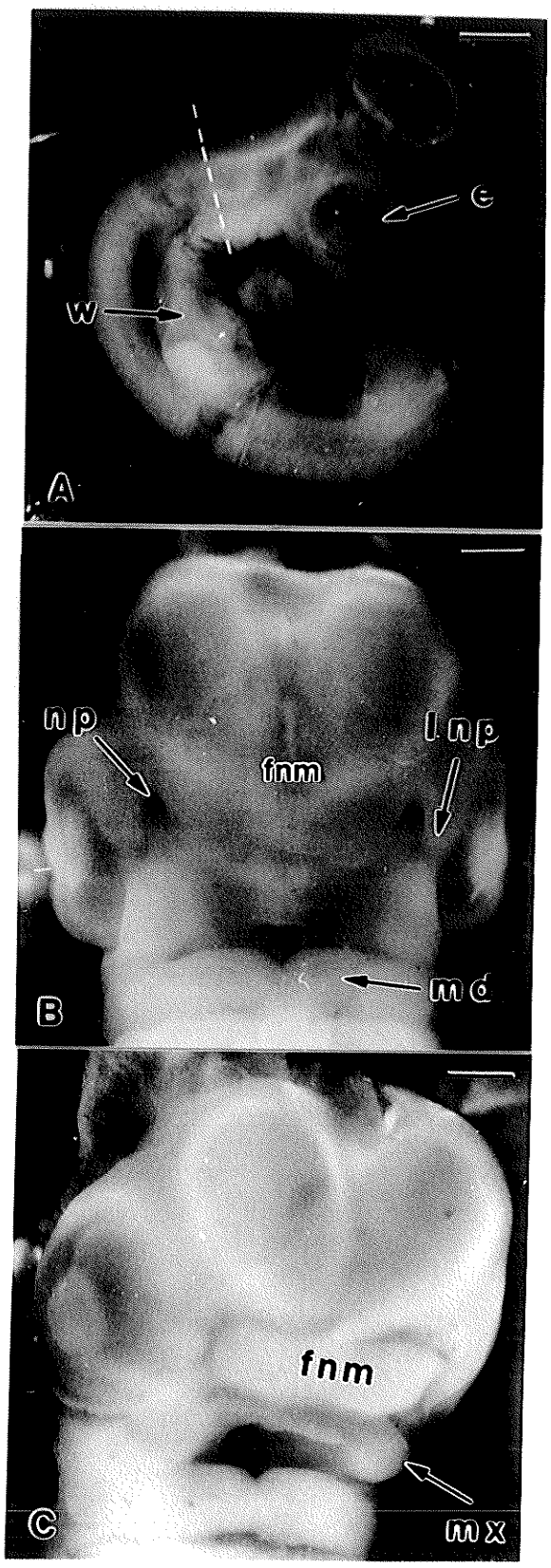
Figure 1.3 **Photographs of a stage 24 chick embryo during the dissection process.**

Key: w = wing, e = eye, np = nasal pit, lnp = lateral nasal process, md = mandible, fnm = frontonasal mass, mx = maxilla.

A. Sagittal view of the embryo as it appears on windowing the egg. The head is dissected from the body below the second branchial arch, at the level of the dashed line.

B. The nasal pit (np) is cut to separate the frontonasal mass (fnm) from the lateral nasal process (lnp). The mandible (md) is inferior to the oral cavity.

C. A 45 degree view of the embryo displays the paired maxillae (mx) at each corner of the stomodeum. The maxilla was either dissected as one piece or divided in two to yield the upper and lower maxilla. The cut within the maxilla was made with the primordium still attached to the face. The upper half was removed and placed in a culture dish and then the lower half was dissected. This ensured that the upper and lower pieces were not confused. Dissection cuts are visible across the frontonasal mass (fnm), through the nasal pits, above and below the maxilla on the right side of the photograph, and below the mandible.


e
w
A
np
fnm
lnp
md
B
fnm
C
mx

CHAPTER II

EFFECTS OF EXOGENOUS bFGF ON CULTURES OF FACIAL MESENCHYME

The facial primordia each give rise to a specific part of the face and each develops a specific arrangement of soft and skeletal tissue. This pattern is set up early on during development but the factors that lead to distinctiveness of the upper and lower beak are unknown. One approach to studying the controls of differentiation and growth is to culture cells from discrete regions of the face *in vitro*. This approach has given much information about cell growth, chondrogenesis, and myogenesis *in vitro* (Langille and Solursh, 1990, 1991; Mina *et al.*,1991; Richman and Crosby, 1990; Wedden *et al.*, 1986).

Fortunately, facial mesenchyme can grow quite successfully in culture. The high density micromass culture technique, developed by Ahrens *et al.* (1977), provides a way to achieve the cell density required for proliferation and differentiation with the lower number of cells that are available from very small tissues such as early embryo facial primordia. Up to stage 24, the primordia are composed of homogeneous, undifferentiated mesenchyme. Cells are isolated directly from the embryo every time a new culture is established. These primary cultures are grown for a very short period (96 hours) and therefore behaviour of the cells should still be quite similar to what is happening *in vivo*.

The type of cells that differentiate reflects the mixed precursor population present in embryonic facial tissue at the time of dissection. Each primordium develops its own reproducible pattern of cartilage *in vitro* that is altered slightly by the kind of media that cells are grown in (Wedden *et al.*, 1986; Ralphs *et al.*, 1989; Langille and Solursh,

1990; Richman and Crosby, 1990; Mina *et al.*, 1991). The mesodermally-derived muscle cells also differentiate in culture, although the patterns of chondrogenesis and myogenesis are not linked (Ralphs *et al.*, 1989). The number of myoblasts which differentiate in cell culture is less than 1% of the total cell number (Richman and Crosby (1990). Therefore most of the cells are mesenchyme rather than mesoderm.

Questions remain regarding the relationship of cell growth *in vitro* to cell growth *in vivo*. The increase in cell number and rate of proliferation seen *in vitro* may relate to the increase in size of the primordium *in vivo*. For example, the frontonasal mass and mandible both grow out extensively *in vivo* and in culture both types of mesenchyme experience large increases in cell number and proliferation. This relationship does not hold true for cultures of maxillary mesenchyme. The maxillary mesenchyme has a significantly higher rate of proliferation than the frontonasal mass and mandible (Richman and Crosby, 1990), yet the maxilla makes the smallest contribution to the chick face.

It is possible that the pattern of chondrogenesis in cultures and the rate of proliferation in culture could be related to the lineage of the cells. The hypothesis is that cells that are derived from a similar lineage will behave similarly in culture. There is evidence, for example, that the cranial half of the maxilla is formed by mostly prosencephalic crest whereas the caudal half of the maxilla is formed by mesencephalic crest (Le Lievre, 1978). The lack of mixing of cells lineages in the stage 24 maxilla is also visible at the molecular level. Transcripts of retinoic acid receptor-β (RAR-β) are concentrated in the cranial half of the maxilla (Rowe *et al.*, 1991). Thus, on the basis of cell lineage and genetic evidence, the maxilla is comprised of two groups of cells.

Micromass culture of each half of the maxilla would demonstrate whether growth characteristics differ. Previous work has concentrated on culturing facial mesenchyme from the whole frontonasal mass, mandible, and maxilla (Wedden *et al.*, 1986; Mina *et al.*, 1991; Langille and Solursh, 1990, 1991; Richman and Crosby, 1990). There has been no description of the behaviour of subdivided primordia in culture.

An additional test of the hypothesis that neural crest lineage is reflected in cell culture is to culture the lateral nasal process. According to neural crest transplantation experiments, the lateral nasal process is comprised mainly of prosencephalic neural crest, as is the frontonasal mass. If this is true, then the lateral nasal process should behave like frontonasal mass in culture.

MATERIALS AND METHODS

I. Incubation and staging of embryos

Fertile White Leghorn chicken eggs (Dept. of Poultry Science, Univ. of Manitoba) were incubated at 38° C, with high humidity, to reach stage 24 (Hamburger and Hamilton, 1951). The embryos were staged one day prior to the experiment to synchronize them. The development could be slowed if required by leaving the advanced embryos at room temperature. Staging was done according to Hamburger and Hamilton (1951) based on external characteristics.

Stage 15, 20, 24, and 28 chick embryos were used in this study. Approximately 2.5 days of incubation were required to reach stage 15 in development. At this stage the head is well developed and the body is straight. The prospective limb area is flat with no external sign of limb buds (Fig. 1.1 A). Stage 20 (approximately 3.5 days incubation period) is reached when the wing bud is a discrete, symmetrical swelling. From this stage onward, the leg buds are distinctly larger than the wing buds. The contour of the mid-trunk is a slightly curved and there is a faint grey pigmentation of the eye (Fig. 1.1 B). A stage 24 chick embryo (approximately 4.5 days incubation time) has a wing bud longer than it is wide and the body is curved from head to tail. The eye pigmentation is now distinct (Fig. 1.1 C). One more day of incubation produces a stage 28 embryo. The 3 developing digits in the wing and the 4 toes on the leg give the limbs a pointed contour. The eye is large and prominent with a nictitating membrane extending halfway over it (Fig. 1.1 D). The chick hatches after 20-21 days of incubation at stage 46 of development.

To stage the embryos, each egg was candled with a fiberoptic light and gently rolled to release the embryo from the inner shell membrane. With the egg positioned horizontally, a small hole was poked in the large end of the egg which allowed the air cell to reposition over the embryo. The egg was windowed to view the embryo and determine the stage.

II. Dissection of embryos

Normal stage 24 chick embryos (Hamburger and Hamilton, 1951) were used for all cell culture experiments. Sterile technique was used to prepare media and all dissection and cell culturing was done in a laminar flow hood.

Each embryo was removed from the egg and placed on a concave dissection slide filled with dissecting media containing 1% Hanks' Buffered Salt Solution (Gibco, adjusted to ph 7.2) and 10% fetal calf serum.

The amniotic sac was removed and the head dissected from the body below the second branchial arch (Fig. 1.2). The tissues dissected were the frontonasal mass, the lateral nasal processes, the mandible, and the maxilla. The maxilla was dissected as either one piece, or cut in half to yield the upper maxilla and the lower maxilla (Fig 1.3). Each of the processes were removed by sterile glass pipette to labelled storage petri dishes filled with dissecting media.

Thirty to forty embryos were required for each experiment to gather adequate tissues for plating. This was primarily due to the very small size of divided maxilla. 1126 embryos were dissected to complete the cell culture experiments for this project.

III. Preparation of tissue for cell culture

The epithelium was removed from the mesenchyme by placing the tissue pieces in 2% trypsin in Hanks' Buffered Salt Solution (Gibco 1:250) at 4° C for 30-60 minutes. The dishes were flooded with serum containing media to stop the trypsin action and then the epithelium was cleanly separated from each piece of mesenchyme with fine forceps, working on a frozen slab. The mesenchyme pieces, from each facial process, were collected with a glass pipette and placed in a labelled centrifuge tube. The tubes were centrifuged at 6500 rpm for 4 minutes to produce a pellet. Excess media was removed, and the pellet was then pipetted up and down 70 times in a measured volume of media to dissociate the cells. The cell number was estimated using a haemocytometer and the final concentration adjusted to 2 x 10^7 cell/ml plating density.

IV. Cell plating

Cells were plated as 2 micromasses (2, 10 μl drops each containing 2 x 10^5 cells) in each well of the four-well tissue culture dishes (Nunclon, Gibco). The initial experiments used defined media for plating, while later experiments used fetal calf serum containing media to plate the cells.

The micromass cultures were allowed to adhere for 1 hour in the incubator at 37° C, 5% CO_2 before flooding with a predetermined media to give a total volume of 500 μl. The media was replaced daily with 500 μl fresh media and the cultures were visually assessed for viability by such criteria as adherence to the plate, density, amount of spread, and the number of floating or detached cells.

V. Composition of media

Two types of media were used, defined media and serum-containing media. Defined media was also used to dilute bFGF in 3 different concentrations. Therefore, the media used were defined medium, fetal calf serum-containing medium, and defined medium with bFGF.

The defined media contained a 60:40 ratio of F-12 Nutrient Mixture with L-Glutamine (Gibco): Dulbecco's Modified Eagle Medium (high glucose) with 4500 mg/l D-glucose + L-glutamine + 110 mg/l Sodium pyruvate (Gibco), 2 mM L-glutamine, antibiotic - antimycotic mixture containing 100 units/ml penicillin G sodium + 100 μg/ml streptomycin sulfate + 0.25 μg/ml amphotericin B as fungizone (Gibco), 5 μg/ml transferrin (Sigma), 100 nM hydrocortisone (Sigma), 5 μg/ml insulin (Sigma), and 50 μg/ml ascorbic acid.

The serum-containing media consisted of a 60:40 ratio of F-12:DMEM with 2 mM L-glutamine, antibiotic as described in the above formula for defined media, and 10% fetal calf serum (ICN).

Media containing bFGF was made fresh daily by adding stock concentrations of bFGF to defined media. The stock concentrations of bFGF were made by reconstituting 97% pure lyophilized bFGF from bovine brain (R&D). The solvent for bFGF consisted of defined media containing 1 mg/ml bovine serum albumin (BSA). Concentrations of 1 ng/ml, 10 ng/ml, and 100 ng/ml bFGF were added to defined media. The initial experiments used the low dosage of bFGF (1 ng/ml) based on previous work done by Richman and Crosby (1990). At this dosage, the cultures which were grown in bFGF

detached from the culture plates and failed to increase in cell number. At a 10 fold increase in concentration, there was improved attachment and increased cell numbers. Only when 100 ng/ml bFGF was added to the defined media did cell numbers equal the base line data of Richman and Crosby.

All additives used in preparing the defined and serum containing media were prepared with sterile technique, aliquotted, and stored at -20° C, except insulin which was stored at 4° C.

VI. Assaying cell number

The cell number was estimated at 48 and 96 hours after plating. The wells were rinsed with defined media to remove serum, and the media was replaced with 200 μl EDTA/trypsin solution (0.1% Trypsin and 0.001M EDTA in Hanks' Buffered Salt Solution). The dishes were incubated for 2 to 10 minutes at 20° C, and then gently agitated to detach the cells from the dishes. Serum-containing media (700 μl) was added to stop the action of the trypsin. The contents were gently pipetted up and down to loosen all the cells of the two micromass cultures and the entire contents of the well were aspirated and placed in labelled centrifuge tubes. The tubes were spun at 6500 rpm for 4 minutes to form pellets. Excess media was removed and the cells were resuspended in a known volume of media. The cell number was then estimated with a haemocytometer.

To dissociate cells at the 96 hour count, for tissues containing cartilage (ie. frontonasal mass, lateral nasal processes, mandible), the wells were rinsed with defined media, 200 μl EDTA/Trypsin added, and the dishes incubated at 37° C until the

micromass spots lifted off the dish (5-15 minutes). Serum containing media (700 μl) was added to stop the trypsin action and the spots were sucked out of each well and placed in a labelled tube. The remaining contents of each well was vortexed and added to the tube. The tubes were centrifuged at 6500 rpm for 4 minutes to form a pellet and excess media was then removed. Collagenase (0.1% in Hanks' BSS, 50-75 μl) was added to each tube and the tubes were incubated at 37° C until the cells could be dissociated, approximately 30 minutes. A measured volume of media was added to each tube and the cells were re-suspended. Cell number was then estimated using a haemocytometer.

Cell number data was recorded for the six populations of cells grown in five different media. Statistical analysis was performed using sample mean, standard deviation, and t-tests (Hassard, 1991).

VII. Detecting cartilage matrix in micromass culture

Micromass cultures incubated for 96 hours were rinsed with defined media, fixed with 1/2 strength Karnovsky's fixative (Karnovsky, 1965) overnight at 4° C, and then stained with 200 μl Alcian blue (ph 1.0, Lev and Spicer, 1964) for 2 hours. The cultures were dehydrated in progressive changes of ethanol (70% to 90% to 100%) for 10 minutes per change. Each well was then partially filled with glycerol.

Photographs of the micromass cultures were taken using a Leica microscope and black and white print film (FP4 Ilford 125 ASA), utilizing bright field to illuminate the clear plastic culture dish from below. The glycerol was removed from the well and the dish was covered with a dark orange filter to improve contrast.

RESULTS

In order to identify distinct cell populations within the face, micromass cultures of several regions of the embryonic chick face were grown in various culture media. Both cell number and pattern of chondrogenesis were evaluated.

This section emphasizes the results that are new, in particular those from the lateral nasal process and divided maxilla cultures. The other primordia (mandible, whole maxilla, and frontonasal mass) were included as internal controls so that the results could be compared to other studies. Each primordia is described and comparisons are made between primordia.

I. Changes in cell numbers of facial mesenchyme grown in micromass cultures with different media

A. Defined media

In the least complex medium, defined medium, there was a decrease in cell number from the initial plating density of 4 X 10^5 cells/well (Fig. 2.1 A). All cultures lost at least 50% of the cells plated between 0 and 48 hours. The frontonasal mass, mandible, and lower maxilla all retained a similar number of cells between 48 and 96 hours. The lateral nasal process, whole maxilla, and upper maxilla all continued to decrease in cell number between 48 and 96 hours.

Although the cell numbers were low in defined media, the cultures were well attached. Cultures grown in defined media were the most difficult to detach to count the cells. Since cells survived in defined media without increases in cell numbers, differences in cell survival were discerned between primordia which were not apparent in media

which allowed large increases in cell number. Frontonasal mass cultures had higher numbers than lateral nasal process in defined media at 48 ($p < 0.05$) and 96 hours ($p < 0.001$). The number of samples in defined media was large enough to overcome randomness within the system and reveal a true difference between primordia (frontonasal mass $n = 13$, lateral nasal process $n = 6$).

Defined media also revealed a definite difference between upper and lower maxilla cultures. Lower maxilla cultures had cell numbers significantly greater than upper maxilla cultures at 48 and 96 hours ($p < 0.001$, $p < 0.002$). After 96 hours of culture in defined media, upper maxilla cultures were not significantly different than lateral nasal process cultures, and lower maxilla cultures were not significantly different than mandibular cultures ($p < 0.05$).

B. Fetal calf serum-containing media

Fetal calf serum increased growth in all types of cultures compared to defined medium (Fig. 2.1 B). The most significant result was that lateral nasal process and frontonasal mass cultures increased 3 and 2.5 fold respectively over plating density. Surprisingly, all other types of mesenchyme decreased in cell number between 0 and 48 hours and then remained constant between 48 and 96 hours. The final cell number in the mandible, maxilla, upper maxilla, and lower maxilla was much higher than in similar cultures grown in defined medium.

C. Defined media supplemented with three concentrations of bFGF

Cell cultures grown in bFGF containing medium tended to detach from the dish. This problem was most severe with doses of 1 and 10 ng/ml bFGF. The type of culture

that had the least problems with detachment was the frontonasal mass, although even these cultures occasionally exhibited lifting from the dish. Thus, I will focus on the results with 100 ng/ml bFGF.

In general, cultures supplemented with 100 ng/ml bFGF increased in cell number to a much greater extent than in defined media (Fig. 2.1 C). Cultures from the frontonasal mass, lateral nasal process, and upper maxilla primordia were the most responsive. Lateral nasal process and frontonasal mass cultures increased 0.5 fold over initial plating density by 96 hours. The upper maxilla cultures were the only other type of culture to increase in cell number above plating density by 96 hours.

Cultures grown in 100 ng/ml bFGF again showed a distinct difference between upper and lower maxilla but the trend was a reversal of the results in defined media. Upper maxilla cultures had more cells than lower maxilla cultures at 48 ($p < 0.02$) and 96 hours ($p < 0.005$).

bFGF data shows a similarity between upper maxilla and lateral nasal process, with no significant difference in cell numbers between the two cultures at 48 or 96 hours ($p < 0.05$), for cultures grown in 100 ng/ml bFGF.

D. Primordia-specific changes in cell number

Data was reorganized by primordia to compare the effects of media on each primordia. The most striking observation in lateral nasal process cultures (Fig. 2.2 A) is the difference in cell numbers between cultures grown in defined media and cultures grown in fetal calf serum-containing media. The 90% reduction for defined media is in

sharp contrast to the 3 fold increase in fetal calf serum after four days of culture. The cell number in fetal calf serum was the highest recorded in the study.

Frontonasal mass was the only primordia to increase in cell number with 1 ng/ml bFGF by 96 hours (Fig. 2.2 B). Cell number with 1 ng/ml bFGF was no different than with 10 or 100 ng/ml at 96 hours. Thus, a plateau of response was reached and increasing the dose of bFGF had no further effect on cell number.

Mandibular cultures did not increase in cell numbers in any media (Fig. 2.2 C). In fact, numbers were substantially below plating density at 48 and 96 hours. There was virtually no response to bFGF until 100 ng/ml was added to the culture medium.

Generally, over the four day culture period, the cell numbers remained low for all three types of maxillary cultures in all media (Fig. 2.2 D,E,F), compared to lateral nasal process and frontonasal mass. One exception was the upper maxilla, in 100 ng/ml bFGF, which increased in cell number above plating density. The whole maxilla appeared to be a combination of upper and lower maxilla, as would be expected.

E. Key points of comparison between facial mesenchyme tissues.

A number of important points became apparent when the cell numbers of primordia were compared. Both lateral nasal process and frontonasal mass cells in culture were very responsive to fetal calf serum-containing media and media supplemented with bFGF.

The maxilla consisted of two separate populations of cells; a bFGF responsive population, mostly in the upper maxilla, and a non-responsive population in the lower maxilla. Upper maxilla cultures had similar cell numbers to lateral nasal process cultures

in defined medium and medium supplemented with bFGF. Lower maxillary and mandibular cultures had equivalent cell numbers in all types of media.

II. Chondrogenic differentiation of facial mesenchyme in micromass cultures with different media

Alcian blue, at pH 1, specifically stains sulphated glycosaminoglycans in cartilage matrix which has formed in the culture (Lev and Spicer, 1964). Generally, cultures had primordia-specific patterns of chondrogenesis in response to various culture media. A low level of background staining of the fibroblast fringe surrounding the culture was also observed. The proportion of the culture occupied by cartilage was not quantified in this study.

A. Defined media

Lateral nasal process and frontonasal mass cultures grown in defined media (Fig 2.3 A,B,C) formed lacy patterns of cartilage matrix. Mandibular cultures exhibited nodules that appeared to coalesce into columns of cartilage. There was no obvious qualitative difference in area of cartilage between the three types of cultures.

Upper maxillary and lower maxillary cultures formed small, centrally located nodules (Fig. 2.3 D,E). No whole maxilla cultures grown in defined media were stained with Alcian blue.

B. Fetal calf serum-containing media

The lateral nasal process, frontonasal mass, and mandible cells formed dense cultures in fetal calf serum-containing media (Fig. 2.4 A,B,C). The lateral nasal process and frontonasal mass cultures formed sheets of cartilage. An outer, dense fringe of

fibroblasts gave the lateral nasal process cultures a "sunflower" appearance. The mandible cultures formed dense, coalesced nodules of cartilage. There was a clear, qualitative increase in the area of the culture occupied by cartilage compared to lateral nasal process, frontonasal mass, and mandible cultures grown in defined medium.

Whole maxilla and upper maxilla cultures produced virtually no cartilage in fetal calf serum-containing media (Fig. 2.4 D,E,F). The lower maxilla cultures had higher levels of background staining than the upper maxilla, making detection of specific cartilage staining difficult (Fig. 2.4 F). The pattern of whole maxilla cultures seemed to be a combination of upper and lower maxilla cultures.

C. Defined media containing bFGF

The general trends for all cultures grown in media containing bFGF were that cultures tended to detach from the plates with time; the cultures were smaller than those in either defined media or fetal calf serum-containing media; the size of the cultures tended to decrease as concentration of bFGF increased; and the nodules of cartilage merged into a sheet as concentration of bFGF increased.

Lateral nasal process and frontonasal mass cultures grown in the lowest concentration (1 ng/ml) of bFGF (Fig. 2.5 A,B) resembled defined media cultures (Fig. 2.3 A,B). These cultures both became more dense at 10 ng/ml (Fig. 2.5 D,E) and 100 ng/ml dosages of bFGF (Fig. 2.6 A,B) and began to resemble cultures grown in fetal calf serum-containing medium.

Mandible cultures at all dosages of bFGF initially formed central nodules. With time, the peripheral fringe detached resulting in small, poorly developed cultures (Fig. 2.5 C,F and Fig. 2.6 C).

All maxillary cultures grown in media containing bFGF only remained attached to the culture dishes if they were initially plated in fetal calf serum containing media. At the lowest concentrations of bFGF (1 ng/ml) the cultures grew poorly (not shown). When the concentration of bFGF was increased to 10 ng/ml the characteristics of the cultures improved to a limited degree (not shown). Changing the culture conditions to a high concentration of bFGF (100 ng/ml) resulted in more favourable growth characteristics but a high level of background staining was detected (Fig. 2.6 D,E,F).

III. Changes in cell number compared to chondrogenesis of micromass cultures

The cell numbers for lower maxillary and mandibular cultures grown in defined media at 96 hours were similar, but chondrogenesis was far greater in the mandible than in the lower maxilla (Fig. 2.3 C,E). This same finding was observed in lateral nasal process and upper maxillary cultures at 96 hours, again with similar cell numbers in defined media and a large difference in cartilage matrix produced in the defined media cultures (Fig. 2.3 A,D). The lateral nasal process developed much more cartilage than the mandible cultures. Thus, the extent of chondrogenesis did not always correlate with cell number data.

DISCUSSION

I. Batch variation in bFGF and fetal calf serum

A. bFGF

The bFGF used in these experiments was purified from bovine brain and was 97% pure. The remaining 3% could consist of other HBGF or various other unidentified substances. bFGF can also be made using recombinant DNA technology. This bFGF is more pure but because it is not synthesized inside a eukaryotic cell, the structure of the protein may be slightly different than native bFGF. I did a short series of experiments using human recombinant bFGF (Gibco) in culture and absolutely no increase in cell number or in chondrogenesis, over cultures grown in defined media, occurred. Thus, this batch of recombinant bFGF seemed to lack biological activity in our system.

The purified native bFGF used in my studies was purchased from the same source as Richman and Crosby (1990) yet the results were different. The major difference was the fact that I needed a 100 fold greater concentration of bFGF to produce an increase in cell number and chondrogenesis in frontonasal mass equal to that obtained by Richman and Crosby (1990). Despite the 100 fold higher dose required, the general trend is the same as reported in Richman and Crosby (1990). The reason for the difference in dose/response is probably related to the cell detachment associated with the specific batch of bFGF used in my study. Cultures grown in defined media did not have problems with detachment and the results were identical to those in Richman and Crosby (1990). This batch of bFGF may have contained trace amounts of some other substance that interfered with attachment.

It is possible that the high dosage of bFGF used was toxic to the cultures, and this resulted in detachment. The concentration of bFGF used in my studies (100 ng/ml) is far greater than would be found *in vivo*. Biochemical analysis of stage 24 chick limbs has shown that the concentration of bFGF is 1.5 ng/mg protein (Seed *et al.*, 1988). Since it was the 1 ng/ml dosage of bFGF that produced the most detachment, while cultures growing in 100 ng/ml bFGF remained attached, it is unlikely that toxicity played a role in cell detachment.

B. Fetal calf serum

Each batch of fetal calf serum is blended from the sera of several calves and there is some variation between batches. My results with fetal calf serum-containing media differ from those of Richman and Crosby (1990). These investigators showed that cell numbers in the mandible cultures grown in fetal calf serum-containing medium doubled after plating, while mandibular cultures in the experiments presented here failed to recover to plating density. There could have been an increased loss of cells at the beginning of the experiment, although this cannot be confirmed as no 4 or 24 hour data was gathered. Also, the mandible cells may not be proliferating which could be shown with S-phase labelling. The maxillary cultures grown in fetal calf serum also had low cell numbers compared to Richman and Crosby (1990). Thus, the batch of fetal calf serum used in the present study probably had different proportions of growth factors than the batch used by Richman and Crosby (1990). Serum contains growth factors such as platelet-derived growth factor (Waterfield, 1989) and insulin-like growth factor while bFGF is a tissue-derived growth factor and is not present in serum (Gospodarowicz and

Moran, 1976; Thomas, 1987; Rifkin and Moscatelli, 1989). In my study, upper maxillary and mandibular cultures had much better response to bFGF than to serum. Thus, factors in serum may be inhibitory to the growth of maxillary cells. Some of the growth factors found in serum could be tested individually in the micromass culture system to see which factors lead to an increase in cell number and chondrogenesis. TGF-β has been shown to have both these effets on cells (Sporn and Roberts, 1988), and is a good candidate for future studies.

II. The relationship of increases in cell number to increases in chondrogenesis

Alcian blue stains the cartilage matrix, not the chondrocytes that produce extracellular matrix. As such, there is no direct relationship between degree of staining and number of chondrocytes. In fetal calf serum-containing media and in media with bFGF, both frontonasal mass and lateral nasal process cultures increased in cell numbers and in the amount of cartilage formed compared to defined media. Although the amount of cartilage matrix was not quantified in this study, there were very obvious differences in area of alcian blue stained matrix between cultures grown in defined media and those grown in the presence of bFGF or fetal calf serum. It is possible that in these two primordia, it is the chondrocytes that make the major contribution to total cell number. The way to test this would be to directly count the proportion of chondrocytes that make up the total cell number.

Interestingly, all types of maxillary cultures had very little cartilage matrix formation in fetal calf serum-containing media, yet cell numbers were the same at 96 hours as mandibular cultures which had extensive chondrogenesis in fetal calf serum. It

would be expected that the maxillary cultures have little or no cartilage as there is no cartilage in the maxilla *in vivo*. There are three possible explanations for this: 1) chondrocytes in the mandible were not proliferating but just differentiating and hence there was no increase in cell number; 2) cell loss in mandible cultures balanced out the increase in number of chondrocytes; or 3) maxillary cultures are proliferating at a higher rate than mandible cultures.

Total cell number reflects the balance between proliferation and cell loss. I did not attempt to determine changes in either proliferation or cell loss, but used cell number as a general assessment of changes in the growth of each primordia. Therefore, I cannot distinguish between a decrease in cell number due to reduced proliferation or increased cell death. Proliferation, or the increased rate of cell division, could be measured by labelling cells which are in the S-phase of cell division or by counting mitotic figures. The pattern of cell death could be established using Nile blue sulfate staining (Sulik *et al.*, 1988).

III. Cell lineage is represented in cell culture

The hypothesis presented in Chapter 1 was that facial cells derived from similar neural crest cell lineages will respond similarly to various exogenous factors applied in a culture system. The data generally does support this hypothesis. The hypothesis was tested in two ways. The cultures of lateral nasal process mesenchyme were compared to cultures of frontonasal mass. Lateral nasal process and frontonasal mass are derived from the same neural crest cells, originating from the region of the prosencephalon. Secondly, cultures of upper and lower maxilla mesenchymal cells were compared. The upper and

lower maxilla are derived from different neural crest cells, arising from the prosencephalon and mesencephalon respectively.

The behaviour of the lateral nasal process and frontonasal mass cultures was very similar in two of the three types of media tested. Behaviour in terms of increases in cell number and chondrogenic pattern was nearly identical in fetal calf serum-containing medium and in bFGF-containing medium. The hypothesis is not supported by cell number data in defined medium where the frontonasal mass had significantly higher cell numbers than lateral nasal process cultures. The chondrogenic pattern in frontonasal mass and lateral nasal process cultures is identical however. Thus, the majority of the data reinforces the hypothesis.

Cell culture of the upper and lower halves of the maxilla demonstrated significant differences in cell number between the two regions. These differences were found when cells were grown in defined medium and in bFGF-containing medium. There was no difference in cell number between upper and lower maxilla cultures when grown in fetal calf serum-containing medium. These results therefore partially support the hypothesis that subpopulations of cells exist within the maxilla. The differences between upper and lower maxilla seen in defined medium and bFGF-containing medium agree with a study by Rowe *et al* (1992) which demonstrated a distinct boundary within the maxilla based on RAR-β transcript expression. The lack of difference between cell number of upper and lower maxillary cultures in fetal calf serum-containing media can be explained by the fact that serum contains a host of growth factors and this may mask specific effects of any one growth factor. In addition, serum does not contain bFGF (Gospodarowicz and

Moran, 1976; Thomas, 1987; Rifkin and Moscatelli, 1989), the factor that does stimulate an increase in cell number in upper maxilla cultures. The response of upper maxilla cells to bFGF was not previously reported (Richman and Crosby, 1990). However, these investigators cultured mesenchyme from the whole maxillary primordium and subtle differences in the response to bFGF would have been masked.

The hypothesis predicts that the upper maxilla will behave more like the frontonasal mass and lateral nasal process while the lower maxilla will be more like the mandible. The bFGF and defined media data are consistent with this idea. Thus the unifying theme is that bFGF seems able to discern those populations that are similar in lineage, whereas fetal calf serum-coantaining medium is not able to do so.

I have shown interesting, regional effects of exogenous bFGF on differential growth of facial mesenchyme *in vitro*. The next step will be to determine whether bFGF is present in the face *in vivo* at the stages of development when the facial primordia are growing.

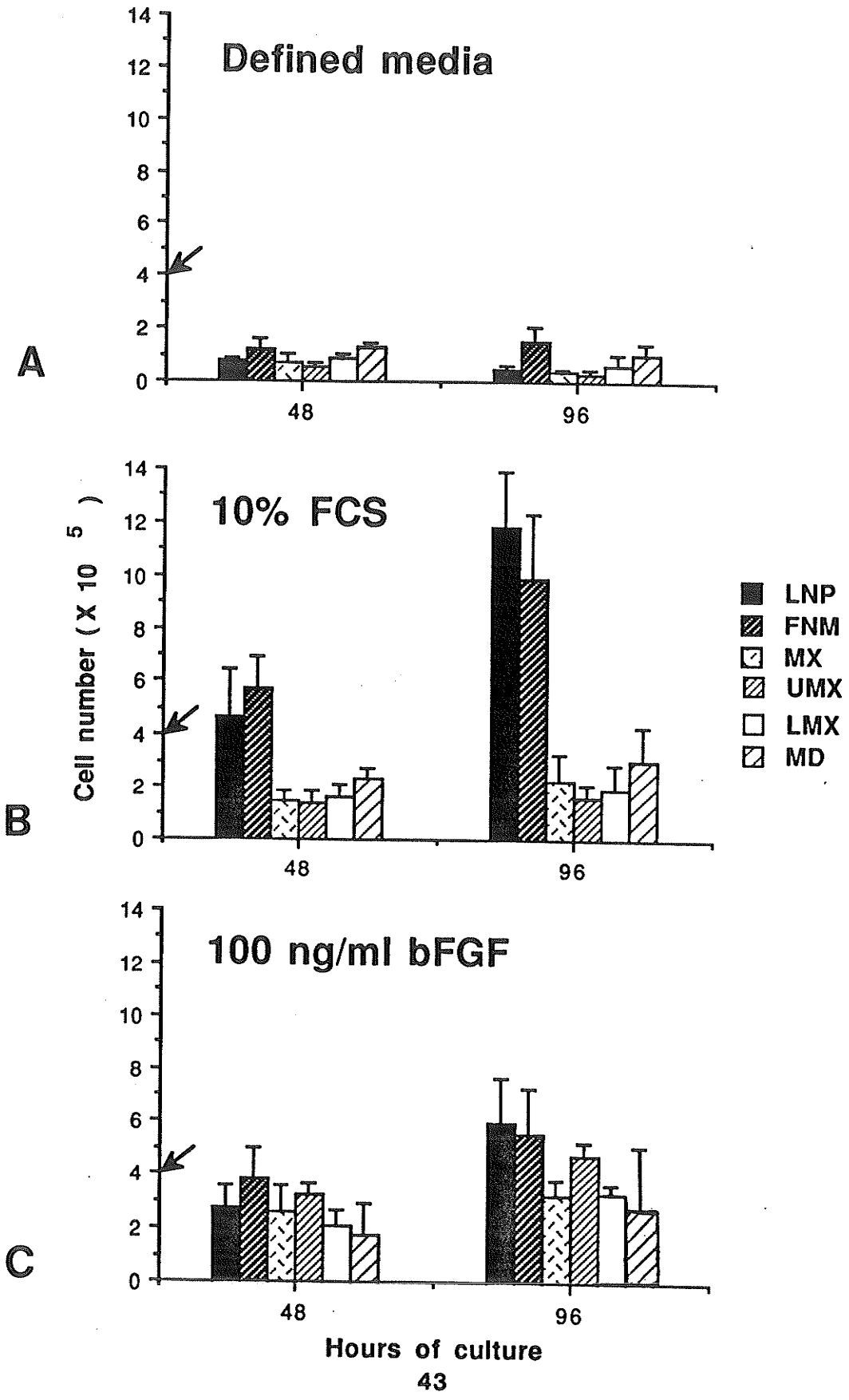
Figure 2.1 Effects of specific media on growth of facial mesenchyme in micromass cultures.

Error bars represent 1 standard deviation about the mean. Arrow = the number of cells initially plated in each well (4 X 10^5 cells). Key: FCS = fetal calf serum, bFGF = basic fibroblast growth factor, LNP = lateral nasal process, FNM = frontonasal mass, MX = whole maxilla, UMX = upper maxilla, LMX = lower maxilla.

A. Defined media. Each bar represents the mean of 5 to 14 cultures. Cultures grown in defined media had low, constant, cell numbers. The small variation from the mean is consistent for all primordia. Although the cell numbers are low, the cells survived in defined media and were well attached to the culture plates.

B. 10% fetal calf serum-containing media. Each bar represents the mean of 5 to 13 cultures. Cultures of lateral nasal process and frontonasal mass cells had much higher cell numbers than the other facial mesenchyme cells at both 48 and 96 hours.

C. Defined media containing 100 ng/ml bFGF. Each bar represents the mean of 3 to 10 cultures. Cell numbers of all primordia grown in high dosage bFGF-containing media were higher than those recorded in defined media, and began to approach cell numbers seen in fetal calf serum-containing cultures.


A
Defined media
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12
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4
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96
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LNP
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MX
UMX
LMX
MD
C
100 ng/ml bFGF
Hours of culture

Figure 2.2 Effects of different culture media on growth of facial mesenchyme in micromass culture.

The data used in Figure 2.1 was re-organized by primordium to compare the effects on primordia by each medium. Error bars represent 1 standard deviation about the mean. Arrow = the number of cells initially plated (4 X 10^5). Key: LNP = lateral nasal process, FNM = frontonasal mass, FCS = fetal calf serum, DM = defined media, bFGF = basic fibroblast growth factor.

A. Lateral nasal process cell cultures. Each bar represents the mean of 3 to 9 cultures. The best response is to fetal calf serum-containing media after 96 hours in culture. Cultures grown in 10 ng/ml bFGF containing medium, counted at 48 hours, had a very large standard deviation. The reason for this variation was that two of the four wells had low cell counts of 2 X 10^5 cells due to detachment of the cultures from the plate. The other two samples had mean cell numbers of about 7 X 10^5 and the cells had excellent attachment to the culture plates.

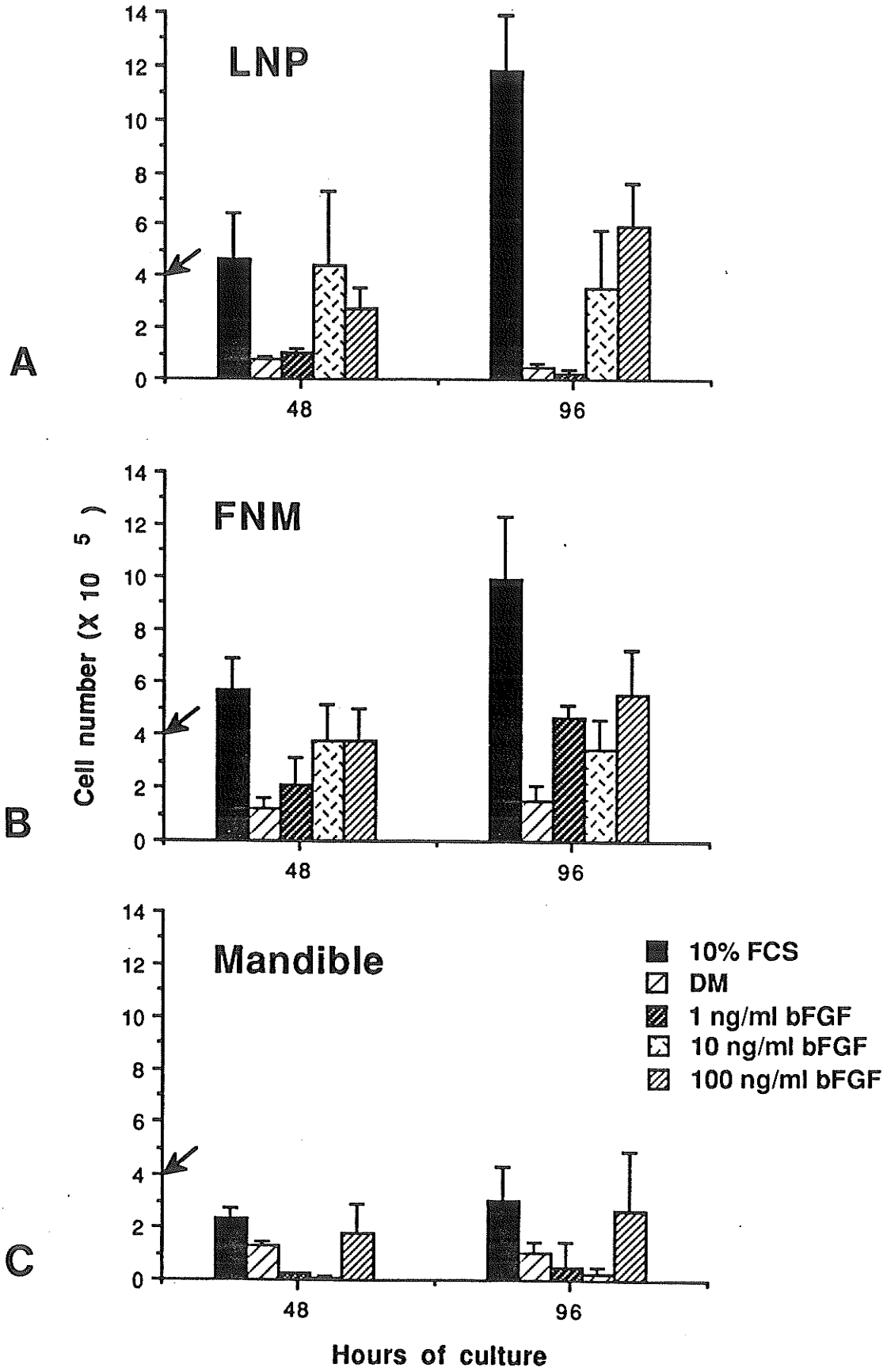
B. Frontonasal mass cell cultures. Each bar represents the mean of 4 to 14 cultures. Once again, the cultures had the highest cell numbers in the fetal calf serum-containing media.

C. Mandible cell cultures. Each bar represents the mean of 4 to 8 cultures. Mandible cultures generally responded much less than either lateral nasal process or frontonasal mass in all media. The cultures grown in 10 ng/ml bFGF-containing medium, counted at 48 hours, had the lowest mean cell number observed in all experiments. The low cell numbers for all cultures grown in low dosage bFGF-containing media is due to detachment of cultures from the culture plates.

D. Whole maxilla cell cultures. Each bar represents the mean of 3 to 6 cultures. The cultures comprised of cells from the entire maxilla had cell numbers which reflected a mid-point between the cell numbers of upper and lower maxilla cultures.

E. Upper maxilla cell cultures. Each bar represents the mean of 3 to 7 cultures. A definite increase in cell number with increased concentration of bFGF is apparent. At 96 hours, the cell number in 100 ng/ml bFGF was significantly higher than in fetal calf serum-containing media.

F. Lower maxilla cell cultures. Each bar represents the mean of 3 to 8 cultures.


A
LNP
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Cell number (X 10 5)
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1 ng/ml bFGF
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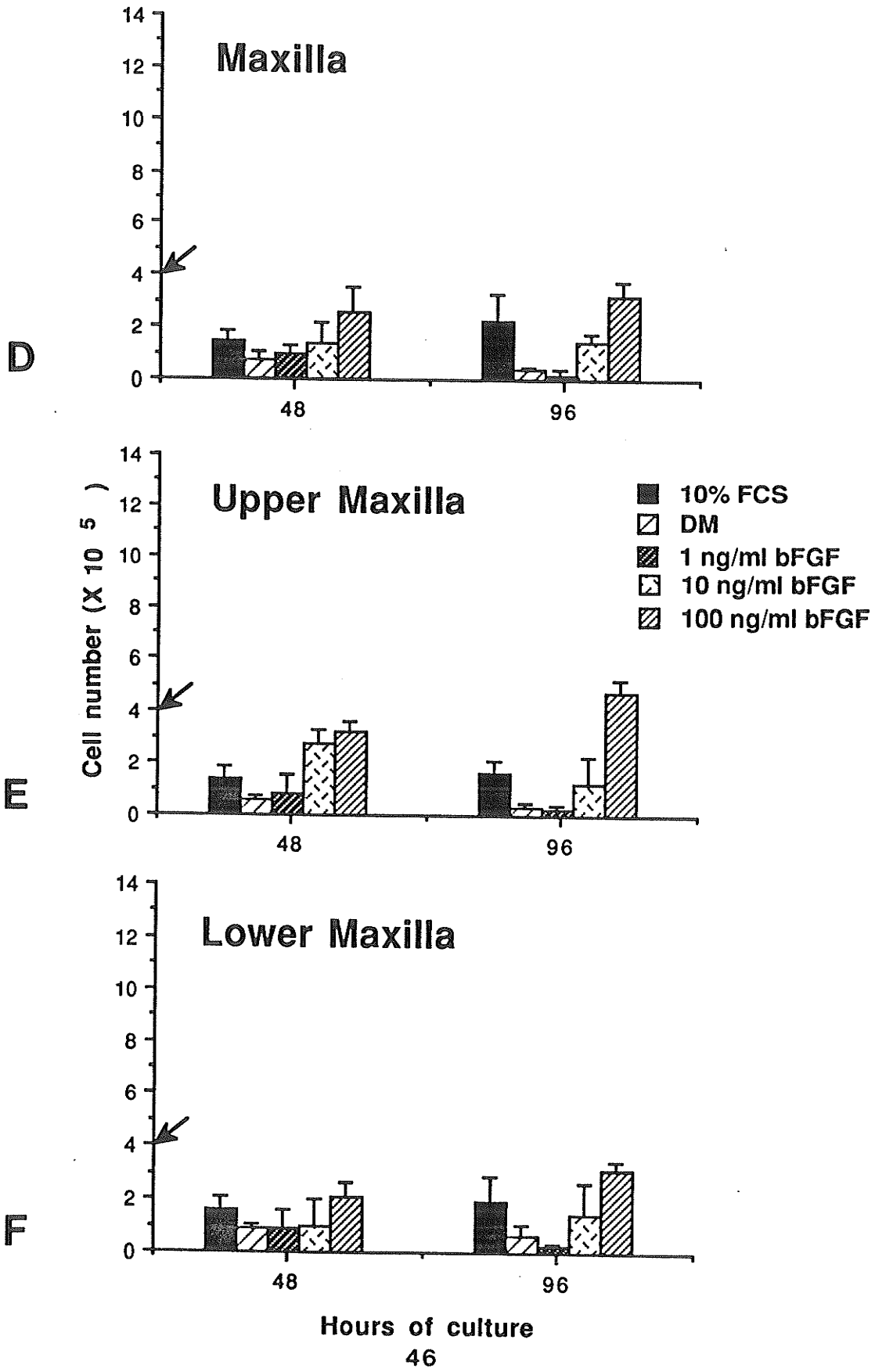
Hours of culture


D
Maxilla
14
12
10
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Cell number (X 10 5)
10% FCS
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1 ng/ml bFGF
10 ng/ml bFGF
100 ng/ml bFGF
F
Lower Maxilla
Hours of culture

Figure 2.3 Micromass cultures grown in defined media, stained with Alcian blue after 96 hours of culture.

Non-specific staining makes the fibroblast fringe visible. The outer edge of each culture is indicated by an arrow. The scale bar represents 1 mm for all figures.

A. Lateral nasal process cells grown in defined medium. Small nodules have developed in a fine, lacy pattern within the central area of the culture. A wide fibroblast fringe comprises half the total diameter of this well-attached culture.

B. Frontonasal mass cells cultured in defined media. The pattern of nodules formed is denser and covers more of the culture area than cultures of lateral nasal process.

C. Mandible cells cultured in defined medium. Small nodules of cartilage are more dense in the centre of the culture. A wide fringe of fibroblasts surrounds the cartilaginous central area. The staining pattern of the cartilage is similar to lateral nasal process and frontonasal mass.

D. Upper maxilla cells grown in defined medium. A few tiny nodules are spread throughout the culture. This culture was not plated in fetal calf serum and attachment is not as good as with the other cultures.

E. Lower maxilla cells cultured in defined media. There are definite small nodules of cartilage in the central area of the culture.

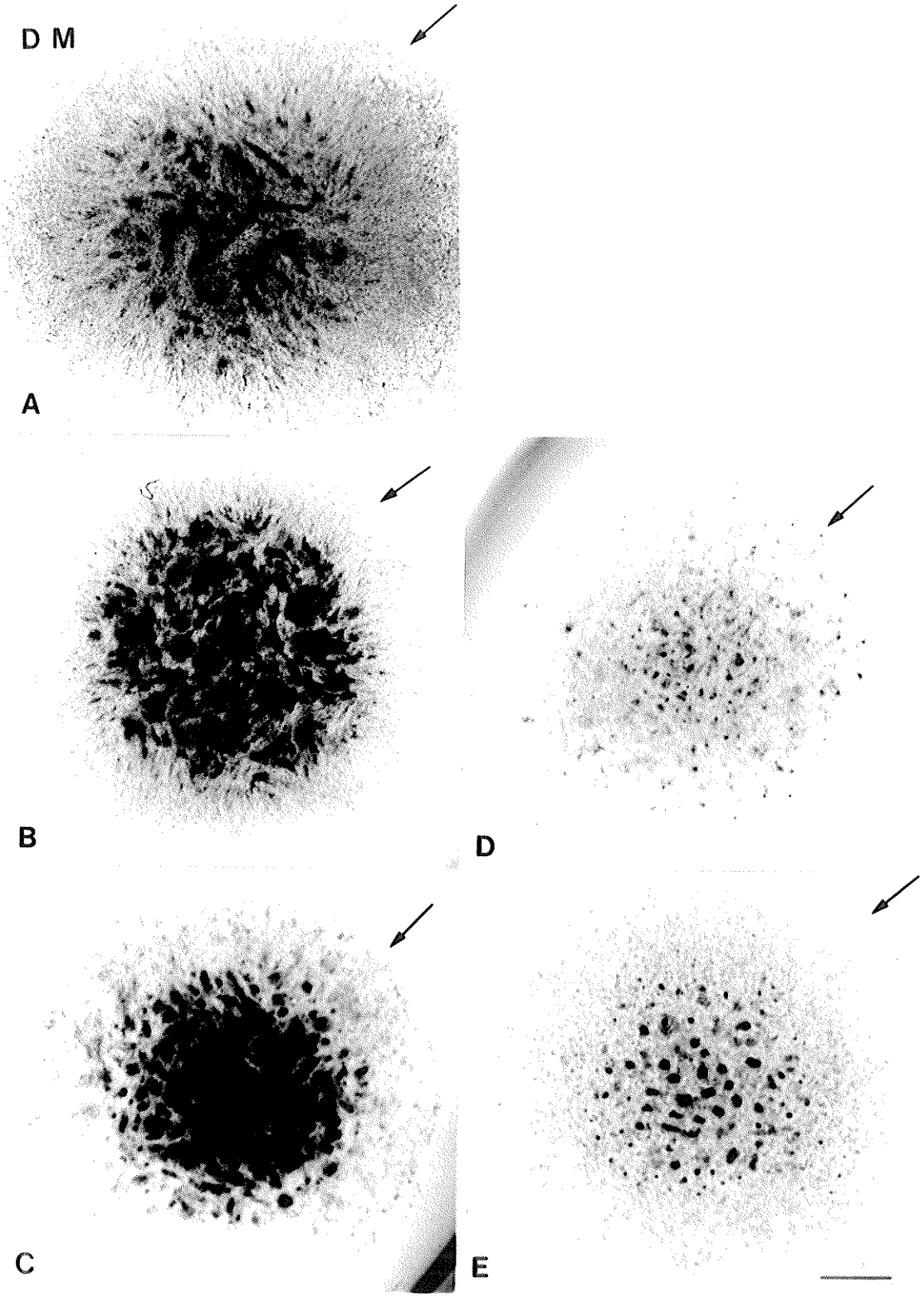

D M
A
B
D
C
E

Figure 2.4 Micromass cultures grown in fetal calf serum-containing media, stained with Alcian blue at 96 hours.

The outer edge of each culture is indicated by an arrow. The scale bar represents 1 mm for all figures.

A. Lateral nasal process cells cultured in fetal calf serum-containing medium. The cartilage pattern is sheet-like with varying density. There is also a wide, dense fibroblast fringe at the edge of the culture. This culture is well attached.

B. Frontonasal mass cells grown in fetal calf serum-containing medium. A dense sheet of cartilage formed covering most of the culture. A narrow fringe of fibroblasts surrounds the cartilage.

C. Mandible cells grown in serum-containing medium. The entire culture consists of dense nodules that have coalesced.

D. A culture comprised of cells from the whole maxilla grown in fetal calf serum-containing medium. The culture has a lot of non-specific staining, some of which may be cartilage. The culture is well attached to the culture dish. The arrow head indicates a stained cartilage nodule.

E. Upper maxilla cells grown in serum-containing medium. The appearance of this culture is similar to the culture of whole maxilla cultured in serum-containing medium. The arrow head points to a stained nodule.

F. Lower maxilla cells grown in serum-containing medium. Once again the appearance is similar to whole maxilla cultures. A few small central nodules may be present.

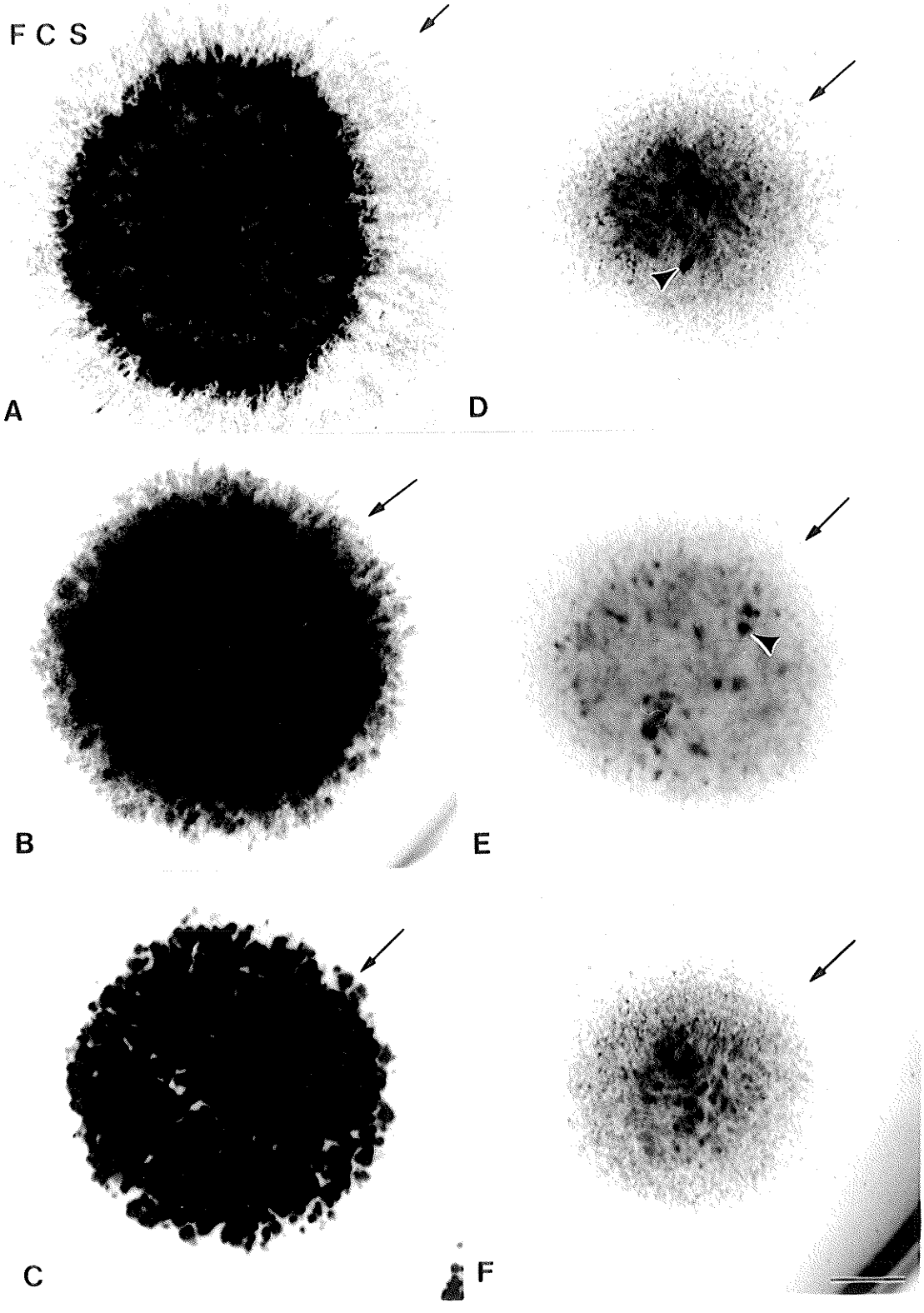

F C S
A
D
B
E
C
F

Figure 2.5 Micromass cultures grown in defined media containing two concentrations of bFGF (1 ng/ml and 10 ng/ml bFGF), stained with Alcian blue after 96 hours of culture.

The outer edge of each culture is indicated by an arrow. The scale bar represents 1 mm for all figures.

A. Lateral nasal process cells grown in defined medium containing 1 ng/ml bFGF. The pattern is similar to that of defined media cultures. This culture was well attached in contrast to cultures grown in higher concentrations of bFGF, and is not representative of lateral nasal process cultures grown in 1 ng/ml bFGF which had a propensity for detachment.

B. Frontonasal mass cells grown in defined medium containing 1 ng/ml bFGF. The cartilage matrix pattern is similar to that in defined media cultures with a dense, lacy pattern.

C. Mandible cells cultured in defined medium containing 1 ng/ml bFGF. Initially, coalesced nodules formed in the central area. With time, the peripheral fringe detached resulting in small, poorly developed cultures.

D. Lateral nasal process cells cultured in defined medium containing 10 ng/ml bFGF. A dense, central sheet of cartilage was formed and the fringe of the culture detached (detachment is indicated with an open arrow). The culture appeared to be multi-layered and reduced in size.

E. Frontonasal mass cells grown in defined medium containing 10 ng/ml bFGF. The cartilage matrix is dense and covers most of the culture.

F. Mandible cells grown in defined medium containing 10 ng/ml bFGF. The nodular pattern is similar to cultures grown in 1 ng/ml bFGF. There is poor attachment to the culture plate.

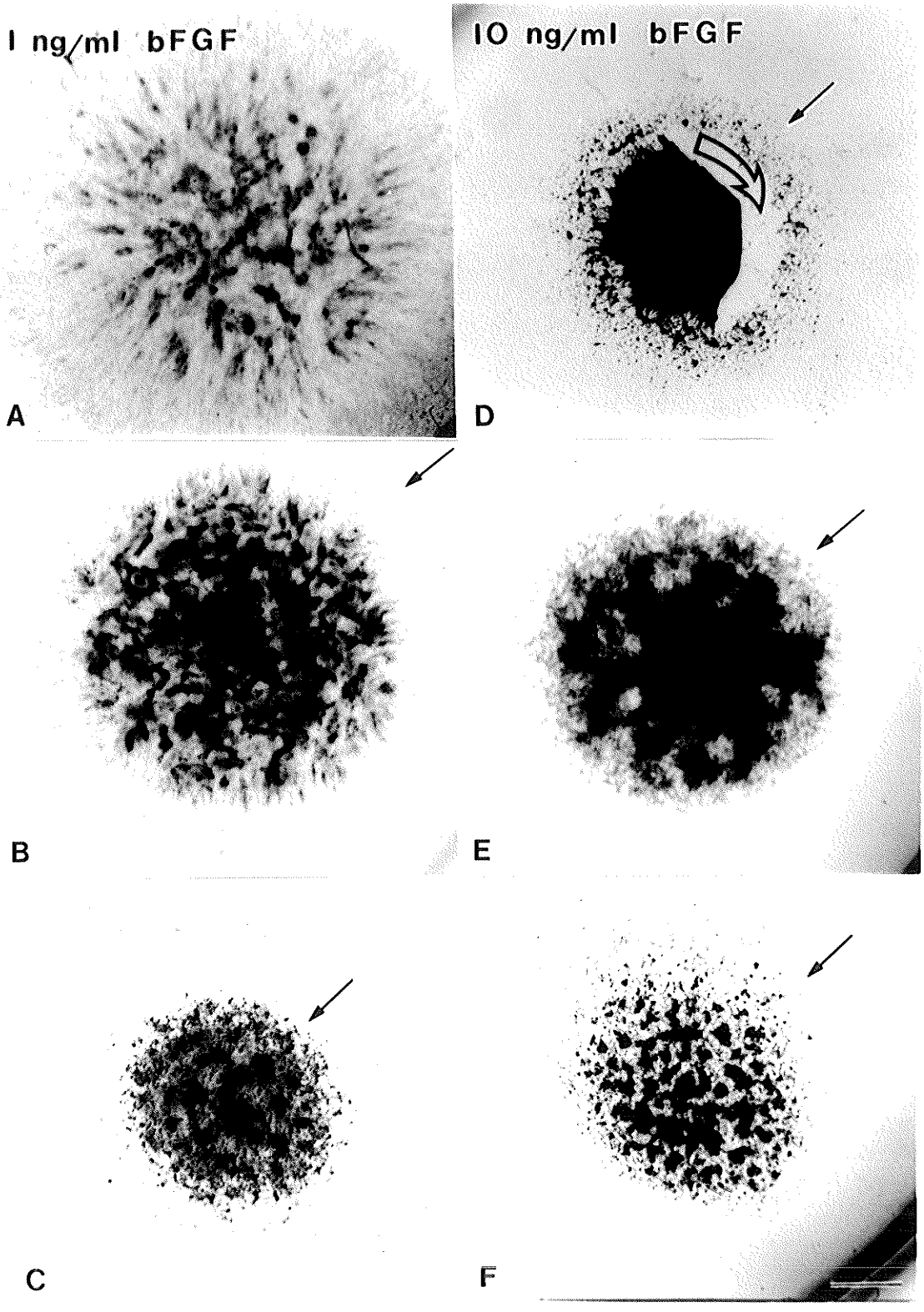

1 ng/ml bFGF
10 ng/ml bFGF
A
B
C
D
E
F

Figure 2.6 Micromass cultures grown in defined medium containing 100 ng/ml bFGF, stained with Alcian blue after 96 hours of culture.

The outer edge of each culture is indicated by a thin arrow. Areas of detachment are indicated with an open arrow. Nodules are indicated with arrow heads. The scale bar represents 1 mm for all figures.

A. Lateral nasal process cells grown in defined medium containing 100 ng/ml bFGF. The central area contains coalesced nodules of cartilage. These aggregations appear to be due to multi-layering of the chondrocytes. The periphery of the culture is detaching.

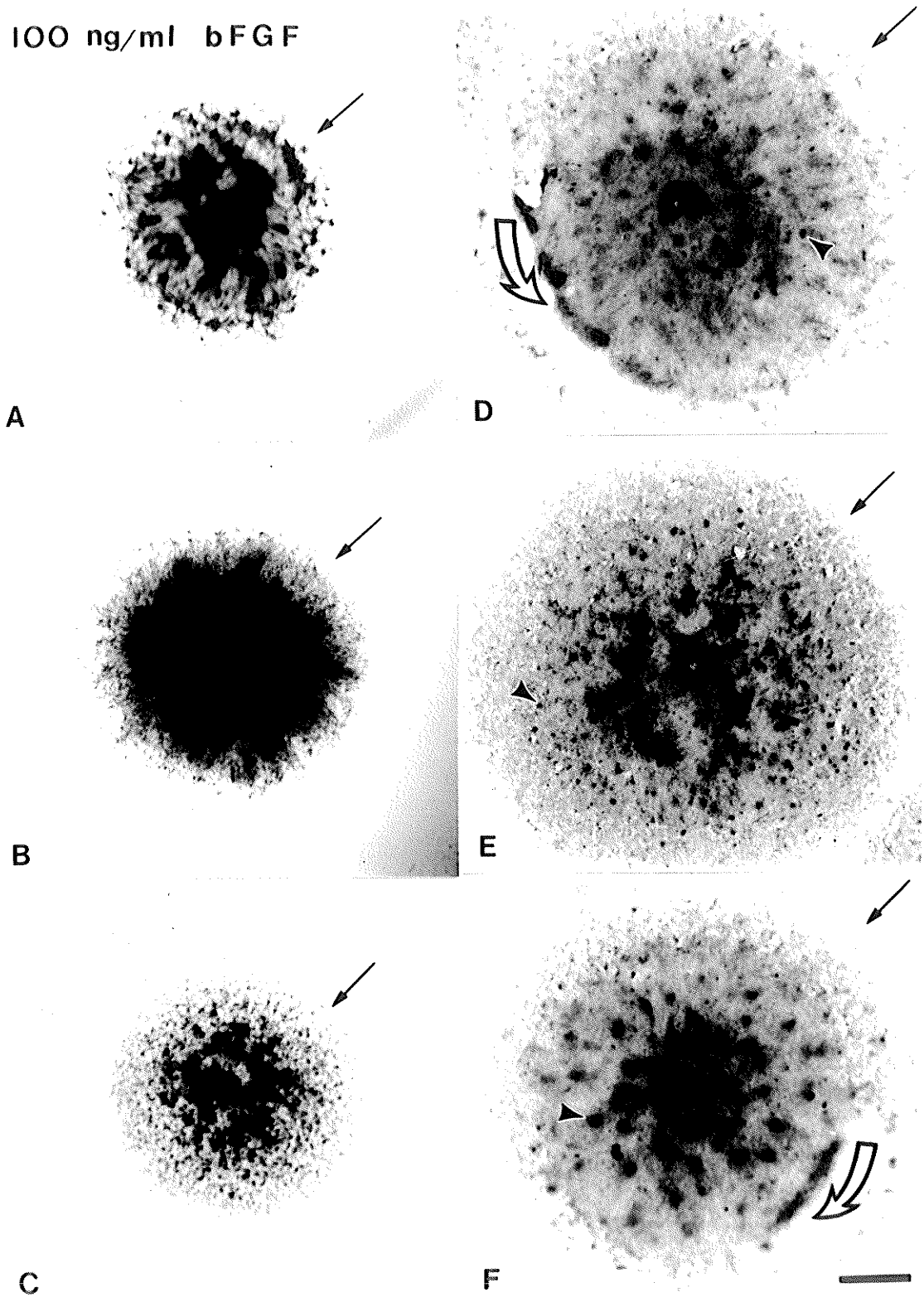
B. Frontonasal mass cells cultured in defined medium containing 100 ng/ml bFGF. A central sheet of cartilage has formed.

C. Mandible cells grown in defined medium containing 100 ng/ml bFGF. Cartilage nodules have formed in the centre of this poorly attached culture.

D. Cells from the whole maxilla cultured in defined medium containing 100 ng/ml bFGF. Tiny nodules that stain with Alcian blue are observed although the entire culture has a lot of non-specific staining. Some detachment of the culture is visible.

E. Upper maxilla cells grown in defined medium containing 100 ng/ml bFGF. A large number of tiny nodules have formed throughout the culture. This culture is well attached.

F. Lower maxilla cells grown in defined medium containing 100 ng/ml bFGF. Large nodules are visible and these are concentrated in the centre of the culture.


100 ng/ml bFGF
A
B
C
D
E
F

CHAPTER III

EXPRESSION OF bFGF PROTEIN IN THE CHICK FACE

While the effects of exogenous bFGF on facial mesenchyme are interesting, the relationship to face development *in vivo* is not necessarily direct. In this chapter I address the matter of endogenous bFGF in the embryonic chick face. Three main questions will be asked: a) is endogenous bFGF present in the face; b) is bFGF concentrated in certain regions of the face; and c) is bFGF uniformly expressed at all stages of development.

I. Endogenous bFGF in the embryo

A number of methods have been used to identify bFGF in embryonic tissues such as chick limb buds and *Xenopus* embryos (Munaim *et al*, 1988; Kimelman and Kirschner, 1987). These methods include protein purification from homogenized tissue using heparin-affinity chromatography. This method does not absolutely confirm the identity of the protein as usually the protein sequence is not obtained. Another more specific method is to use Western Blotting. The protein is purified from tissue, run on a gel, and blotted. An antibody is then used to identify the band that contains bFGF. Size and quantity of the protein can be determined using this method. Another method for detecting bFGF in tissues uses immunocytochemistry. The great advantage is that the distribution of the protein at the tissue and cellular level can be described. Correlations can then be made between distribution and role in development. The disadvantage of immunocytochemistry is that not all antigenic sites are exposed in tissue sections and many antibodies will not recognize the one or two exposed sites. In addition, quantitative assesments of the amount of staining are difficult to make.

I have chosen to look at bFGF distribution in the face with immunocytochemistry. The presence of endogenous bFGF has been confirmed in variable amounts in all tissues or cells examined to date (Joseph-Silverstein *et al.*, 1989; Kalcheim and Neufeld, 1990; Anderson *et al., 1991; Gonzalez* et al., *1990; Kardami and Fandrich, 1989)* although no studies have specifically looked for bFGF in the face. Since this method has not been completely worked out, one of the aims of this chapter is to develop the conditions for studying bFGF protein expression in embryonic facial tissue.

II. Selection of antibodies

Antibodies are produced by two methods. Monoclonal antibodies can be made using a cloned line of cells that produce a single immunoglobulin. These antibodies have a single specificity and one level of binding affinity. I used polyclonal antibodies, made by a second technique in which an animal is injected with a foreign antigen and then the resulting antibodies are collected and isolated from the plasma.The antigen is injected into rabbits which stimulates a variety of lymphocytes to produce a number of clones of antibody (Larsson, 1988).

Unlike monoclonal antibody sera, polyclonal antisera are never identical even though the immunogen is the same. The antisera can contain antibodies which were present before immunization, or antibodies which develop against impurities in the antigen preparation (Ormerod and Imrie, 1989). Although each animal immunized produces different strains of antibodies, this can be an advantage when sera containing an antibody of particularly high affinity binding and high specificity is produced.

When making polyclonal antibodies, the bFGF antigen injected must be very different from the animals' innate bFGF or an antibody against the bFGF antigen cannot be produced without causing an auto-immune response within the animal. One approach is to use only part of the bFGF protein and conjugate it to a more highly antigenic protein. Kardami and Fandrich (1989) created good immune responses to bFGF conjugated with Keyhole Limpet haemocyanin. Not only does this approach lead to greater antigenicity but it also allows antibodies to be made against specific parts of the protein rather than to the whole protein. There are regions of homology between members of the HBGF family so antibodies to whole bFGF could cross react with other HBGF's. The antibodies used in this study were created by conjugating the first 24 amino acids of bFGF to Keyhole Limpet haemocyanin (provided by Dr. E. Kardami).

III. Preservation of antigens in tissue sections

bFGF is very sensitive to proteolysis and heat, making it a difficult protein to work with, as immunocytochemical work requires that tissues be fixed prior to staining. The objective of fixing the tissue is to preserve the antigenicity while preserving the structure of the tissue and allowing the tissue to be permeable to the antibodies. Most antigens are soluble in the buffers used to dilute antibodies and to rinse tissue sections. As such, they must be fixed in the tissues with fixatives such as formaldehyde, gluteraldehyde, ethanol, acetone or methanol. If the fixative destroys the antigen or makes it inaccessible to staining, a more gentle fixative such as paraformaldehyde may be used. Occasionally, the staining must be done without prior fixation. However, this technique tends to result in tissue sections with poor morphology as the structure is not

preserved. Embedding procedures can also damage delicate tissues such as early embryonic tissue. bFGF is a difficult antigen to preserve and my goal in this set of experiments was to define optimal conditions for staining facial mesenchyme.

IV. Choosing a detection system for localizing bound antibody

There are a number of methods used to detect antigens in tissue sections (Lacey, 1989; Larsson,1988). In the direct method, labelled antibody is applied directly to the sections to bind to the antigen. The label makes the antibody visible, thereby allowing the bound antigen to be located. These labels may be fluorescent, gold with silver enhancement, biotinylated or radiographic (Ormerod and Imrie, 1989).

I chose to use the indirect method, which uses an unlabelled primary antibody and a labelled secondary antibody, which is then used to detect the primary antibody. The indirect method is more sensitive than direct methods because more than one secondary antibody molecule can react with each molecule of primary antibody. Another advantage is that only one preparation of labelled secondary antibody is required for a series of primary antibodies. In this study, a series of primary antibodies (S1, S2, S3, S4) raised in rabbits were used with a single secondary antibody (anti-rabbit antibody).

In order to further amplify the signal, I used a biotin - avidin system in which biotin is conjugated to a second antibody and to flourescein labelled streptavidin. The four binding sites of the labelled avidin have a high affinity for biotin, which increases the visibility of the bound antibody.

The distinct advantage of using a fluorescent label over visible coloured detection systems is its sensitivity. The bright fluorescent staining stands out clearly against the

dark background while in coloured detection systems the coloured reaction products are often not much different than background staining of the tissue. A disadvantage of immunofluorescent staining is that it fades with time while coloured stains are permanent.

MATERIALS AND METHODS

I. Incubating and staging embryos

Fertile White Leghorn chicken eggs were placed in an incubator at specified intervals to produce embryos at stages 15, 20, 24, and 28 (Hamburger and Hamilton, 1951). The embryos were staged as described in Chapter II.

II. Preparation of tissues for sectioning

Heads were dissected in cold phosphate buffered saline (PBS, 2 gm/l potassium chloride, 2 gm/l potassium phosphate, 80 gm/l sodium chloride, 17 gm/l sodium phosphate dibasic hepta hydrate). To prepare the tissue for frozen sections, one of the following two protocols was used (Fig. 3.1): 1) the heads were fixed overnight in fresh 4% paraformaldehyde in PBS at 4° C and then rinsed in PBS and sunk in 30% sucrose in PBS at 4° C or; 2) not fixed but placed immediately in 30% sucrose in PBS at 4° C for 2 hours. In both cases, the heads were then embedded in cold OCT compound (Lab-Tek Products) and either frozen in ethanol with dry ice and stored at -20 °C, or snap frozen in liquid nitrogen and stored at -70° C. Storage time prior to sectioning was usually overnight with the exception of two experiments where embedded heads were stored for three days and twenty days respectively.

Heads prepared for wax sections (Fig. 3.1) were fixed overnight in either fresh 4% paraformaldehyde in PBS at 4° C or 95% EtOH at 4° C, rinsed in PBS for 30 minutes, and then dehydrated in a series of ethanols: 50/50 EtOH/PBS for 15 minutes; 2 changes of 70% EtOH for 15 minutes each; 90% EtOH for 30 minutes; 2 changes of 100% EtOH for 30 minutes each; and then cleared with 2 changes of 100% Toluene for

30 minutes each. The specimens were carried through 3 changes of filtered, low melting point paraffin wax at 60° C for 20 minutes each, and then transferred to molds. The paraffin blocks were stored overnight at 4° C until sectioned.

III. Preparation of slides

Glass slides were prepared in four ways: 1) acid-cleaned by dipping in 10% HCL in 70% EtOH for 10 seconds, double distilled water for 10 seconds, 95% EtOH for 10 seconds, and then placed in racks and dried in a 150° C oven for 1 hour; 2) acid-cleaned and coated by dipping in a 2% solution of 3-aminopropyltriethoxysilane (TESPA, Sigma) in acetone for 10 seconds, 2 changes of 100% acetone for 10 seconds, double distilled water for 10 seconds, and baked dry at 42° C for 1-1.5 hours; 3) acid-cleaned and coated by dipping in 0.6% gelatin and air dried overnight; 4) acid-cleaned, TESPA coated, dipped in 1% gelatin to apply a second coating, and air dried overnight.

IV. Sectioning and preparation for staining

Paraffin embedded embryos were sectioned at a thickness of 5 μm, floated on slides, and left on a 40° C hot plate overnight to attach to the glass slides (see flow diagram, Fig. 3.1). Serial sections of stage 20 and 24 heads were arranged on each slide. In order to directly compare the effect of fixation on staining, heads fixed in paraformaldehyde and alcohol were placed on each slide.

Paraffin was cleared from the sections by 2 changes of 100% xylene for 2 minutes each and two changes of 100% EtOH for 5 minutes each. At this point each section was isolated by a circle drawn around it with a Dakopen pen (Dakopatts, Dimension Labs) to limit the amount of antibody required to incubate each section. The sections were then

rehydrated by immersing them for 2 minutes each in 95% EtOH, 70% EtOH, 50% EtOH, and rinsed with 3 changes of PBS for 2 minutes each.

Frozen sections were cut at a thickness of 10 μm, placed on prepared slides, and stored on ice in a hydrated container. Serial sections from one developmental stage were arranged on each slide. The sections were then circled with the Dakopen pen. At this point one of four procedures was employed: 1) the sections were stained; 2) the sections were rinsed 3 times in PBS for 5 minutes each and then stained; 3) the sections previously fixed were refixed by immersing in 1% paraformaldehyde for 10 minutes, rinsed and stained; 4) the sections not fixed prior to embedding were fixed in 1% paraformaldehyde for 10 minutes, rinsed and stained.

V. Immunocytochemical Staining

All of the sections were stained in an identical manner (Fig. 3.2). The primary bFGF antibodies obtained from E. Kardami (S1, S2, S3, S4) were raised against a synthetic peptide containing the first 24 amino acids of the 146 amino acid form of bFGF derived from bovine brain conjugated to keyhole limpet hemocyanin. Whole serum was used to incubate the sections. The polyclonal antibodies were not affinity purified prior to use. The primary bFGF antibodies and pre-immune sera were diluted 1:1000 with 1% BSA and 0.01% sodium azide in PBS. Drops (10-20 μl) of primary antibody or pre-immune were placed on each section and incubated at 4° C overnight in hydrated containers. They were then rinsed 3 times in PBS for 5 minutes. Slides were incubated for 1 hour at room temperature in the secondary antibody, biotinylated anti-rabbit IgG (Amersham), diluted 1:20 with BSA/sodium azide/PBS. Slides were then rinsed 3 times

in PBS for 5 minutes each and subsequently incubated in Streptavidin-Fluorescein (Amersham) diluted 1:20 in BSA/ Sodium azide/ PBS for 20 minutes at room temperature. The slides were stained for 10 seconds in 10 μg/ml Hoechst dye 33342 (Calbiochem-Behring Corp.) for 10 seconds. Slides were then rinsed 6 times in PBS for 5 minutes each, dried, sections circled with a diamond pen on the back of the slide and then coverslipped. The mountant consisted of a mixture of glycerol-PBS (9:1) containing 1 mg/ml diparaphenoldiamine (DPDA, Sigma). Coverslips were sealed with clear nail polish and then stored in light proof containers at 4° C.

Sections were examined with either a Zeiss or Nikon photomicroscope equipped with a fluorescence attachment. Photographs were taken with colour slide film (Kodak Ektachrome ASA 400) and black and white print film (HP5 Ilford ASA 400). Dark field photographs were taken on a Leica microscope with black and white print film (FP4 Ilford ASA 125).

RESULTS

In order to establish conditions for optimal staining with bFGF antibodies, a number of variations in experimental conditions were tested. The results of various coating, fixation, and embedding methods are reported as well as the staining pattern in the face at four distinct developmental stages. A total of 46 embryos were successfully sectioned and stained including 8 stage 15, 12 stage 20, 17 stage 24, and 9 stage 28 embryos.

I. Optimizing the integrity of tissue sections

Retention of the sections to slides was optimized by testing several methods of fixation as well as several coatings for the glass slides. The most successful coating was a combination of TESPA and gelatin. This regimen retained the sections on the slides for the entire staining procedure. Fixation by paraformaldehyde prior to sectioning also improved adherence.

The embedding method also affected the success of sections sticking to the slides, with wax sections being superior to frozen. Frozen sections were difficult to retain on the slides intact, especially when the tissue was snap frozen in liquid nitrogen. This method of freezing tissues lead to brittleness and subsequently the blocks fractured on sectioning. A more gradual freezing process in dry ice/ethanol produced much better sections.

Fixing the slides after sectioning appeared to improve tissue adherence to the slides. This technique was used for a small sample of slides (n=4) with tissue that were

fixed in 4% paraformaldehyde overnight, prior to sectioning. Refixing did not change the staining patterns but appeared to improve the adherence to the slides.

II. Effects of tissue preservation on staining pattern

While tissue preservation is central to evaluation of the staining, it is also very important to preserve antigenicity of the bFGF protein. Wax sections adhered very well, but the heat of embedding introduced artifacts in the staining pattern. S1 antibodies stained the mesenchyme more intensely than the epithelium in wax sections (Fig. 3.3 A). In contrast, frozen tissues not fixed prior to sectioning had brighter staining in the epithelium (Fig. 3.3 B). The sections were viewed on the microscope using phase contrast to ensure that differences in stain intensity were due to expression of antibody and not increased cell concentration in either epithelium or mesenchyme (not shown). The increased staining in the epithelium is not due to increased cell density of this tissue layer. When the same antibody was applied to frozen sections, which had been fixed in 4% paraformaldehyde overnight, the epithelium and mesenchyme were stained equally (not shown). Therefore, staining of the epithelium is lost upon fixation. Also, wax sections had extracellular staining in addition to intracellular staining. In contrast, frozen sections appeared to have only intracellular staining.

Wax sections were also incubated in S2 antibodies and pre-immune serum (not shown). S2 staining was much less intense than S1. Pre-immune serum slides exhibited a dark orange background stain. Both S2 and pre-immune serum stained the epithelium and mesenchyme evenly. In wax sections, the method of tissue fixation (either 4% paraformaldehyde or 95% ethanol) did not affect the staining pattern.

Another effect of fixation on staining pattern became apparent at high magnification. In unfixed tissue, the nucleoli did not stain resulting in dark intracellular areas being visible, even at lower power magnification (Fig. 3.4 A). I did not confirm the nuclear character of these dark areas with Hoechst staining as the Zeiss microscope was not equipped for this wavelength. Cells which were fixed in paraformaldehyde overnight, had homogeneous staining throughout the cell with multiple bright punctate stained areas (Fig. 3.4 B). Since this pattern was observed in all sections incubated in primary antibodies and pre-immune serum, the punctate stains are an artifact of fixation.

The method of preservation had profound effects on the level of background or non-specific fluorescence. Wax sections had the highest level of non-specific staining in pre-immune sections while frozen, non-fixed sections had the lowest. The best conditions for adherence of sections, preservation of antigenicity, and low level background staining were a) fixation in 4% paraformaldehyde over night, b) freezing the next day, c) placing sections on TESPA/gelatin coated slides.

III. Staining pattern with different antibodies

The type of antibody used had an effect on the intensity of staining. The S1 antibody consistently had more intense staining than all other antibodies (Fig. 3.5 B,C). S2, S3, and S4 were all very similar in their ability to stain the tissue, with a moderately bright staining pattern visible in all sections (Fig. 3.5 E,F; Fig. 3.6 A,B,C,E,F,G). The sections stained with pre-immune (Fig. 3.7 B,D) did not display the specific green stain of sections incubated in primary antibodies (Fig. 3.7 A,G). The dark orange stain observed in pre-immune sections is background fluorescence.

IV. Staining pattern at different stages of development

There was no visibly discernable difference in the intensity or pattern of staining between stages (Fig. 3.8 A-L). Stage 15 embryos that were not fixed prior to sectioning were heavily damaged with sectioning, but tissue fragments stained the same as older stages that were similarly treated (see also Fig. 3.5).

V. Staining pattern in different facial primordia

Frozen tissues had homogeneous staining throughout the entire mesenchyme. There was no regional concentration of staining in any area of the face at all stages of development (Fig. 3.9 A-F; 3.10 A-G).

The wax sections produced a tantalizing artifact when stained with S1. There appeared to be more intense staining in the frontonasal mass, lateral nasal process, and the maxilla of stage 20 and 24 tissue sections (Fig. 3.11). However, this finding was not confirmed in tissues that were frozen in OCT compound.

DISCUSSION

I. Immunocytochemical techniques affect staining pattern

Preservation of the delicate tissues of early chick embryos to allow detection of endogenous bFGF resulted in trying various embedding and fixation methods. The immunocytochemical technique could be improved by: 1) reducing the concentration of paraformaldehyde; 2) reducing the fixation time to a few hours rather than overnight; 3) fixing sections on slides after sectioning. Despite the technical difficulties in retaining antigenicity with intact tissue sections, bFGF-like protein was detected throughout the embryos and specifically in all facial primordia from stage 15 to stage 28.

Staining patterns were observed which were specific to fixation and embedding techniques. When tissue was embedded in wax and stained with S1 antibody, the mesenchyme stained much more intensely than the epithelium. The reverse pattern was seen in frozen specimens which were not fixed prior to embedding. The lack of epithelial staining in wax sections could be attributed to loss of bFGF during processing. A study done by Kalcheim and Neufeld (1990) also used wax embedded tissues. Various mesoderm-derived structures such as the limb bud, mesenchyme dorsal to the neural tube, vertebral muscles and cartilage were stained. The surface ectoderm was not stained with their bFGF antibody, a result similar to my experiments. This artifact produced by the embedding method provides evidence that bFGF is bound with different affinities to different tissues. The binding of bFGF within the mesenchymal cell is potentially stronger than the binding of bFGF to epithelium. Mesenchymal bFGF is probably bound

to cell surface receptors (Heuer *et al.*,1990; Wanaka *et al.*, 1991) whereas epithelial bFGF is more likely to be associated with HSPG's.

II. Antibodies are specific for bFGF

The antibodies used in my experiments were raised in rabbits against a synthetic peptide containing residues 1-24 of the truncated, 146 amino acid, bovine brain bFGF conjugated to Keyhole limpet haemocyanin. The region of the amino-terminal of bFGF exhibits negligible homology with other members of the FGF family of growth factors. To show that the antibodies used (S1, S2, S3, and S4) do recognize bFGF, the antibodies could be pre-absorbed with sepharose-immobilized [1-24] bFGF before adding to tissue sections (Kardami, 1990). This treatment tests for non-specific fluorescence and should result in the loss of immunofluorescence pattern elicited by the antibodies.

The use of whole, unpurified bFGF antibody serum could account for the lack of specific, regional staining within the embryo. The polyclonal serum contains large amounts of albumin and other proteins which can interfere with antibody binding. To reduce non-specific staining, the serum could be used at a higher dilution ratio and/or the serum could be increasingly purified until a higher regional specificity was achieved.

III. Local action of bFGF in tissues can be modified by the environment.

bFGF *in vivo* is associated with the extracellular matrix. The basement membrane underlying the epithelia always contains bFGF (Gonzalez *et al.*, 1990). bFGF accumulates in extracellular matrix due to its high affinity for heparin (Moscatelli *et al.*, 1991). Data from this laboratory (Leon-Delgado, 1992) demonstrated specific regional concentrations of HSPG in condensing cartilage and in the basement membrane region

of the chick face. My data shows no correlation between the distribution of HSPG and bFGF. Rather, bFGF is expressed everywhere in the face and does not seem to be concentrated in regions that contain HSPG. One possible explanation for this lack of association of HSPG and bFGF is that my antibody cannot distinguish between short and long forms of bFGF.

Both low (16-19 kD) and high (over 20 kD) molecular weight forms of bFGF have been characterized (Brigstock *et al.*, 1990). Cytosol contains the shorter, 18 kD form of bFGF whereas nuclei contain 18, 22.5, and 24 kD forms of bFGF (Brigstock *et al.*, 1990). The expression of different forms of bFGF also varies with stage of development (Giordano *et al.*, 1992) and with the source of tissue (Sommer *et al.*, 1987). It is possible that one form of bFGF is found in the epithelium while another is found in the mesenchyme, or that the chondrogenic regions express one type of bFGF. The association of one form of bFGF with a particular tissue would suggest that each form fulfils a different function in development.

It is possible that bFGF is more concentrated in certain locations. A relative high concentration of bFGF in the frontonasal mass could reflect an autocrine effect of bFGF on frontonasal mass growth. In other words, bFGF is synthesized by the same group of cells that respond to this growth factor. My data did not show any increased concentration of bFGF, except in the epithelium. However, other antibody preparations might demonstrate regional concentrations elsewhere in the mesenchyme. Higher concentrations of bFGf are associated with differentiation, whereas lower concentration are known to stimulate proliferation (McAvoy and Chamberlain, 1989). Thus it is

possible that regions of condensing cartilage such as the prenasal cartilage in the frontonasal mass will show increased concentration of bFGF compared to the peripheral mesenchyme. Local variations in the concentration of bFGF could be very important to elongation of cartilage rods and the development of a protrusive beak.

bFGF activity may be modulated by other growth factors, such as TGFβ. Both bFGF and TGFβ are localized within the facial primordia. *In vivo* TGFβ is expressed in the maxilla of mice (Williams *et al.*, 1991). The close proximity of these two growth factors suggest they could interact with each other. Effects of bFGF on TFGβ gene expression have been demonstrated (Noda and Vogel, 1989). Moreover, bFGF and TGFβ both stimulate chondrogenesis in micromass culture and have an additive effect when present together in culture medium. Also, TGFβ overcomes the inhibition of myogenesis caused by bFGF (Schofield and Wolpert, 1990). Thus, interactions between bFGF and other growth factors or molecules found in the face are certainly possible.

Figure 3.1. Protocol for the preparation of tissue for immunocytochemical staining.

The embedding technique used, either frozen or wax, divided the tissues into two groups. Similar preparation headings for these two groups are highlighted in bold outlines for the embedding, sectioning, and staining procedures. The frozen tissues were fixed prior to embedding, or after sectioning. The staining procedure is depicted in a flow diagram (Fig. 3.2).

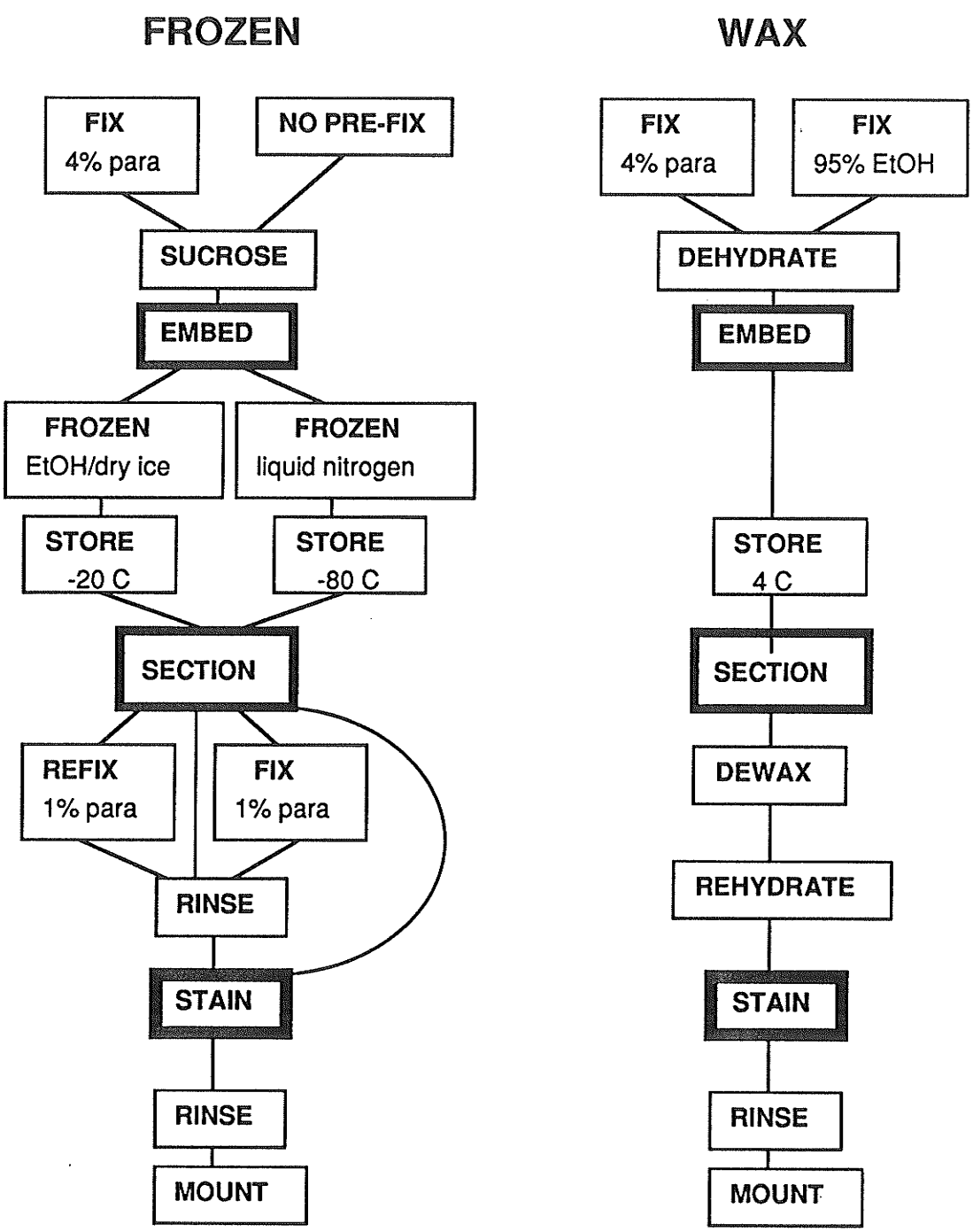

FROZEN
WAX
FIX
4% para
NO PRE-FIX
SUCROSE
EMBED
FROZEN
EtOH/dry ice
FROZEN
liquid nitrogen
STORE
-20 C
STORE
-80 C
SECTION
REFIX
1% para
FIX
1% para
RINSE
STAIN
RINSE
MOUNT
FIX
4% para
FIX
95% EtOH
DEHYDRATE
EMBED
STORE
4 C
SECTION
DEWAX
REHYDRATE
STAIN
RINSE
MOUNT

Figure 3.2 Immunocytochemical staining sequence.

All sections were stained in an identical manner. Tissues were initially incubated with a rabbit bFGF primary antibody (S1,S2,S3, or S4) or a negative control (pre-immune). The primary antibody was enhanced by incubating all slides with a secondary antibody, biotinylated anti-rabbit antibody, followed by incubation in streptavidin fluorescein. Hoechst stain is a nuclear-specific stain. The mounting media contained diparaphenoldiamine (DPDA) which reduces the loss of fluorescence.

STAINING

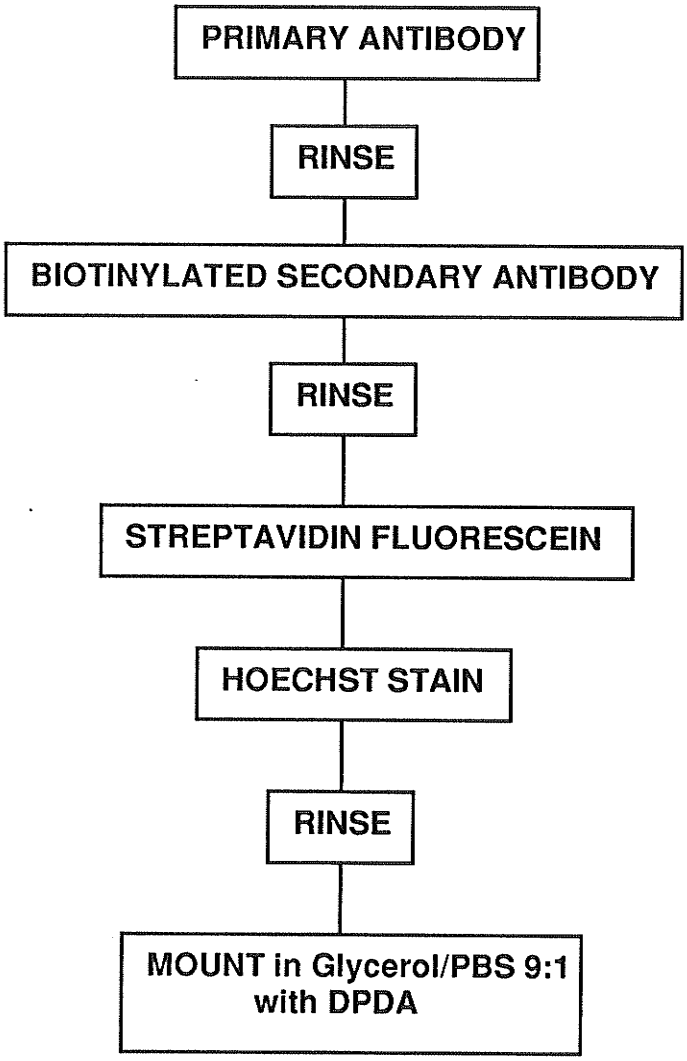

PRIMARY ANTIBODY
RINSE
BIOTINYLATED SECONDARY ANTIBODY
RINSE
STREPTAVIDIN FLUORESCEIN
HOECHST STAIN
RINSE
MOUNT in Glycerol/PBS 9:1
with DPDA

Figure 3.3 The effects of tissue preservation on staining pattern

Stage 24 chick embryos sectioned through lateral nasal process. Key: S1 = primary antibody S1, epi = epithelium, mes = mesenchyme. The scale bar represents 0.02 mm for both figures. Figures A and B viewed with FITC excitation.

A. The fixed tissue was embedded in low-melting temperature wax. The mesenchyme stains more intensely than the epithelium. There is extracellular staining as well as intracellular staining.

B. The non-fixed tissue was embedded in frozen OCT compound. The epithelium stains more intensely than the mesenchyme. Both sections were viewed with phase contrast and the staining pattern is not due to increased cell density in the tissue layer exhibiting the intense stain.

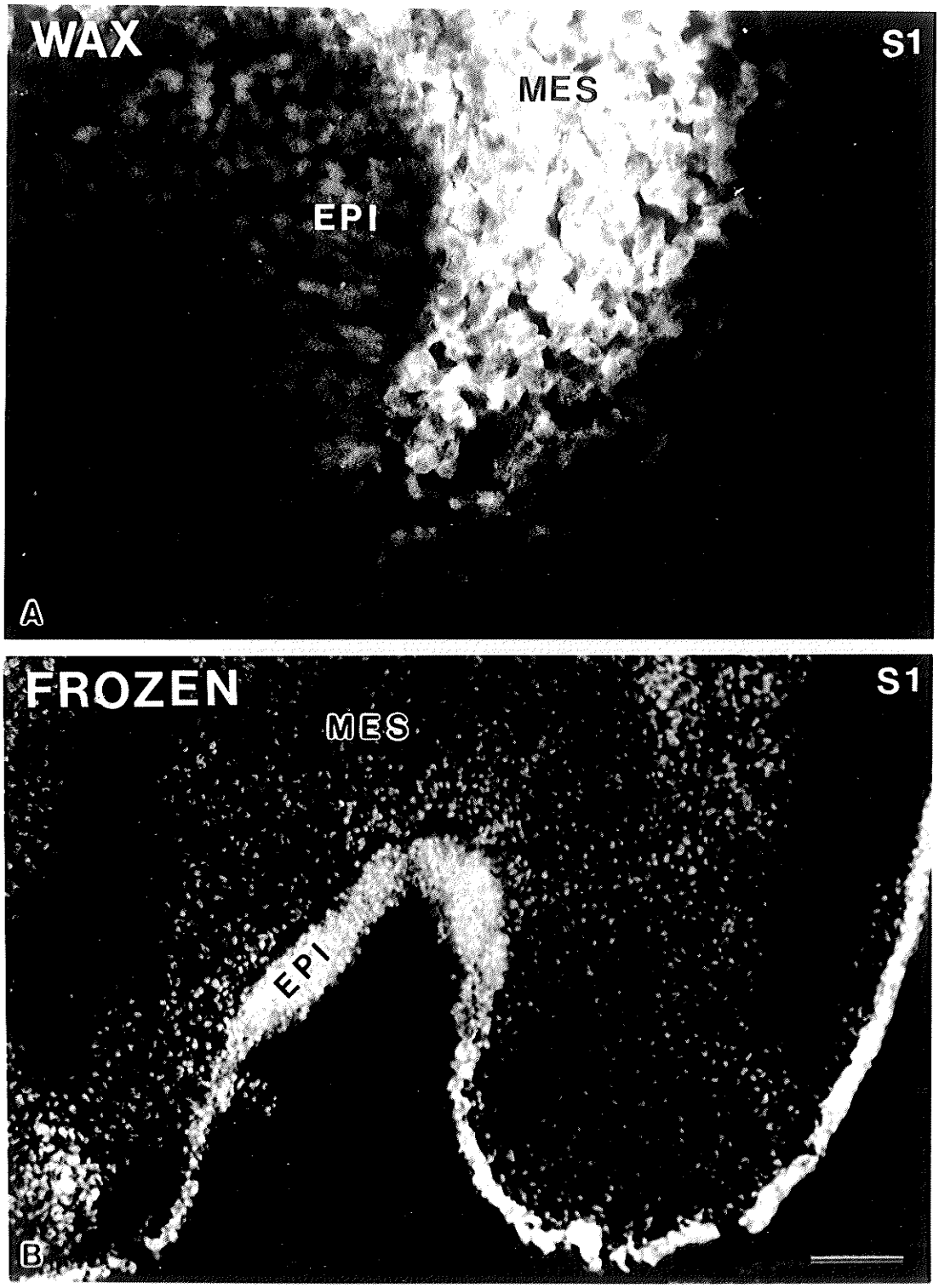

WAX
S1
MES
EPI
A
FROZEN
S1
MES
EPI
B

Figure 3.4 A high magnification view of effects of fixation on staining pattern.

These sections were prepared using frozen sections stained with S3 antibody. The scale bar represents 0.01 mm. Figures A and B viewed with FITC excitation.

A. The tissue used in this section was not fixed prior to embedding and sectioning. The arrowheads point to nucleoli which appear as dark, unstained intracellular areas.

B. The tissue used in this section was fixed in 4% paraformaldehyde overnight, prior to embedding and sectioning. The cells have homogeneous staining throughout with multiple, bright, punctate stained areas (indicated by arrowheads).

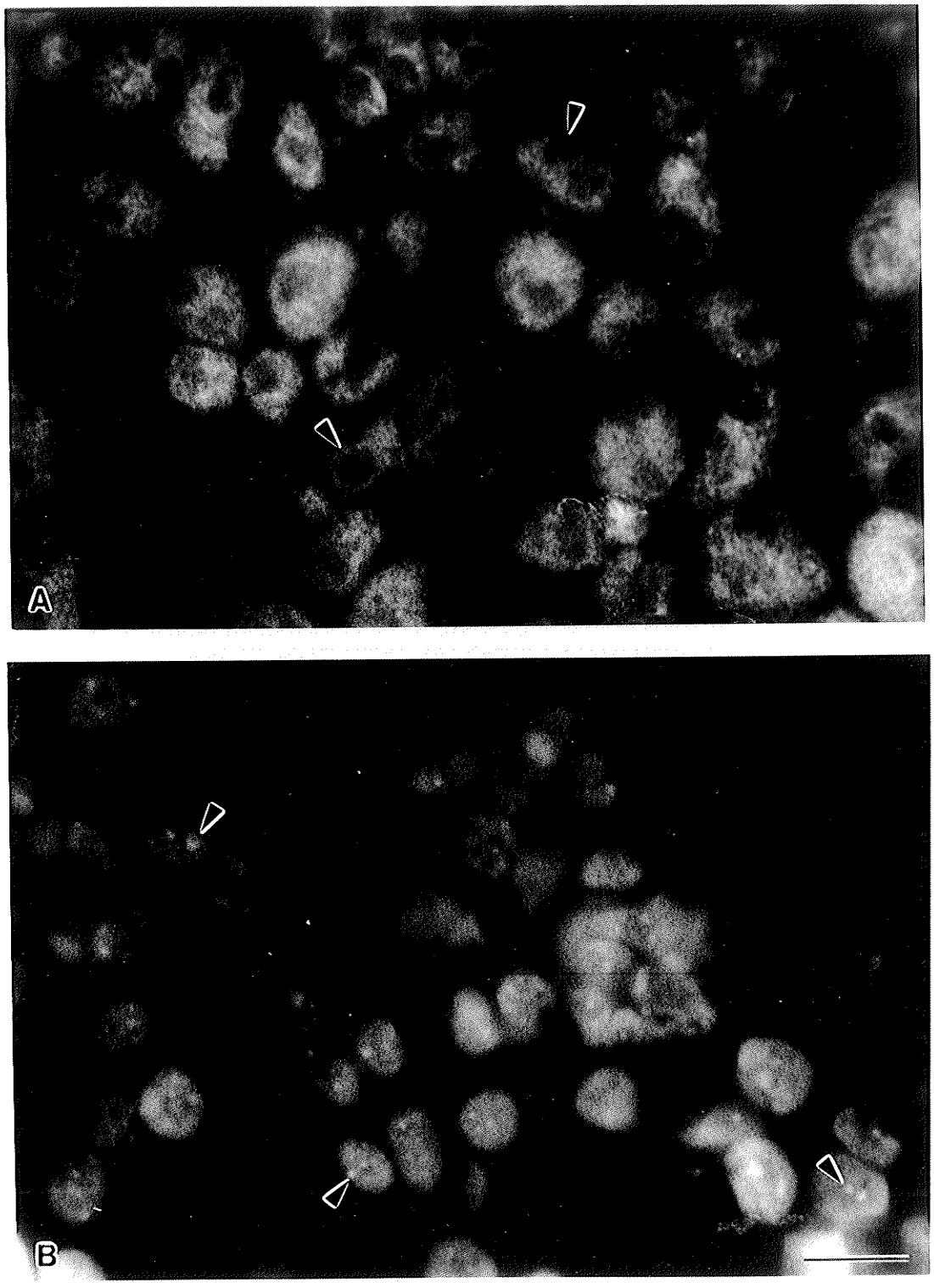

A
B

Figure 3.5 A stage 15 embryo, frozen and stained with different antibodies.

All photomicrographs of cells are from frontnasal mass. Key: e = eye, b = first branchial arch, fnm = frontonasal mass. The scale bar represents 0.5 mm in figure A, 0.1 mm in Figure B, E, and H, and 0.02 mm in figure C, D, F, G, I, and J.

A. Darkfield view of stage 15 embryo in sagittal section. Arrow head points to first branchial arch.

B, C, D. Serial photographs taken of the same section, stained with S1. Figures B and C, viewed with FITC excitation, show intense staining with S1 compared to S2. Figure D is a phase contrast view of Figure C.

E, F, G. Serial photographs taken of the same section, stained with S2. Figures E and F were viewed with FITC excitation. Figure G is a phase contrast view of Figure F.

H, I, J. Serial photographs taken of the same section, stained with pre-immune serum. Figures H and I, viewed with FITC excitation, show reduced staining compared to S1 and S2. The pre-immune sections had a dark orange stain compared to the bright green stain of primary antibodies. Figure I is a phase contrast view of Figure J.

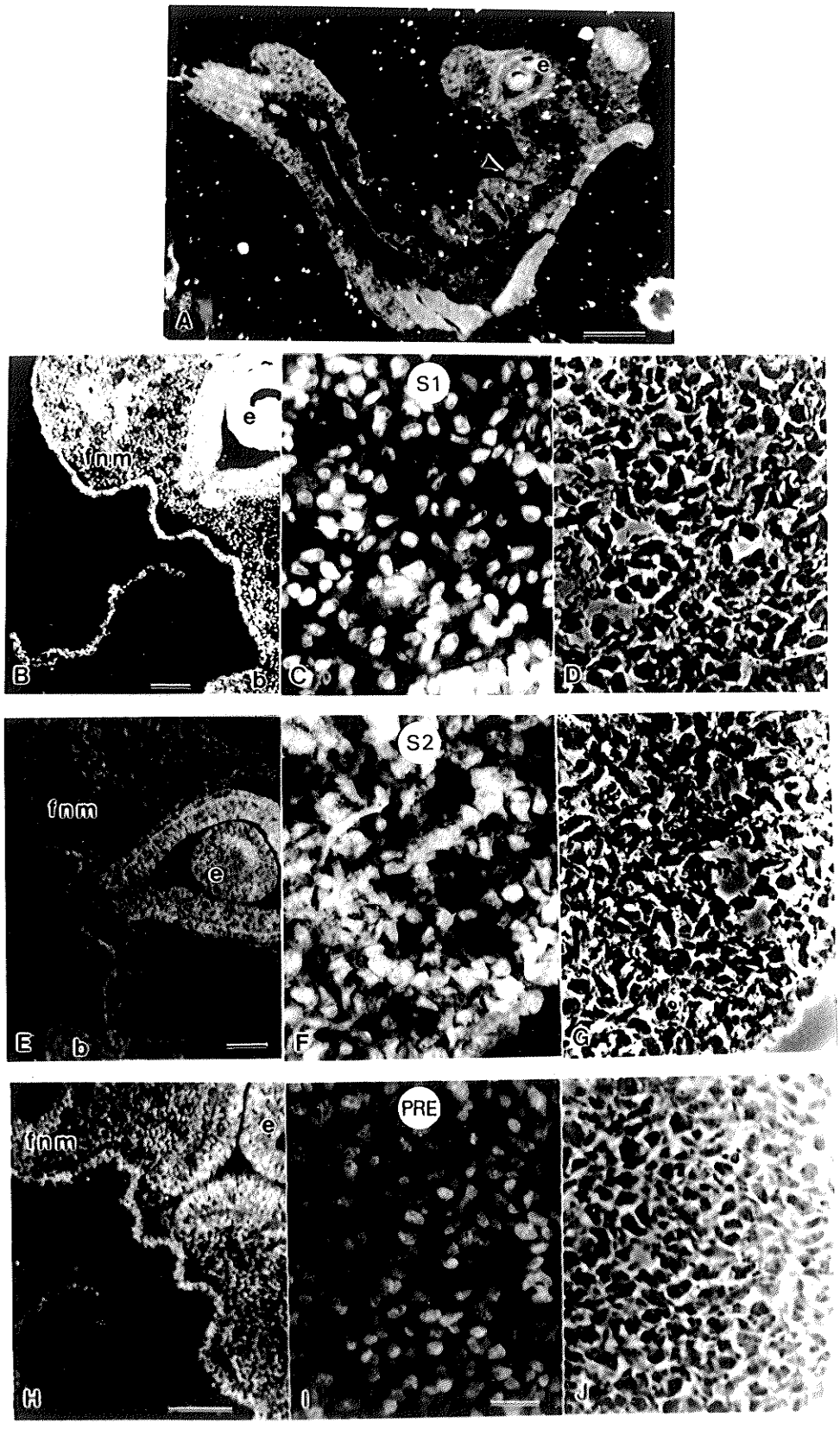

A
e
B
fnm
e
b
S1
C
D
fnm
e
E
b
S2
F
G
fnm
e
H
PRE
I
J

Figure 3.6 The staining pattern with different bFGF antibodies and two fixation methods.

Stage 24 embryos were embedded in frozen OCT compound. The scale bar represents 0.02 mm for all figures. All figures were viewed with FITC excitation. Figures A, B, C, and D were prefixed and figures E, F, G, and H were not fixed prior to sectioning.

A. Fixed, frozen tissue stained with S2 antibody.

B. Fixed, frozen tissue stained with S3 antibody.

C. Fixed, frozen tissue stained with S4 antibody.

D. Fixed, frozen tissue stained with pre-immune serum.

E. Non-fixed, frozen tissue stained with S2 antibody.

F. Non-fixed, frozen tissue stained with S3 antibody.

G. Non-fixed, frozen tissue stained with S4 antibody.

H. Non-fixed, frozen tissue stained with pre-immune serum.

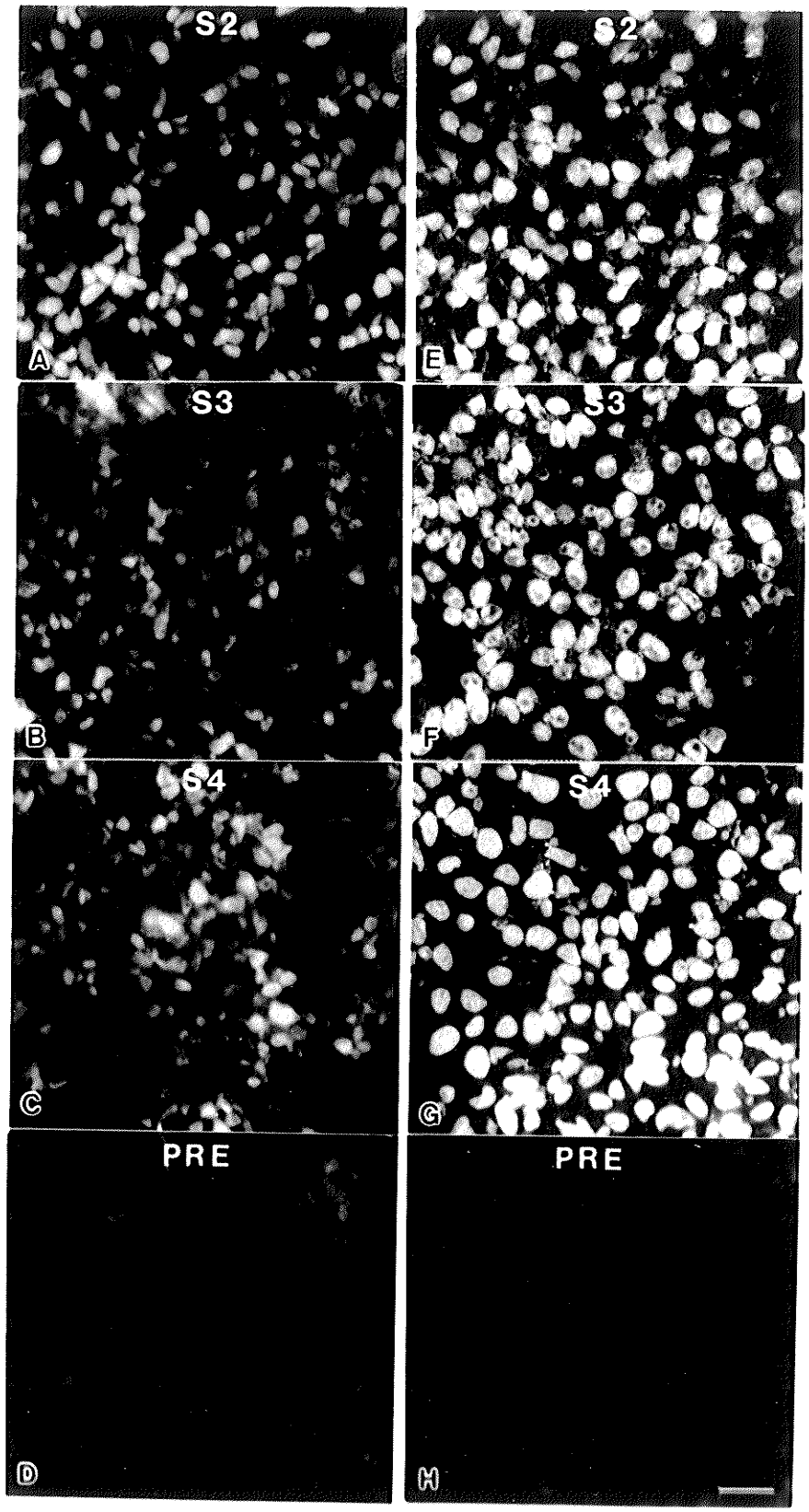

S2
A
S2
E
S3
B
S3
F
S4
C
S4
G
PRE
D
PRE
H

Figure 3.7 A colour plate of sections stained with immunofluorescent S1 antibody, or pre-immune serum.

All tissues were frozen by embedding in OCT compound.The scale bar represents 0.02 mm. All figures were viewed with FITC excitation.

A. This tissue was not fixed prior to sectioning and was stained with S1 antibody. The fluorescent staining is bright green against the dark background. Dark, non-stained nucleoli are within the cells, and are indicated with an arrow head.

B. This tissue was not fixed prior to sectioning and was stained with pre-immune serum. The photograph was taken with an automatic shutter speed which has over exposed the film. The cells have minimal staining.

C. This tissue was fixed in paraformaldehyde overnight and the cells have homogeneous stain throughout with some brighter punctate areas of stain within the cells.

D. This tissue was fixed prior to sectioning. The pre-immune serum was less bright on viewing, with a yellow - orange colour. The photograph has been overexposed by the automatic shutter setting.

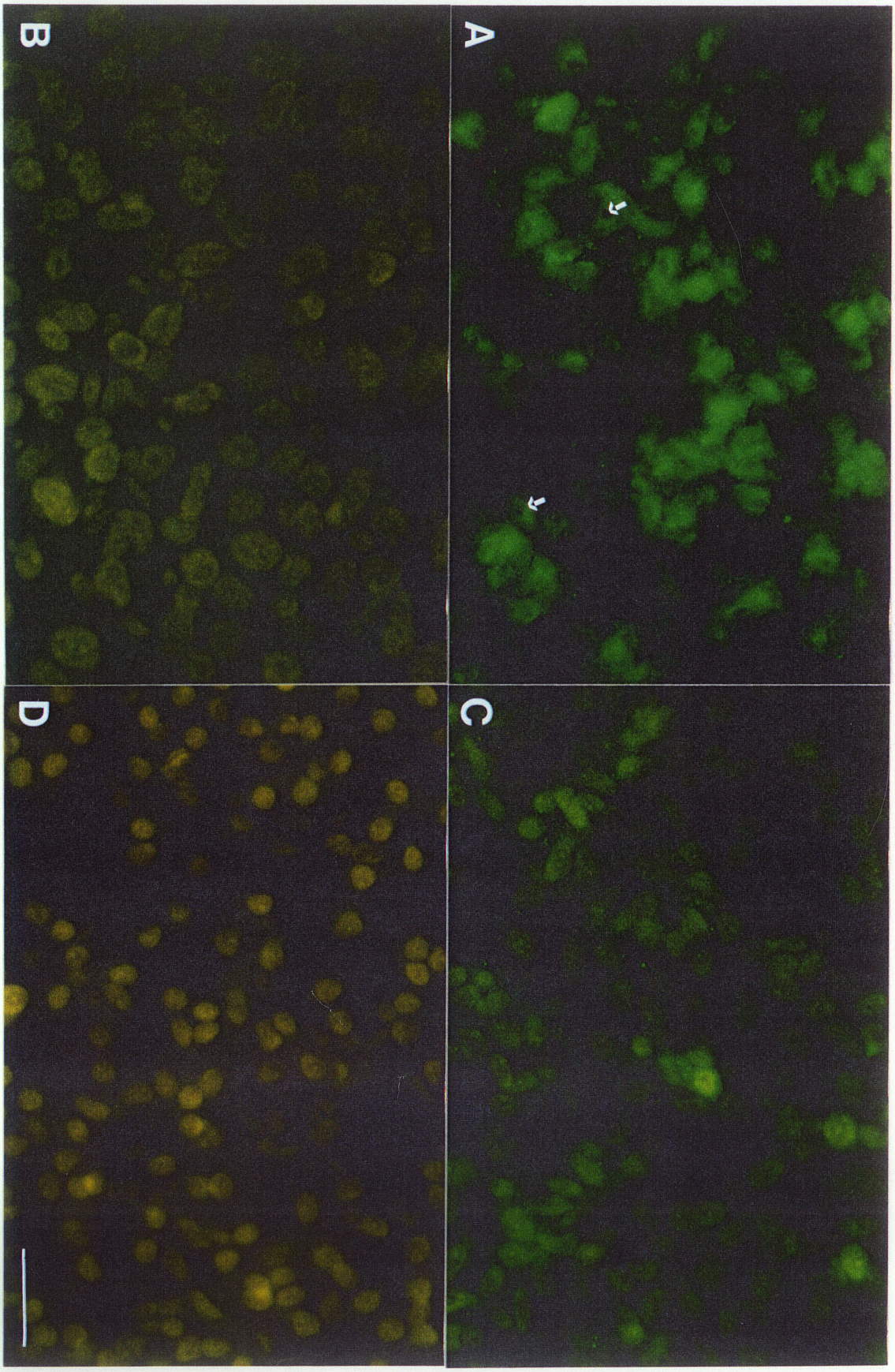

A
B
C
D

Figure 3.8 Embryos of different developmental stages stain equally.

All sections were stained with S3 antibody. All high magnification views of cells are from the frontonasal mass. Key: e = eye, b = first branchial arch, lnp = lateral nasal process, np = nasal pit, fnm = frontonasal mass, mx = maxilla. The scale bar represents 0.01 mm in figures A, D, G, J, and 0.02 mm in remaining figures.The left and middle columns are viewed with FITC excitation. The right hand column is viewed with phase optics.

A, B, C. Serial photographs of stage a 15 embryo. The white dashed lines indicate the area of figures B and C. Figure C is a phase contrast view of figure B.

D, E, F. Serial photographs of a stage 20 embryo. The white dashed lines indicate the area of figures E and F. Figure F is a phase contrast view of figure E.

G, H, I. Serial photographs of a stage 24 embryo. White dashed lines indicate the area of figures H and I. Figure I is a phase contrast view of figure H.

J, K, L. Figure J is a representative photograph of a stage 28 embryo. Figures K and L are high power views of cells in the frontonasal mass of an adjacent section. Figure L is a phase contrast view of figure K.

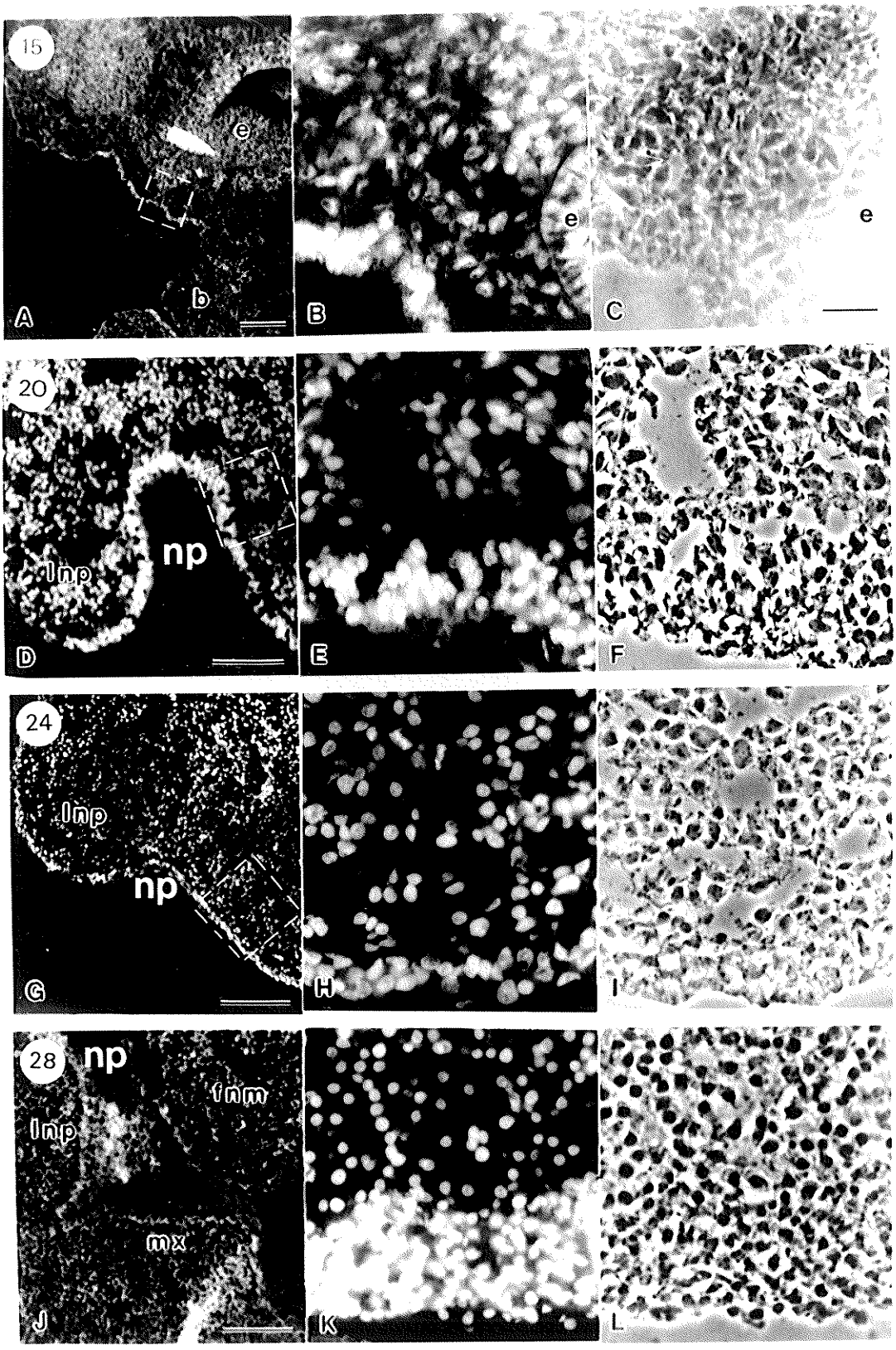

15
e
b
A
B
e
C
e
20
np
lnp
D
E
F
24
lnp
np
G
H
I
28
np
lnm
lnp
mx
J
K
L

Figure 3.9 A stage 24 embryo, not fixed prior to sectioning, stained with S1 antibody.

Key: FNM = frontonasal mass, LNP = lateral nasal process, np = nasal pit, MD = mandible, MX = maxilla. The scalebar represents 0.1 mm for figures A and B, and 0.02 mm for figures C, D, E, F. All figures viewed with FITC excitation.

A, B. The epithelium is more intensely stained than the mesenchyme. The tissue was torn on sectioning.

C, D, E, F. Cells from each facial primordia indicate even staining of each area in the face. The apparent increase in intensity in figures E and F is due to increased cell density.

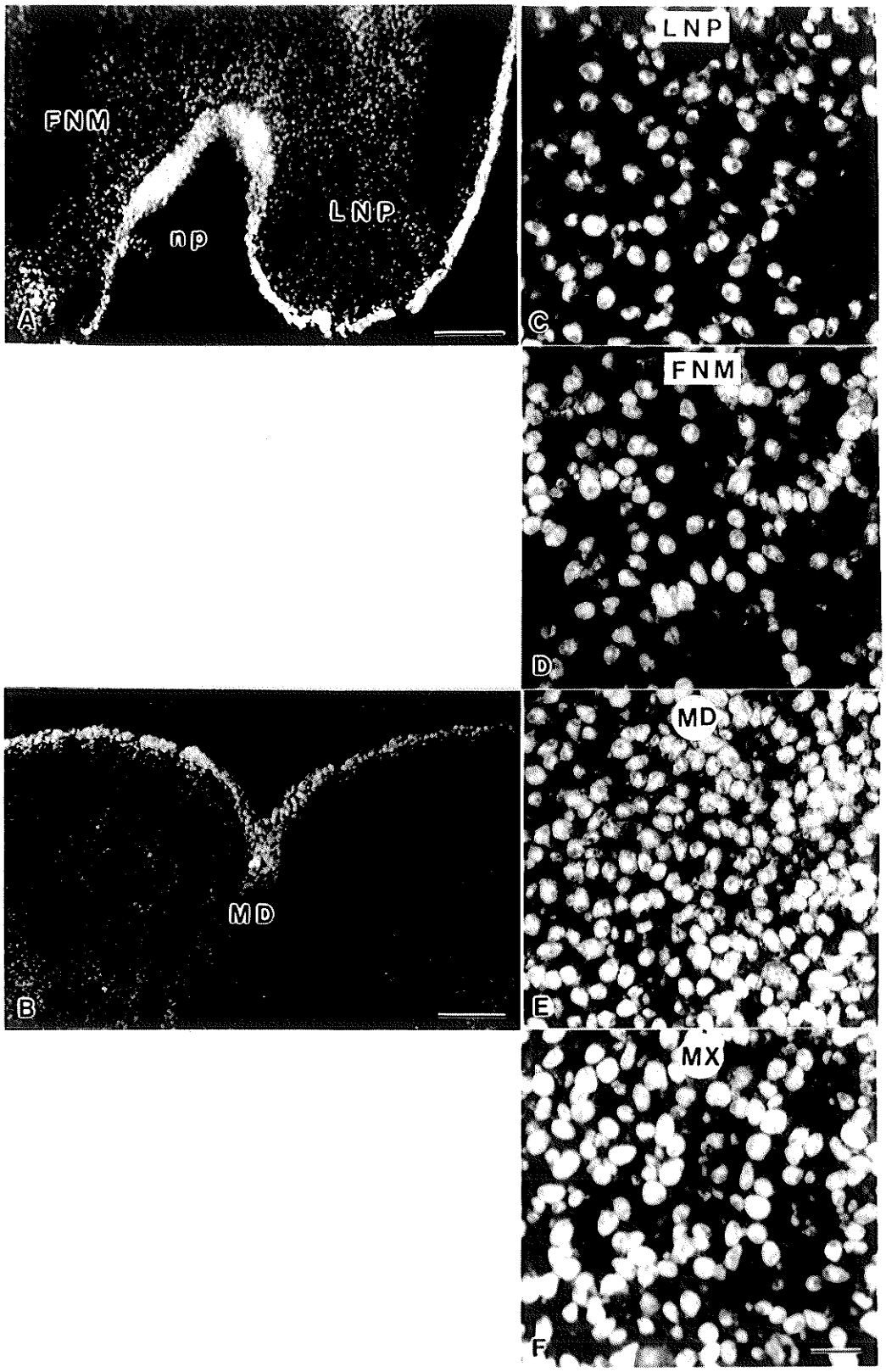

FNM
LNP
np
A
LNP
C
FNM
D
MD
B
MD
E
MX
F

Figure 3.10 A stage 28 embryo with even staining in all facial primordia.

The embryo used for these sections was fixed, frozen, and stained with S2. Key: np = nasal pit, fnm = frontonasal mass, lnp = lateral nasal process, mx = maxilla, md = mandible. The scale bar represents 0.5 mm for figure A, 0.1 mm for figures B and C, and 0.02 mm for figures D, E, F, and G. Figures B - G are viewed with FITC excitation. A is a dark field view to illustrate the integity of the tissue.

A. A dark field view of the embryo. White dashed lines indicate areas of higher power photographs for figures B and C (Figure B corresponds to #1 and Figure C corresponds to #2).

B, C. Higher power views of figure A indicate even staining of primordia with the S2 antibody.

D, E, F, G. Cells are from each facial primordia in stage 28 embryo. Figure F has an area of folded tissue which appeares to have more intense staining due to increased cell density.

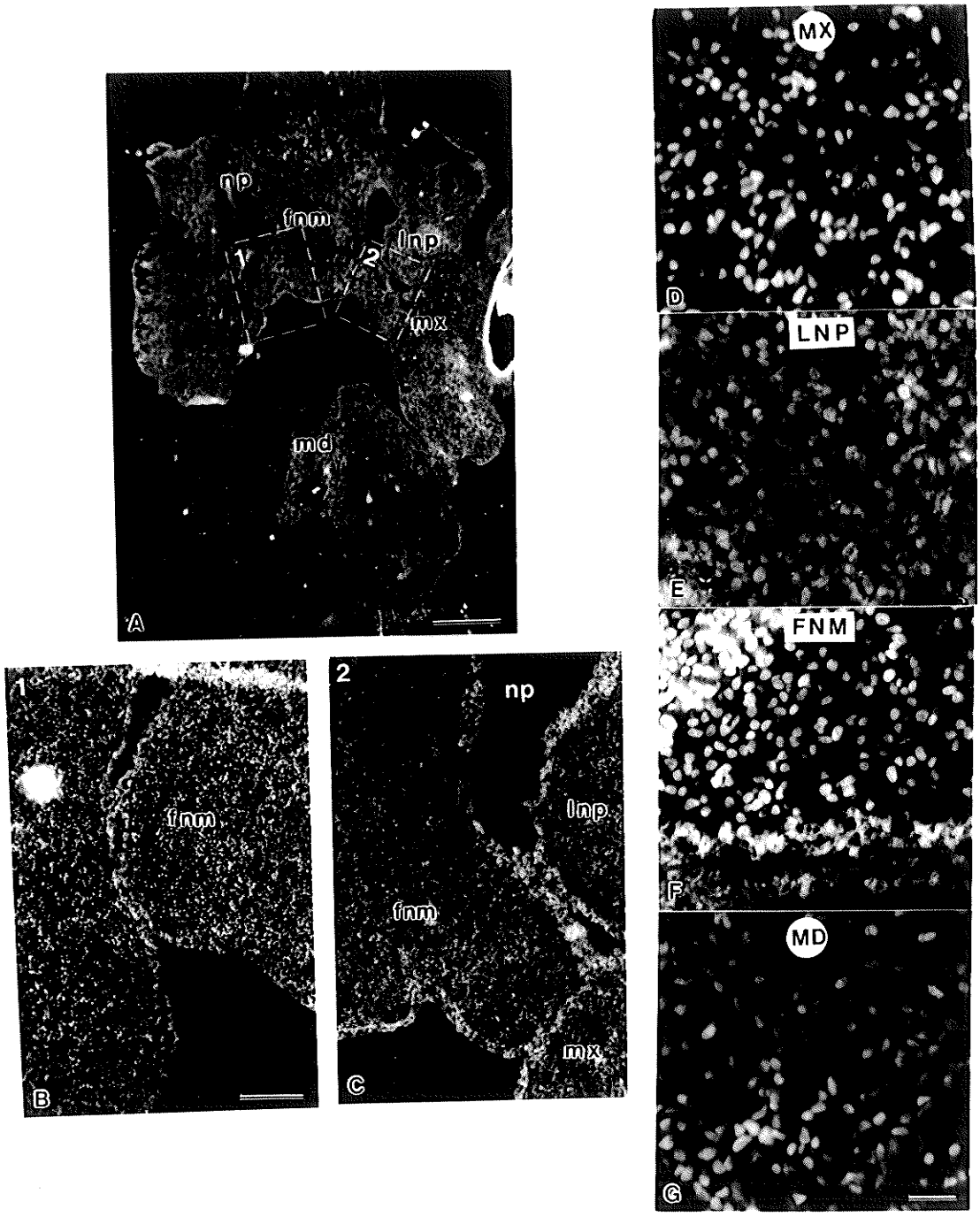
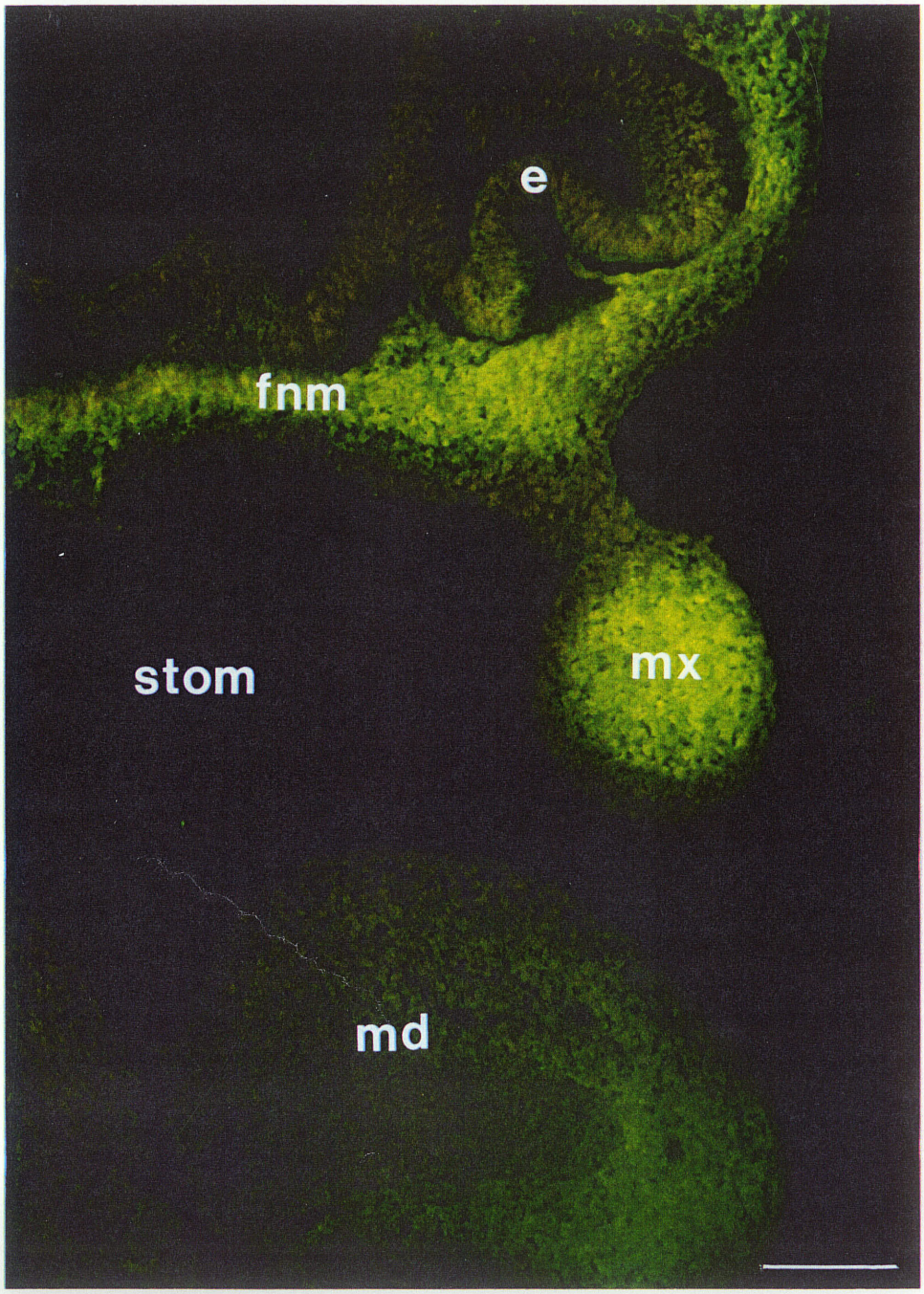

np
fnm
1
2
lnp
mx
md
A
1
fnm
B
2
np
lnp
fnm
mx
C
MX
D
LNP
E
FNM
F
MD
G

Figure 3.11 A colour photomicrograph of a wax embedded, stage 24 embryo stained with S1.

The figure was viewed with FITC excitation. The scale bar represents 0.1 mm. Key: e = eye, fnm = frontonasal mass, mx = maxilla, md = mandible, stom = stomodeum.

All wax embedded embryos, stained with S1, exhibited a regional pattern of staining. There is increased intensity of staining in the mesenchyme of the maxilla, frontonasal mass, and area surrounding the eye compared to staining in the mandibular primordium.


e
fnm
stom
mx
md

CHAPTER IV

GENERAL DISCUSSION

I have demonstrated that the addition of bFGF to cultures of facial mesenchyme produces primordium-specific responses relative to their neural crest lineage and that antibodies to bFGF are bound to facial epithelium and mesenchyme. The major question which persists is what is the normal role for bFGF from early to late stages of development *in vivo*? In this section, I will suggest several experiments that extend my results and address the *in vivo* role of bFGF.

I. Experiments that test the function of bFGF *in vivo*.

I have shown the specific effects of bFGF in cultured cells. In order to demonstrate whether bFGF has a similar effect *in vivo*, a local release of bFGF within each primordia could be achieved using bead implants (Tickle *et al.*, 1985; Leon-Delgado, 1992). Small beads could be presoaked in various concentrations of bFGF and placed under the epithelium, through small incisions within the facial primordia. The beads could slowly release bFGF in the face. If the facial mesenchyme *in vivo* responds like cultures, then differential proliferation and differentiation of the mesenchyme will produce changes in growth patterns in the face. The prediction is that the upper beak, derived from frontonasal mass, lateral nasal process, and upper maxilla, will have increased outgrowth whereas the mandible will not respond.

Organ culture can also be used to get nearer to the *in vivo* role of bFGF. This approach combines some of the advantages of cell culture, such as using defined medium and purified growth factors, with the advantages of whole embryo culture. A significant

advantage of organ culture is that whole primordia can be studied and morphogenesis is closer to what occurs *in vivo*. The organ culture of chick trunks with attached limb buds was reported very recently (Niswander and Martin, 1993). Several growth factors were added to defined medium and several members of the HBGF family (Hst-1 and bFGF) were able to stimulate outgrowth of limb bud mesenchyme. In the same system, bone morphogenetic protein-2 was found to be inhibitory. It is possible that a similar system could be used for the early face provided that results could be seen within a 48 hour period. After this time, cell death and tissue degradation occurs. The results of Niswander and Martin (1993) are very exciting because they support the role of bFGF in the outgrowth of another part of the chick embryo, the limb bud. The same system could probably be used to test the effect of antibodies to bFGF. This "loss of function" experiment (as in Amaya et al., 1991; Mitrani *et al.* 1990) would reveal whether endogenous bFGF is required for outgrowth.

The effects of bFGF on growth of facial mesenchyme may be related to epithelial-mesenchymal interactions known to occur in facial primordia. The epithelium controls the outgrowth of mesenchyme (Wedden, 1987) and the mesenchyme determines shape of the face (Richman and Tickle, 1989). I have shown that bFGF is expressed at high levels in the epithelium in tissues that were least altered by fixation and embedding techniques. It is possible that the major reservoir of bFGF protein is in the epithelium and that by adding exogenous bFGF to facial mesenchyme in culture, one is replacing the bFGF normally supplied by the epithelium. The results of Niswander and Martin (1993) suggest that FGF-related growth factors released from epithelium are important

for outgrowth of limb mesenchyme. Organ culture of facial primordia with and without epithelium would reveal whether exogenous bFGF can substitute for facial epithelium.

The reduced response of mandibular cultures to bFGF in my studies relative to lateral nasal process and frontonasal mass suggests that factors other than bFGF may be able to stimulate growth of mandibular mesenchyme. My results showed that serum was able to stimulate chondrogenesis in mandibular mesenchyme, thus the factors contained in the serum could be studied next. These factors include platelet-derived growth factor, transforming growth factor-β, and insulin-like factor. The mandible should be subdivided prior to culture to allow different subpopulations to be revealed.

II. Experiments that examine interactions between bFGF and other factors present in facial mesenchyme.

Many other molecules are known to be expressed in the embryonic face (reviewed in Richman, 1992) and many of these should be further investigated. I have shown that bFGF-like protein is detected in the chick face and RAR-β is expressed in some of the same regions of the face (Rowe *et al.*, 1991, 1992). The interactions of bFGF and retinoic acid could be tested by a combination *in vivo/ in vitro* experiment. After application of retinoic acid to the embryo, the various primordia constituting the face could be cultured in media with and without bFGF. Culturing the two halves of the maxilla after retinoic acid treatment, would show if there are changes in the response to bFGF due to the irreversible effects of retinoic acid. The prediction is that the FGF responsive cell population is the same group of cells that express RAR-β.

III. Refining the fate map of cranial neural crest: Planning of future experiments based on this revised map.

Future studies that involve subdivision of facial primordia will require a more detailed fate map of cranial neural crest than is currently available. My results indicate that subpopulations of neural crest cells remain true to their origins, and that cells of one lineage tend to group together in specific regions of the face. The upper maxilla, lateral nasal process and frontonasal mass fuse to give rise to the upper beak. It is not unreasonable to expect that the cells in the upper maxilla arise from the same level of the neural crest as cells from the lateral nasal process and frontonasal mass. Unfortunately, the proportion of the primordium at stage 24 that is contributed by prosencephalic crest and mesencephalic crest has not been accurately determined. Le Lièvre (1978) did not concentrate on the results at stage 24, but rather focused on the neural crest derivations of the fully developed chick skull. A study using injected, fluorescent dye, as described in Lumsden *et al.* (1991), could be carried out with dye being injected into the prosencephalon-mesencephalon region and dyed cells followed into the facial primordia up to stage 24. If the DiI is too dilute by this stage of development, quail-chick chimeras could be constructed.

The injection studies of Lumsden *et al.* (1991) revealed boundaries within the mandible similar to those proposed for the maxilla. At stage 15, the cranial half of the mandible is composed primarily of mesencephalic cells whereas the caudal half is derived from neural crest cells at the level of the first rhombomere. The prediction is that cell number and chondrogenesis in cultures of the two halves would exhibit distinct

characteristics. Comparing divided maxilla cultures to divided mandibular cultures could further establish common lineage of cells within these primordia since the caudal part of the maxilla and the cranial part of the mandible share a common lineage.

In humans, there is an immense variety in facial form, some of which occur as a result of problems in differential growth of facial components. For example, if the maxilla fails to grow out, or the mandible has excessive growth, a prognathic facial type develops. If the maxilla develops excessively or the mandible fails to achieve normal growth, the typical "buck-tooth" appearance of a retrognathic facial type occurs. Facial development in humans is very similar to chicks, in the early embryo stage (Sperber, 1976; Moore, 1982; Riviere, 1983; Ten Cate, 1985). The factors which stimulate or inhibit normal differential growth in the chick may prove useful therapeutically, in the future, for treating malocclusion in young children. In some futuristic clinic, missing or reduced growth factors may be supplemented to stimulate growth of specific parts of the face which would not otherwise develop in a 'normal' growth pattern.

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