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THE ROLE OF BMP-2 AND TGF-β DURING EARLY DEVELOPMENT AND PATTERNING OF THE AVIAN MANDIBLE AND LIMB BUDS

by

Steven M. Kolodziejczyk

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
December, 1998

DALHOUSIE UNIVERSITY

FACULTY OF GRADUATE STUDIES

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "The Role of BMP-2 and TGF-β During Early Development and Patterning of the Avian Mandible and Limb Buds"

by Steven Kolodziejczyk

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dated: November 2, 1998

External Examiner

Research Supervisor

Examining Committee

DALHOUSIE UNIVERSITY

DATE: SEPTEMBER, 1999

AUTHOR: Steven M. Kolodziejczyk

TITLE: The Role of BMP-2 and TGF-β during early development and patterning of the avian mandible and limb buds.

DEPARTMENT: Biology

DEGREE: Ph. D. **CONVOCATION: Fall YEAR: 1999**

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Dedication

This thesis is dedicated to my parents, Jozef and Helena Kolodziejczyk, who always encouraged me in all my academic pursuits, no matter how strange. My father's encouragement often took the form of an idiom which, roughly translated from the Polish says "that which you learn you do not have to carry on your back". In other words, learning is never a millstone around one's neck. I want to thank my wife Katharine for her support and encouragement through all these long years. She stuck by me when all of good sense would have given up. Her ability to challenge my mind and keenly analyze my blitherings is appreciated more than I can say here. She is a fine intellect and has been an inspiration to me in many ways. Lastly, and most importantly, I dedicate this thesis to my daughter Helen Eve, the greatest little girl ever. She has brought me more joy than I could have imagined, and her curiosity and quest for answers to sundry questions is perhaps the greatest compliment I could ever hope to receive. I love you deeply and completely.

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Abstract

The TGF-β superfamily of growth factors is important to the proper development and differentiation of organisms. The molecules themselves are highly conserved between phyla and are found in virtually all organisms studied. During embryogenesis, superfamily members are implicated in cell and tissue differentiation directly and as components of a larger signal cascade ultimately leading to the production of a fully formed organism. They are suggested as candidate inductive molecules, supplying the information that will specify cell type and eventually lead to tissue formation. TGF-β and BMP-2 are also of great importance in the adult and continue to be expressed throughout the life of the organism. The role of these molecules during very early development, however, is not completely understood.

The chick mandible and limb bud are similar in many respects. Each begins as a mass of undifferentiated mesenchyme overlain by epithelium. From these beginnings, two very different structures form. Members of the TGF-β superfamily localize to both regions of the embryo early in development. To determine the distribution and role of these proteins, as well as their function is the aim of this study. By first establishing the presence of BMP-2 and TGF-β, both superfamily members, in the chick limb and mandible, and then by inhibiting the function of the cytokines with neutralizing antibodies, it was hoped to learn about their importance during embryogenesis.

It appears that both factors are required to initiate and maintain pattern formation and regulate growth in both early mandible and limb bud. Both TGF-β and BMP-2 are found in the developing limb and mandiblular primordium. Both factors are detectable throughout the developmental stages studied. In addition, their appearance in other regions of the chick during embryogenesis reinforce their developmental importance. Furthermore, the removal of the protein by antibody neutralization has profound effects upon development of limb and mandible, resulting in aberrant growth in antibody injected embryos. Limb and mandibular explants subjected to treatment with TGF-β or BMP-2 neutralizing antibodies grow significantly taller than untreated controls, indicating a growth regulatory role for both factors. Explants treated with the highest doses of either TGF-β or BMP-2 antibody exhibit skeletogenesis earlier than controls, as measured by HBQ staining. This suggests that, in addition to guiding early patterning in the limb and mandible, these factors may play a new role later in the developmental program and direct formation of cartilage and bone in the embryo.

Abbreviations

aa = Amino acids

Ab = Antibody

AER = Apical ectodermal ridge

BMP = Bone morphogenetic protein

DAB = Diamino benzidene

D.p.c. = Days post-coitum

DEPC = Diethyl pyrocarbonate

Dpp = Decapentaplegic gene

ECM = Extracellular matrix

EDTA = Ethylenediaminetetraacetic acid

En = Engrailed gene

FGF = Fibroblast growth factor

FITC = Fluorescein isothyocyanate

HBQ = Hall and Brunt's quadruple stain

Kb = Kilobases

PBS = Phosphate buffered saline

PDGF = Platelet-derived growth factor

SDS = Sodium dodecyl sulfate

Shh = Sonic hedgehog gene

TGF = Transforming growth factor

TBS = Tris buffered saline

Vg1 = Vegetal 1 gene

Xbra = X brachyury gene product

Xgsc = X gooscoid gene

ZPA = Zone of polarizing activity

Note: genes and transcripts are indicated by italics. Gene products are indicated in regular font.

Acknowledgements

I would like to thank my supervisor, Brian K. Hall, a man for whom I have the utmost respect and a scholar in the truest sense of the word. His drive to collect, collate and disseminate information and his obvious love of language and its use are an inspiration. Thank you Steve Smith, one of the cleverest people I know. Steve is also a man whose sense of humor and love of puns and bad jokes kept me laughing when I needed it most. Dave Coltman, my partner in excess has been an example of what a good scientist is, as well as being able to heft a good pint when required. Thank you to Tom Miyake for help with sundry technical problems and also for kindly supplying mouse sections for part of the analysis within, as well as being a fine friend. Thank you to Alma Cameron, our lab tech, a very stable and kind person who has helped me more than she need to. Thanks to the technical support staff at Dalhousie, who helped me get much of this thing together. You are too numerous to mention but have been immensely helpful. Thank you all.

Chapter 1: General Introduction

During the course of embryogenesis, increasingly complex structures are created from seemingly less complex precursors. The morphogenetic events that occur as organisms develop are precise both spatially and temporally. In other words, for a given type of organism, the timing of developmental events and the appearance of morphological structures are precisely the same from embryo to embryo, given identical developmental conditions. At the cellular and molecular level, these processes appear to be under the control of a plethora of interacting molecular signals generated by the embryo. Molecular communication between cells can result in the induction of new cell types. In general, such cell-cell signaling falls into one of two categories. Interactions may take place over a short-range, which includes direct cell contact, or a long-range, which encompasses distances from one to several cell diameters. The generation of a specific molecular signal may, depending on factors such as concentration, trigger not only the differentiation of, but the release of secondary signals from, the responding cells. Secondary signals may initiate differentiation of yet other cells. Such processes are responsible for many of the developmental events seen during embryogenesis. In fact, the generation of patterns leading to the formation of complex structures (such as the head and limbs) in vertebrates derives from relatively simple molecular interactions shared by distantly related organisms. The first step in the development of structures involves the generation of positional information, possibly in the form of morphogen gradients acting over short distances. These signals may interact with inhibitors acting over longer distances. The interaction of these signals and local cellular responses may initiate secondary cell states which, in turn, supply their own positional information (Meinhardt, 1996).

The process of induction, whereby one cell type influences the differentiation of another is well known at the level of tissues. Molecules mediating inductive processes are varied and their effects and modes of action only partially understood. The development of the mandible from undifferentiated primordia and the induction of mandibular bone in the chick is one such example. The patterning and growth of the mandible and the arrangement of internal structural elements are well known from histological studies. The importance of mandibular epithelia for chondro- and osteogenesis is well-established (Hall, 1978, 1992; Hall and Coffin-Collins, 1990; Pinto and Hall, 1991). The role of specific molecular signals responsible for patterning and formation of the mandible, and the rest of the vertebrate craniofacial skeleton, are slowly being elucidated.

The inductive interactions involved in formation and growth of vertebrate limbs are more clearly understood. The initial patterning and subsequent development have been subjects of intense study such that it can now be shown that growth along each of the limbs' three axes is controlled by specific secreted factors produced by defined regions of the primordium (discussed below). The overall growth and development of the limb, the formation of the digits and the fate of interdigital tissue are becoming clearer.

The early development of the vertebrate limb and mandible is initially similar; both share comparable structural components, including cartilage and bone, both require inductive signals originating, at least in part, in the epithelium in order to develop properly, and many inductive interactions are controlled by related morphogens. While the limb and mandible clearly use many different cytokines, among those in common are members of the TGF-β superfamily, including the TGF-βs and bone morphogenetic proteins (BMPs). In the chick, for example, *in situ* studies have revealed *BMP-2* and *-4* transcripts

in the early facial primordia (Francis-West et al., 1994). The appearance of both transcripts in specific regions of facial epithelium and subsequent expression in associated mesenchyme suggest a role for these factors during epithelial-mesenchymal inductive interactions and outgrowth of facial primordia. While mRNA transcripts have been found, detection and localization of BMP proteins during this critical developmental phase have not been done. Similarly, *BMP-2* transcripts have been visualized in the early developing chick wing bud, during the time that initial polarities are being established, specifically the anterioposterior axis (Francis et al., 1994). At later stages, BMP-2 and –7 may be involved in the patterning of limb bone (Kawakami et al., 1996). BMP-2, BMP-7 and TGF-β mRNA transcripts are also expressed in a variety of epithelial and mesenchymal tissues in the mouse and are believed to be intimately involved in patterning and morphogenesis during murine embryogenesis (Lyons et al., 1989,1990, 1995; Luo, et al., 1995).

The expression of growth factor transcripts at times corresponding to the initial appearance of limb and mandibular primordia and their subsequent patterning and growth suggest an important developmental role for members of the TGF-β superfamily. The spatial and temporal localization of mRNA transcripts and the proteins themselves would support this view. The manipulation of these factors within the embryo or its parts may yield information about some of the functions of the cytokines during embryogenesis.

1.1. The chick embryo

The chick embryo presents an ideal system for studying developmental processes, including the patterning and morphogenesis of the head and limb components. The embryos are readily available, simple to grow and easy to manipulate. Other model systems such as mice and zebrafish are used widely

to study embryogenesis but are primarily useful for genetic analysis. Avian embryos, on the other hand, lend themselves to transplantation and *in ovo* manipulation that are difficult to accomplish in other systems. Moreover, avian embryos are useful for cell, tissue and organ culture, *in situ* hybridization, and gene transfer as well as for classical histological techniques. Whole embryos may be grown in shell-less culture for long-term study. Embryos cultured in this manner are easily visible and accessible to manipulation. One disadvantage of shell-less culture is that the embryos cannot be grown to hatching, but this is not a problem if the study involves only earlier aspects of development. Embryos can be grown in the shell and manipulated through a 'window' cut into the eggshell just above the embryo. Advantages include the ability to grow the animal to hatching and that supporting structures (shell, shell membranes, and chalazae) are left intact, allowing normal physiological conditions to be maintained. In studies of bone development, the shell provides a source of calcium for osteogenesis (Bronner-Fraser, 1996). Avian embryonic development is similar in many respects to that of mammals. The mechanisms involved in cell-cell interactions and signal transduction are similar and signaling molecules, including receptor ligands, are often nearly identical. Furthermore chick developmental stages are well established and easily identified (Hamburger and Hamilton, 1951; Lillie, 1952).

Avian development differs from mammalian in that the embryo does not originate as a three-dimensional blastula but rather as a two-dimensional 'disk' of cells adhering to the yolky substrate. One of the first identifiable features of the developing chick is the primitive streak, readily visible by stage 4, corresponding to about 18 hours of development. It appears as a dark streak of cells approximately 1.8 mm in length (Lillie, 1952) (for staging table, see Appendix). Present are the so-called primitive groove and primitive pit along with Hensen's node. All three of these features progress anteriorly during

development. Cells migrate and enter through the primitive pit and descend to different levels, giving rise to mesoderm and endoderm. Notochord begins to appear by about stage 5 and consists of a rod of tissue extending anteriorly from Hensen's node. By stage 6 (24 hours of development), the blastoderm begins to fold anterior to the notochord, raising the developing head from the surface of the yolk and giving rise to three-dimensional morphology. As this process continues and the primitive streak moves posteriorly, progressively more of the embryo is raised from the yolk surface and the gut is floored in, freeing the embryo from the yolk. Shortly after this stage, neural folds begin to form anteriorly and neurulation begins, proceeding in an anterior to posterior direction. By stage 11, corresponding to 40-45 hours of development, the three primary vesicles of the brain are visible, as are the optic lobes, heart and at least 12 pairs of somites (Fig. 1). At stage 15, or 50-55 hours of development, cranial and cervical flexure has progressed so that the free head of the embryo rests on its side (usually its left). At this stage, the first visceral arch, or mandibular arch, is visible, as are slight flattenings of tissue, which will give, rise to the fore and hind limb buds (Fig 1). The optic cups, which will develop into the eyes, are prominent. Numerous somites are visible although counting them, especially those found anteriorly, is difficult due to the overlying ectoderm. At stage 18, or 3 days of development, flexure is more pronounced. The optic cups are completely formed and the large heart and second and third visceral arches are apparent. Limb buds are enlarged, with the leg buds appearing larger than the wing buds (Fig. 1). The tail bud is clearly visible and may have a slight bend toward the head. At stage 24, the limb buds are distinctly longer than they are wide, although digits are not yet apparent (Fig. 1). Mandibular development is well advanced, and the mandibular arches develop two protuberances, indicative of an underlying mandibular patterning mechanism. Slightly later, by stage 26 (5 days), digit formation in the limb appears as thickened tissue separated by faint grooves. Growth and development proceed until, 21 days

after incubation, a fully formed chick is ready to hatch.

1.2. Induction

Induction is the process whereby the developmental fate of one cell type is determined by interaction with a second cell type. This direction of cell destiny is the result of molecular messages exchanged between cell groups (Slack, 1993; Greenwald and Rubin, 1992). The many cell types essentially arise from interactions between just two kinds of cells – epithelium and mesenchyme. Simple epithelia are two-dimensional sheets of cells attached to each other and to a basement membrane by adhesion molecules. Epithelial cells possess apical-basal polarity and thus can function as barriers and are capable of vectorial transport. Their ability to form three-dimensional structures such as tubes and sacs allows for compartmentalization of organismal components into organs and systems. In *Drosophila*, for example, epithelial organization in large part determines animal form (Tepass, 1997). Mesenchymal cells, unlike epithelial cells, are not organized into cohesive sheets but rather occur as dispersed populations of cells residing within a common meshwork of tissue-specific extracellular matrix components (Hall, 1992).

Induction can occur at regions in the embryo where epithelia and mesenchyme come together, the epithelial basement membrane defining the boundary between cell types. Signals exchanged between epithelium and associated mesenchyme are responsible for most major developmental events including the establishment of major embryonic axes, patterning, cell differentiation, organogenesis, and cell growth regulation (Sawyer and Fallon, 1983; Maclean and Hall, 1987). These events can be more generally classified as differentiation, morphogenesis and growth.

If induction involves the generation of a message by one cell type and its reception by a second cell type to yield a third, what is the nature of this signal? The derivation of intricate structures from a single cell appears, at first glance, to require a highly complicated system. In fact, the generation of patterns leading to complex structures such as the vertebrate head and limbs may result from relatively simple interactions between cells. Cells undergoing patterning, growth and differentiation in developing vertebrate embryos can receive positional or inductive information from a number of molecular signals. These include the products of the homeobox genes and various cytokines, as well as non-peptide factors such as retinoids (Thaller and Eichele, 1987; Duboule, 1992; Jessell and Melton, 1992; Tickle, 1995; Bitgood and McMahon, 1995; Newman, 1996). The variety of signaling molecules involved in induction is large and includes many growth regulatory cytokines: platelet-derived growth factor (PDGF), epidermal growth factor (Durkin et al., 1988), insulin-like growth factors (IGF), immunomodulators (including the interleukins (IL), tumor necrosis factors (TNF), colony stimulating factors (CSF) and interferons (Goldring and Goldring, 1991). Of special interest are members of the FGF family and the TGF-β superfamily, the latter including the TGF-βs, activins, Dpp, Vg-1 and the BMPs (Goldring and Goldring, 1991; Jessell and Melton, 1992; Slack, 1993). Even extracellular matrix (Govindarajan et al., 1995) components which include glycoproteins, glycosaminoglycans and proteoglycans regulate development by supplying specific differentiation signals, acting as barriers or filters to other molecules or supplying structural support and morphology. Recently, a group of enzymes, the metalloproteinases, have been found to be important in developmental processes (Olsen, 1996). At least one metalloproteinase is BMP-1, an enzyme responsible for activating latent TGF-β and BMP-2 to –7 as well as directly regulating the assembly of matrix proteins. Indeed, some superfamily members can bind tightly to ECM components *in vitro*, for example collagen IV. Cytokine availability and activity

can thus be locally regulated (Davis, 1988; Paralkar et al., 1991). Growth factors are of interest because they act directly on the cell itself, through specific receptors on the surface, to elicit a response (Kolodziejczyk and Hall, 1996; Jaye et al., 1992).

Inductive processes take place in one of three ways; over long distances, over short distances, or through direct contact between cell types. Long-range interactions are defined as those that occur over distances of between 200 to 400 μm, or approximately 10 to 20 cell diameters. Short-range inductions occur over distances of 20 to 40 μm (Hall and Hörstadius, 1988). Both short- and long-range inductions involve diffusible signaling molecules secreted by one cell and travelling to a target cell. Short-range induction may result from the deposition of a signaling molecule directly into the extracellular matrix (Govindarajan et al., 1995) where it can be accessed by the responding cell, termed matrix-mediated induction. Direct contact on the other hand requires that the responsive cell physically contact the inducing cell either by extending cellular processes through the basement membrane or by degrading the ECM in order to create junctional connections (Hall and Hörstadius, 1988).

One of the best studied examples of induction is mesoderm formation in the amphibian *Xenopus*. Among vertebrates, the formation of mesoderm is probably one of the first inductive interactions to take place during embryogenesis (Nieuwkoop et al., 1985; Smith, 1987, 1989). Early in the development of amphibian embryos, at the morula stage, essentially only two cell types exist; prospective ectoderm and prospective endoderm. Dissection and culturing of the animal pole cells in isolation from vegetal pole cells prior to the 64 cell stage of development leads to the production of epidermal and endodermal cell types respectively. If this procedure is carried out later in development however, both epidermis and mesoderm form from animal pole cells.

In *Xenopus*, the secretion by the animal cap of activin, a growth factor belonging to the TGF-β superfamily, induces mesoderm formation. The diffusion of activin from its source throughout the embryo appears to establish a morphogen gradient (McDowell et al., 1997). Expression of the genes *Xgooscoid* (*Xgsc*) and *Xbrachyury* (*Xbra*) is directly dependent upon the concentration of activin (Gurdon et al., 1994). Animal caps dissected from *Xenopus* blastulae grown in association with exogenous sources of activin expressed both genes in a concentration dependent manner. At these high concentrations of activin, cells adjacent to the source expressed *Xgsc* while those farther away expressed *Xbra*. Lower concentrations resulted in the expression of *Xbra* in adjacent cells and no expression of either gene at greater distances from the source. A similar gradient effect is seen for BMP-4 during patterning of *Xenopus* embryos. Unlike the activin gradient that is generated by diffusion, that of BMP-4 in the gastrula appears to result from the actions of the BMP-4 inhibitors noggin and chordin (Jones and Smith, 1998). By looking for expression of Myf-5, a marker of dorsolateral mesoderm, it was determined that high concentrations of BMP-4 will induce ventral tissues while lower levels influence the formation of dorsolateral tissues. At high concentrations, BMP-4 may induce ventralization by inhibiting activin directly (Jones et al., 1992). Recent evidence indicates that head formation in *Xenopus* may be under the influence of BMP and *Wnt* (Glinka et al., 1997). Inhibition of BMP and *Wnt* signalling in *Xenopus* embryos results in a complete secondary anteroposterior axis complete with secondary head formation. The function of the head-organizer in *Xenopus* embryogenesis may be to inhibit *Wnt* and BMP activity, possibly through the action of *cerberus*, a head inducer (Glinka et al., 1997).

The diverse roles played by BMP and related proteins range from patterning to induction, differentiation and apoptosis, all critical to embryogenesis. Members are expressed in a variety of tissues at different times. Early in development, it appears that some members of the superfamily are involved in dorsoventral patterning of the embryo and in the induction of mesoderm. In fact, in *Xenopus*, maternal RNA localized in the vegetal pole of the egg encodes for Vg1, a member of the superfamily, possibly involved in initial axis formation and mesoderm induction (Weeks and Melton, 1988). Suzuki et al. (1994) constructed a dominant negative *Xenopus* BMP-2/-4 receptor that exhibited normal ligand binding. Intracellular signal transduction, however, was blocked. Ventral blastomeres expressing this receptor were respecified to form dorsal mesoderm. In addition, a second dorsoventral axis was created, implying that BMPs are required to establish normal body pattern in *Xenopus* (Dale et al., 1992; Suzuki et al., 1994). Activins, a subfamily of the group, appear to induce the axial mesoderm to form notochord, neural tissue, muscle and eye structures in *Xenopus* embryos (Thomsen et al., 1990; Asashima et al., 1990). Interestingly, these structures are primarily anterior, so activins may be involved in patterning of the embryo along the anteroposterior axis, although this specification may be previously established (Sokol and Melton, 1991).

Xenopus is not alone in demonstrating that TGF-β superfamily members induce differentiation and pattern formation. Differentiation of the dorsal neural tube in the chick for example requires contact with epithelium expressing BMP-4 or BMP-7 (Liem et al., 1995). Positioning and early development of teeth in the mandibular arch of the mouse appears to require a combination of two growth factors acting at the same time (Neubuser et al., 1997). Nephrogenesis in the mouse requires an inductive signal in the form of BMP-7 (Luo et al., 1995; Vize et al., 1997). Expression of *Pax-9*, a marker of prospective tooth mesenchyme, is inhibited by BMP-2 and -4 but stimulated by FGF-8. Domains of expression

are wide but overlapping. Areas where a precise concentration of these growth factors are found correspond to regions where future tooth growth will be initiated. Such evidence suggests that the induction of at least some tissue types is mediated by the interactions of more than one signaling molecule and may be initiated by differential concentrations of these factors.

A recent interesting example of the control of patterning in organ systems is seen in the zebrafish. During development of the zebrafish heart, radial symmetry is replaced by left dominance, as the heart changes from a symmetrical tube to one that curves to the left and then loops back to the right. This change in symmetry is associated with a differential expression of *BMP-4* transcripts. Initially, BMP-4 expression is radially symmetric but changes during embryogenesis to left predominant. Blocking the action of BMP-4 with a dominant negative BMP-4 receptor construct (a truncated receptor incapable of downstream signaling) prevents bending in any direction while mutants expressing high levels of BMP-4 expression on the right side of the heart ultimately exhibit 'right-looping' (Chen, et al., 1997).

1.3. Origin and morphogenesis of the embryonic chick mandible

The mandible originates from the first branchial arch, initially an outpocketing of tissue located caudally to the maxilla and head of the embryo (Fig. 1). Cranial neural crest cells are produced during neurulation, originating from the junction between the neural plate and epidermal ectoderm of the forming neural tube (Fig. 2). The epithelially-derived neural crest cells change shape and elongate, lose adhesion with their neighbors and break free from the neural crest, becoming mesenchyme (Atchley and Hall, 1991). During early development of the chick, cranial neural crest cells migrate from the region of the mesencephalon, approximately at the level of somites 4 and 5, to the mandibular

primordium (Langille, 1994). In the chick embryo, neural crest movement begins between H.H. stages 8.5 to 10, corresponding to the 5 and 10 somite stage respectively. Neural crest cells begin to populate the first arch at approximately stage 15 (Le Douarin, 1974, 1982). The majority of the craniofacial skeleton, associated muscle and connective tissue and mammalian dentition arise from neural crest-derived cells although head mesoderm and oral ectoderm also contribute components (Hall and Hörstadius, 1988; Langille, 1994).

The skeletal components of the chick mandible consist primarily of bone but include a cartilaginous component, Meckel's cartilage, which manifests as a rod running through the center of each half of the jaw. Mandibular bone elements surround Meckel's cartilage. Most of the skeleton comprising the skull and face form through intramembraneous ossification. These include the bones of the dermatocranium, the bones surrounding the eyes and mouth and the mandible. This so-called membrane bone forms directly, with no cartilageneous precursors. The bones of the base of the skull and the rest of the body (with few exceptions) form through a different method, termed endochondral ossification. In this process, bone is formed by first proceeding through a cartilaginous phase in which hypertrophic chondrocytes, after laying down an extracellular matrix of type II and X collagen, transform into, or are replaced by, osteogenic progenitor cells. Ultimately, the cartilage model becomes mineralized and replaced by bone (Hall, 1987; Richman, 1994). In the mandible, for the most part, bone formation does not first proceed through a cartilage intermediate. Instead, mesenchymal cells differentiate directly into osteoblasts, forming membrane bone (Richman, 1994). Unlike the situation in mammals, avian Meckel's cartilage is not replaced by bone, save for the distal and proximal ends which undergo endochondral ossification (Romanoff, 1960; Hall, 1987; Atchley and Hall, 1990). The distal end forms the tip of the beak while the proximal end becomes the articular process. In mammals, the mandibular

cartilage rod is resorbed and replaced by bone shortly after birth (Richman, 1994).

Neural crest-derived mandibular mesenchyme is induced by overlying epithelium to undergo chondrogenesis and osteogenesis (Tyler and Hall, 1977; Hall, 1978). Induction of bone and cartilage is precisely regulated temporally. Furthermore, epithelial-mesenchymal contact is an absolute requirement for these processes to occur *in vivo*.

Studying embryogenesis of the chick mandible can show that the epithelium exerts a developmental influence on mesenchyme. Separation of epithelium from mesenchyme can be accomplished by incubating excised mandibular arches with either the chelating agent EDTA or the proteases pancreatin and trypsin (Fig. 3). Treatment with EDTA breaks connections between the basal surface of the epithelium and its basement membrane. Incubation in EDTA followed by dissection is used to separate epithelium from the arch, leaving the basal lamina as a layer overlying the mesenchyme. Mesenchymal tissue prepared in this manner will undergo osteogenesis in the absence of epithelium if the explant is of the correct developmental stage. Incubating arches in a solution containing proteolytic enzymes digests the extracellular matrix of the basal lamina, leaving intact the mesenchymal portion of the mandibular arch. The epithelial layer can then be removed by dissection, leaving behind isolated mesenchymal tissue free of epithelium and basal laminar components. Isolated pre-osteogenic mandibular mesenchyme derived from 3- to 4-day-old embryos does not undergo osteogenesis *in vitro* unless cultured in association with mandibular, wing- or hind-limb-bud epithelia (Hall, 1978). Mandibular mesenchyme isolated from 4.5 to 5 day old embryos however will undergo osteogenesis in the complete absence of epithelium (Tyler and Hall, 1977). Experiments such as these underscore the fact that the induction of

osteogenesis is stage specific. These results indicate that differentiation of neural crest-derived mesenchymal cells into bone during the development of the chick mandible is dependent upon an inductive signal originating in the overlying epithelium and that this signal is localized in the basal lamina (Tyler and Hall, 1977; Hall, 1978). In addition, once the target cells of the mandible have received this message over a sufficient period, osteogenesis will proceed regardless of the nature, or indeed the presence, of the epithelium. Furthermore, there exists a temporal 'window' during which the inductive process can take place.

Molecular messages originating in the epithelium at a specific time in embryonic development of the mandible are responsible for mesenchymal cell proliferation and differentiation. *In vitro*, sodium fluoride (NaF) or EGF induce osteogenesis in mandibular mesenchyme after 6 to 8 days of culture, but only in the presence of serum (Hall, 1992). NaF initiates osteogenesis *in vitro* (Farley et al., 1983). This result suggests that factors present in the serum supplement are necessary in addition to either of the mitogens to induce osteogenesis. Furthermore, a combination of both EGF and NaF for only a single day is sufficient to begin the differentiation of mesenchymal cells into bone even in the absence of serum (Hall, 1987, 1992). It is conceivable that these factors, alone or in combination, stimulate the synthesis of a differentiation factor by the mesenchyme. They may be responsible for cell proliferation but not necessarily for differentiation. It is of interest that laminin and entactin, components of epithelial basement membranes, contain EGF-like domains (Sasaki et al., 1987; Durkin et al., 1988). Mesenchymal cells in contact with the basal lamina of the epithelium will derive at least some proliferative cues directly from the ECM. NaF, on the other hand, has not been detected *in vivo* during differentiation and *in vitro* may only mimic the effects of some other factor. It has been shown that endogenous EGF may regulate some aspects of mandibular development.

Inhibition of EGF signaling with either antisense oligonucleotides or disruption of EGF receptor kinase activity retards or alters mandible and tooth development in the mouse (Shum et al., 1993).

In birds and reptiles, a ring of membrane bones, the scleral bones, forms around the developing cornea. By performing transfilter experiments on preosteogenic scleral tissue, Pinto and Hall (1991) showed that a diffusible factor originating in the epithelium is required by the mesenchyme for osteogenesis to take place. Varying pore size as well as distances between tissues showed that the inductive process in this case was long range, acting over 150 to 300 μm. Pore sizes as small as 0.03 μm did not prevent induction. Pinto and Hall determined the molecular mass of the diffusable molecule(s) to be between 3500 and 6000 Daltons. In transfilter experiments on chick mandibular tissue, a pore size of 0.2 μm, which is sufficient to prevent cell-cell contact, did not prevent induction in mandibular mesenchyme (Van Exan and Hall, 1984). However, contact may be a requirement in the initiation of chondrogenesis. Prechondrogenic mesenchyme combined with inductive epithelium will not initiate chondrogenesis if the filter used in the recombination is less than 0.8 μm in thickness (Smith and Thorogood, 1983).

1.4. Origin and morphogenesis of chick limb buds

Limb bud primordia originate from mesodermally derived mesenchymal cells located at specific regions along the flank of the embryo. Limb development occurs in distinct phases (Richman, 1994). Initially, at around stage 15, specific limb 'fields' where limb growth will begin arise on the flank of the embryo. Shortly thereafter (at stage 16) ventral ectoderm in these regions begins to thicken and develop into the apical ectodermal ridge (AER) (Saunders, 1948). The AER is a specialized portion of epithelium along the rim

of the developing limb bud that influences proliferation of the underlying mesenchymal cells and maintains them in an undifferentiated state. Mesenchymal cells which exist in many regions of the developing embryo in addition to the limb buds respond to specific signals, including those generated by the AER to establish identities and positions. Cells derived from somitic mesoderm will form muscle while those derived from lateral plate mesoderm will ultimately give rise to cartilage and bone (Young et al., 1993). Only when the mesenchymal cells leave the region of AER influence can they differentiate and form the prechondrogenic condensations which precede skeletogenesis (Summerbell and Lewis, 1973). Budding and outgrowth of limb primordia begin at stage 17, with limb elongation and growth continuing through stage 24. Chondrogenic condensation formation follows at stage 25, after which skeletal elements, cartilage and bone, are produced.

Limb growth patterning is polarized along all three axes. The AER, a region of specialized pseudostratified epithelium appearing at stage 16, is responsible for proximodistal growth of the limb bud. Experimental evidence suggests that the AER is a source of fibroblast growth factor (FGF), which is responsible for limb bud elongation (Fallon et al., 1994; Niswander et al., 1993). Outgrowth of the limb bud is influenced directly by morphogens originating in the AER, specifically members of the FGF family of growth factors. Most recently, *FGF-8* transcripts have been found to localize to the AER in early mouse limb development and have emerged as candidates for the AER produced signal (Crossley et al., 1996). FGF-8 appears to upregulate and maintain *sonic hedgehog* (*shh*) levels. *Shh* is closely linked to expression of FGF-4 activates *BMP-2* expression in the limb (Laufer et al., 1994). The coexpression of *Hedgehog*, a gene closely related to *shh*, and BMP genes in various organs of the developing mouse indicates that development of many embryonic structures results from interactions between multifunctional gene families, although

coexpression does not comprise proof (Bitgood and McMahon, 1995). FGF-4 has been localized to the AER, but appears later than FGF-8. Another member, FGF-2 is suspected of being the patterning morphogen released by the AER (Fallon et al., 1994).

Signals governing patterning in the different axes of the developing limb may interact in a complex fashion. Studies using a wingless chick mutant, *Jwg*, indicate that the expression of *FGF-8*, a marker of the AER, was initially delayed and then reduced in the developing bud. Furthermore, limbs without *FGF-8* expression become bi-dorsal and express reduced levels of *shh*. Implantation of FGF-8 expressing cells into the limb bud results in rescue of wing bud outgrowth and maintains *shh* levels (Ohuchi et al., 1997).

The so-called 'zone of polarizing activity' (ZPA) (Tickle et al., 1982), a region of mesenchymal cells located in the posterior of the limb bud, controls anteroposterior growth. Grafting of posterior limb bud mesenchyme (Tickle et al., 1982) to a position in the anterior region of the bud produce digit duplication and posterior structures in an anterior position (Tickle et al., 1975). This effect is mimicked by the application of retinoic acid to the anterior region of the limb bud (Tickle et al., 1982). ZPA activity appears to be linked to the expression of *shh* (Riddle et al., 1993). In addition, polarizing activity can be mimicked by direct application of *shh* protein to the limb bud (Lopez-Martinez et al., 1995). *Talpid*^{3} (*ta*^{3}) chick mutants exhibit polydactyly consistent with abnormal anteroposterior patterning. In normal limb buds, anteroposterior patterning is controlled by a signal emanating from posterior mesenchyme, the polarizing region. Grafts of this region to a more anterior position on the developing limb bud cause respecification and the production of posterior structures, including digit duplications (Tickle et al., 1975). The polarizing activity of limb mesenchyme in *ta*^{3} mutants, unlike normal chicks, extends across the entire a-p axis, with the

polarizing activity of posterior mesenchyme lower than that of normal buds (Francis-West et al., 1995). The expression of *BMP-2, -4* and *–7* in mutants is uniform across the a-p axis, unlike the situation in normal limbs, where expression is confined to specific regions of the limb.

The expression pattern of BMP genes in mutants correlates with the polarizing activity of the mesenchyme. Previous studies suggested that BMP-2 might be responsible for a-p patterning in the limb bud (Francis, et al., 1994). The expression in mutant chick embryos lends support to the idea that *BMP-2, BMP-4,* and *BMP–7* (or heterodimers) may be involved with pattern determination during development.

Dorsoventral patterning may be mediated by molecular signals emanating from the dorsal and ventral ectoderm of the limb bud. The dorsalizing signal is supplied by the expression of the *Wnt-7a* gene and localized to the dorsal ectoderm (Parr and McMahon, 1995). Production and maintenance of18 ventral limb characteristics may be the result of the homeobox-containing gene *engrailed* (*En-1*). Mice ectopically expressing *En-1* exhibit limbs with a double dorsal phenotype. Replication competent retroviral vectors can be used to allow ectopic expression of murine *En-1* in early chick dorsal limb bud, resulting in repression of *Wnt-1* expression, a gene closely related to *Wnt-7a* (Logan et al., 1997).

1.5. TGF-β superfamily

Many members of the TGF-β superfamily of growth and differentiation factors have been implicated in embryogenesis. These include the bone morphogenetic proteins (BMP), transforming growth factor- (TGF-β), and activin

families as well as molecules such as *Drosophila* decapentaplegic (dpp), nodal and dorsalin (Kingsley, 1994; Kolodziejczyk and Hall, 1996). The superfamily is comprised of at least 24 members, although new candidates are regularly being discovered (Kolodziejczyk and Hall, 1996). BMPs were initially isolated from demineralized bone matrix by Urist (1965) and named for the ability of the matrix to form ectopic bone when implanted subcutaneously into rat muscle. Many superfamily members induce bone formation in the rat ectopic assay system (Kolodziejczyk and Hall, 1996).

Superfamily members are expressed in virtually all phyla and are found in organisms as diverse as *Caenorhabditis elegans*, *Drosophila*, *Xenopus*, and mammals including humans (Massagué, 1990; Kingsley, 1994). For example, human BMP substitutes for *Drosophila dpp*, another superfamily member, in transgenic flies and restores dorsoventral patterning (Padgett et al., 1993). Conversely, the *Drosophila* proteins dpp and 60A, both superfamily members closely related to BMPs, induce ectopic bone formation in the rat (Sampath et al., 1993).

BMP-2 and *–4* have been localized in the developing face of both the chick and the mouse. *In situ* hybridization reveals that in mice, *BMP-4* expression is localized in the epithelial layer of the developing face at day 9.5 of development. The signal is transient and decreases until about day 11.5 when it resides only in the dental lamina, follicle and papilla. BMP-2 mRNA on the other hand appears later, at around day 12, and is found exclusively in the neural crest-derived mesenchyme (Bennett et al., 1995). Similarly in the chick, *BMP-4* transcripts are found in the epithelia of all facial primordia early in development (stage 20). Expression subsequently moves to localize in mesenchymal cells at the distal tips of facial primordia. *BMP-2* mRNA expression initially appears in the frontonasal mass (stage 16), localized to epithelia and subsequently occurs

throughout the underlying mesenchyme of the maxillary and mandibular processes (Francis-West et al., 1994). The initial appearance of BMP mRNAs in epithelia and subsequent expression in the mesenchyme may indicate a role in an epithelial-mesenchymal interaction typical of inductive processes. BMP-2 saturated beads implanted into cultured stage 22 mandibular mesenchyme do not however induce bone formation but produce localized cell death, suggesting that BMP-2 is not an inducer of osteogenesis in the chick mandible (Ekanayake and Hall, 1997).

In ovo implantation of beads saturated with BMP-4 at the tip of a developing hind limb digit results in localized cell death around the area of the bead and digit bifurcation (Macias et al., 1996). Implantation of TGF-β 1 or 2 saturated beads however induces ectopic cartilage in regions of mesoderm normally programmed to undergo apoptosis. This was accompanied by the down regulation of *BMP-4*, *msx-1* and *msx-2* gene expression. This indicates that closely related members of the same cytokine superfamily may elicit reciprocal effects. In addition, it is likely the growth factors must operate in a coordinated manner *in vivo* in order to produce a normal limb. PDGF applied to cultured stage 24 chick limb bud cells inhibits the formation of cartilage nodules, as measured by the decreased synthesis of sulfated proteoglycans, a marker of chondrogenesis (Chen, 1992). The inhibitory effect is reversed by exogenous BMP-4. Further, TGF-β 1 and TFG-β 2 inhibit the formation of cartilage in cultured chick limb bud mesoderm, a process stimulated by BMP-4 (Chen et al., 1991).

Embryonic duck hind limb buds transfected to express a dominant negative BMP receptor exhibit one of three phenotypes: syndactyly (webbed digits), truncated digits, or transformation of feathers to scales (Zou, 1996). Interdigital cell death is necessary to free the digits of the developing limb but

by blocking the BMP signal at the level of the receptor, this process is inhibited, producing syndactyly. The transformation of normal dorsal hind limb scuta or scales into feathers indicates that a single growth factor may play multiple developmental roles even within the same embryonic region. BMPs appear to be involved in limb patterning. Kawakami et al. (1996) were able to show that by dominant negative BMP receptors expressed in developing chick limb bud did not alter the pattern of bone within the limb but did result in distal and posterior truncations.

1.6. Experimental outline

The research aim was to study the role of TGF-β and BMP-2 in the patterning and development of chick mandible and limb buds, focussing on the time of initial appearance of primordia, subsequent growth and patterning until the onset of chondro- and osteogenesis. The first step was to establish that *BMP-2* and *TFG-β* mRNA as well as their associated gene products (proteins) are present during this period of early chick embryogenesis. Initially, northern blots were used to analyze chick tissue for BMP-2, -4, -5, -7 or TGF-β transcripts. The expression of cytokine mRNA would suggest a possible developmental role for these factors. Emphasis was placed on mandibular tissue, especially comparisons of the epithelial and mesenchymal levels of mRNA from different stages of development. Having established the presence of BMP and TGF-β transcripts in the embryo at the time of interest, immunohistochemistry was used to localize the proteins within the mandible, limb buds and other regions of the embryo. Frozen and paraffin embedded sections were analyzed and a variety of visualization methods used to ascertain the regions of growth factor expression. Temporal patterns of cytokine expression were analyzed through immunostaining tissue sections from a range of developmental stages. The transient expression of growth factor protein

during critical developmental stages of specific tissues would suggest a role for cytokines in embryogenesis.

In order to investigate the specific role of growth factors in the development of the embryo, cytokine-specific antibodies and phosphatase inhibitors were used to interfere with signal transduction. Injection of inhibitors directly into the perivitelline (air) spaces of fertilized chick eggs was used as a method to determine their effects and the range of effective concentrations. Analysis of gross morphological changes was accompanied by microscopic analysis of stained sections from affected and control embryos. Finally, either explanted limb buds or mandibular tissues from various developmental stages were grown in culture. Explants were treated with growth factor neutralizing antibodies to determine effects on development and growth rate. Explants were fixed, sectioned and immunostained for the presence growth factors. Sections were also treated with Hall and Brunt's quadruple stain (HBQ) to determine the extent of skeletal element development (Hall, 1986).

The data yield information about the role of growth factors in regulating growth of limb and mandibular primordia as well as their localization and possible functioning in other areas of the chick embryo. These factors have been implicated in cartilage and bone modeling, and they may also function to direct growth in many regions of the early vertebrate embryo.

Figure 1: Representative stages of early chick embryo development indicating major morphological structures and also flexure and rotation of embryo. Figures are drawn to indicate correct relative sizes. o.l. = optic lobe; s = somite; w = wing (fore limb) bud; l = leg (hind limb) bud; h = heart. Mandibular (1st visceral) arch is indicated by (*) in figures (B) and (C) and by 'm' in (D). Anterior is toward the top of the page in A – C and toward the left in (D). Scale bar = approximately 3 mm.

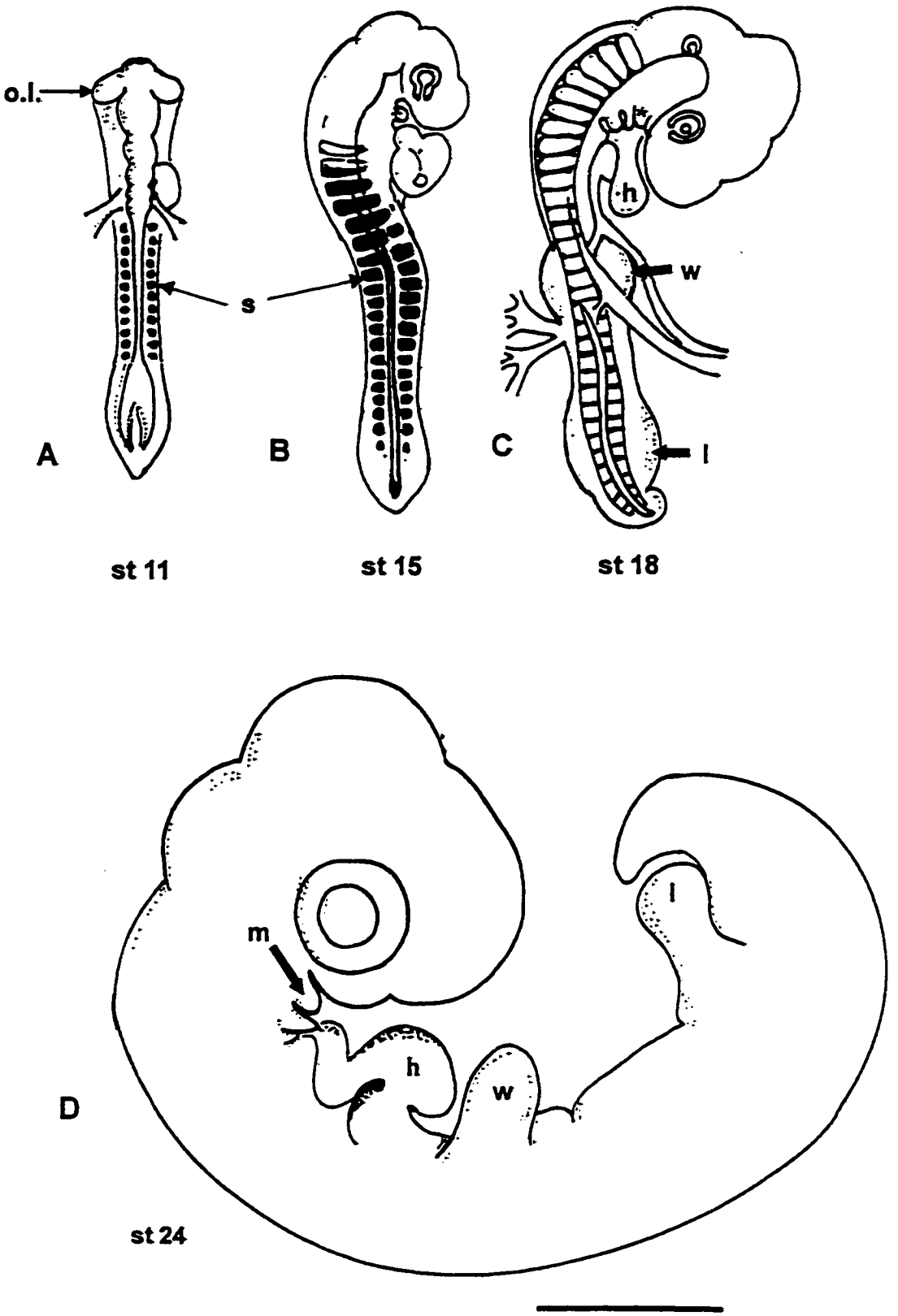
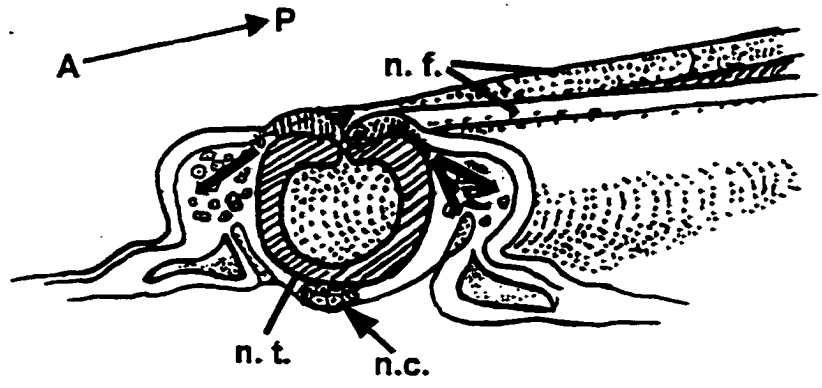


Figure 1

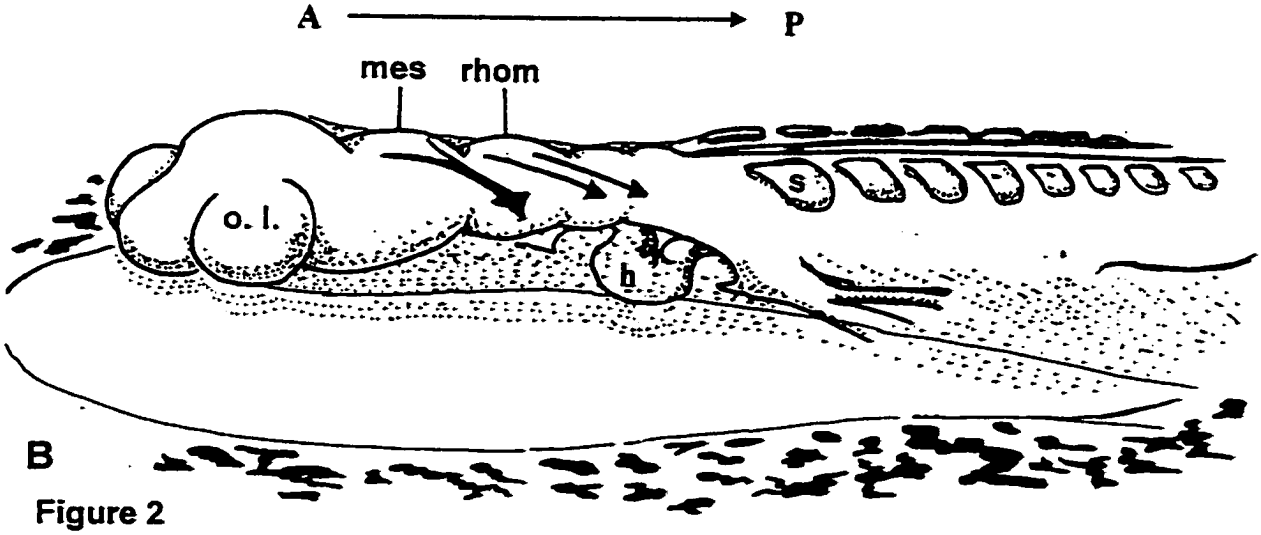
Figure 2: Illustration of source and migration of neural crest cells during chick embryogenesis.

A) Cross section through neural tube of stage 11 chick embryo. Arrows in figure indicate migration of neural crest cells from region of fusing neural tube, at junction of neural and epidermal ectoderm. Arrow above figure indicates direction of neurulation, where A = anterior and P = posterior. n. t. = neural tube; n. f. = neural fold; n. c. = notochord.

B) Oblique view of embryo, with arrows indicating movement of neural crest cells from region of anterior neural tube into mandibular primordia and elsewhere along flank (arrows). Clear area under anterior of embryo represents the lifting off of the future head from the blastoderm and formation of the sub-cephalic pocket. Arrow above figure indicates the direction of neurulation. A = anterior; P = posterior; mes = mesencephalon; rhom = rhombencephalon. h= heart; s = somite.



A



B

Figure 2

Figure 3: Separation of mandibular epithelium from underlying mesenchyme, after pre-treatment with either the chelating agent EDTA or the proteolytic enzymes pancreatin and trypsin. After incubation of mandibular explants in either of the two solutions, the epithelium can be mechanically removed by careful dissection using fine (#5) forceps. In the case of pre-treatment with EDTA, the basal lamina remains intact and covers the mesenchymal cells. Incubation of explants with proteolytic enzymes, on the other hand, digests the extracellular matrix components of the basal lamina so that after dissection, only 'naked' mesenchyme remains. e = epithelium; b. L. = basal lamina; m = mesenchyme; e.c.m. = extracellular matrix components.

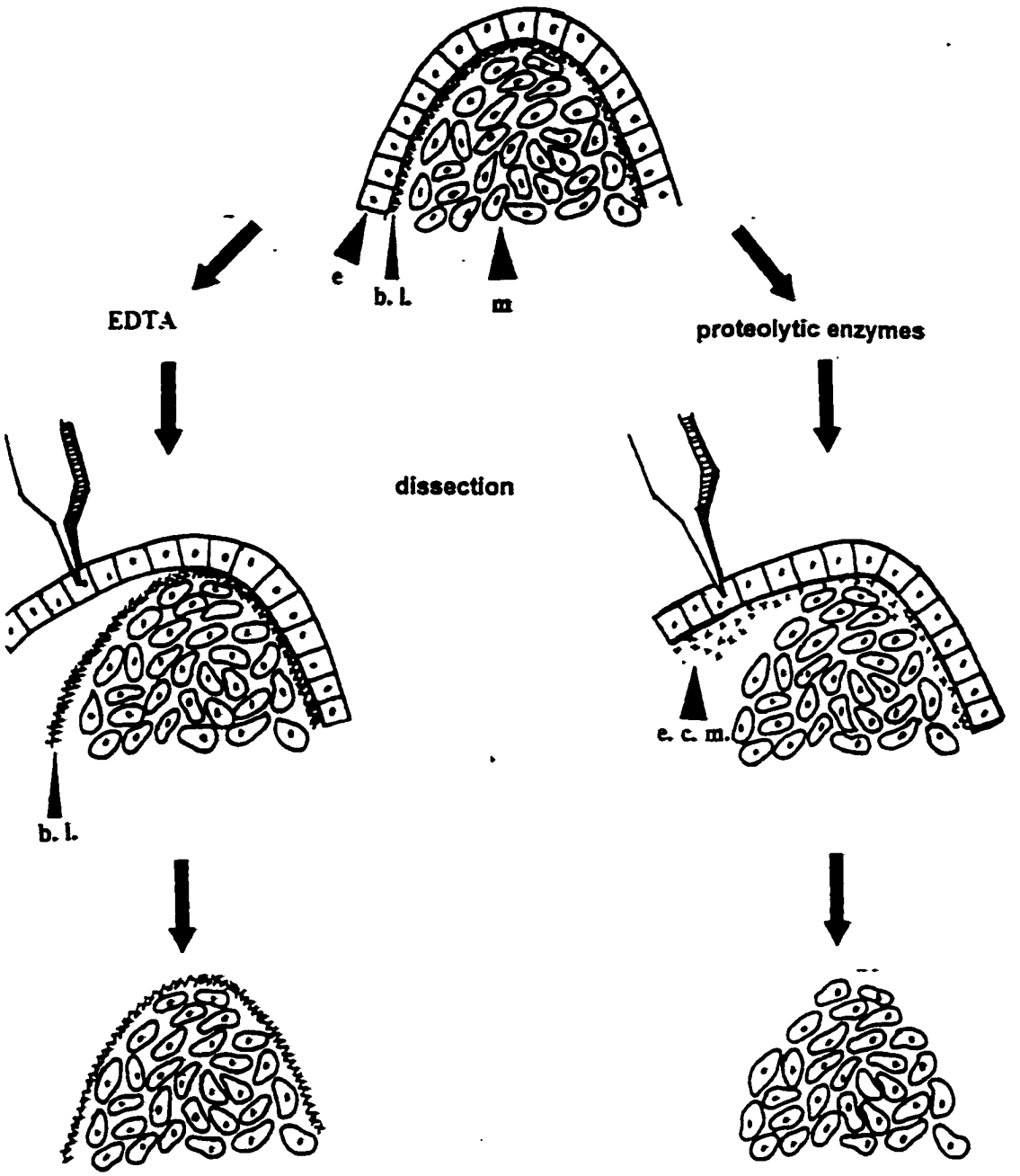


Figure 3

Chapter 2: Detection of TGF-β and BMP-2 mRNA transcripts and protein in avian embryos.

2.1. Introduction

In order to investigate the role of TGF-β superfamily growth factor members on development and patterning, it was important to first establish their presence in the embryo during early development, approximately between stages 14 and 30. To this end, it was decided to first look for the presence of mRNA specific to the factors being investigated. Two methods were chosen; northern blotting to look for the presence of TGF-β and BMP mRNA and western blotting to determine the ability of BMP-2 antibody to identify BMP-2 protein.

The TGF-β superfamily of growth and differentiation factors is composed of closely related proteins that have profound influences on the growth and development of vertebrates and invertebrates. Members of the superfamily are found in virtually all phyla and retain strong sequence similarities between distantly related organisms. The number of superfamily members is constantly changing as new factors are found. The superfamily contains at least 24 members and include the activins, bone morphogenetic proteins (BMPs), and the TGF-β isoforms (Kolodziejczyk and Hall, 1996).

All members share structural features. The precursor protein can be divided into several regions that vary in length, depending on the specific superfamily member. A short, hydrophobic leader sequence (15 – 25 a.a.) is followed by a pro-domain (50 – 375 a.a.) which varies greatly in length among superfamily members and may be important for protein folding and oligomerization (Hammonds et al., 1991; Kingsley, 1994) and finally the sequence coding for the mature protein (110 – 140 a.a.). The mature protein is

released after proteolytic cleavage at a conserved cluster of basic residues. Within the mature region there are seven cysteine residues that are conserved in superfamily members (Fig. 6).

Conserved cysteines within each monomer are involved in intrachain folding and form the so –called 'cysteine knot' which is a feature of the superfamily, an eight member ring held together by disulphide bonds. This conformation links several β-sheets together and may allow superfamily members to resist denaturation by heating, pH, or other severe conditions (Daopin et al., 1992; Schlunegger et al., 1992). The monomers are approximately 'hand-shaped' and can link heel to fingers to form dimers held in place by disulphide bonds (Fig. 6B). It is known that TGF-β suprfamily members form homodimers readily (Rosen and Thies, 1992). Recently, however, the possibility of heterodimer formation between members has been demonstrated. BMP-2 and BMP-7, for example, can form heterodimers that, *in vitro*, are several times more potent that homodimers, as measured in an *in vitro* alkaline phosphatase induction assay (Israel, et al., 1996).

Whether such configurations exist *in vivo* remains to be determined. Similarly, the *in vivo* size of the mature BMP-2 protein and its oligomeric forms has not been described to date.

2.2. Materials and Methods

2.2.1. Northern blots

2.2.1.1. BMP and TGF-β clones

BMP-2 clones were supplied by Dr. Paul Brickell, University College, London, U. K. Briefly, the complete coding region (772 b.p.) of mature chicken *BMP-2* was cloned into plasmid vector pBluescript KS^+. Clones were originally

prepared by reverse transcription of embryonic chick limb RNA. The recovered cDNA was then amplified by polymerase chain reaction. Primers consisted of 24 oligonucleotide long sequences identical to those found in *Xenopus*, human and mouse. The fully sequenced BMP-2 clones share approximately 97% amino acid identity with *Xenopus*, human and mouse (Francis, et al. 1994). Clones corresponding to the mature coding regions of mouse TGF-β1 were obtained from Dr. Su Wen Qian (Laboratory of Chemoprevention, NIH, Maryland) as 1085 base pair inserts in pBluescript KS^+. Human and chicken TGF-β 1 are identical and differ from mouse TGF-β 1 by only one amino acid (Derynck, et al. 1986).

2.2.1.2. Probe generation and labeling

Plasmids containing BMP-2 or TGF-β clones were linearized with Hind III. Antisense RNA probes were synthesized with T3 RNA polymerase. Riboprobes are generally more specific than cDNA probes in detecting transcripts on northern blots. Sense RNA probes were used as controls. BMP-2 sense probes were synthesized by first linearizing the clones with Bam HI followed by incubation with nucleotides in the presence of T7 RNA polymerase. In the case of TGF-β, antisense strands to be used as probes were created by first linearizing the clone with Hind III and adding T3 RNA polymerase. Control probes for TGF-β were first linearized with Xba I and probes were synthesized using T7 RNA polymerase. Prior to transcription, linearized probes were phenol extracted, precipitated in ethanol and dissolved in autoclaved, distilled water. Probes were radioactively labelled according to the protocols outlined in BRL technical bulletin 8033-1 (Gibco BRL, Canadian Life Technologies Inc., Burlington, Ontario). Briefly, approximately 200 µCi of [α^{32}P] UTP was lyopholized to 2 µl in an eppendorf tube. To the tube at room temperature was added 2 µl 5X T7 polymerase buffer (Gibco BRL), 1µl of NTP stock (consisting

of 10mM each of ATP, CTP, and GTP), 1 µl of 50 mM dithiothreitol (DTT), and 0.5 µg of DNA template. Distilled, autoclaved water was added to achieve a final volume of 9 µl. The reaction mixture was incubated for 5 minutes at 37° C. After this time, 1 µl of the appropriate RNA polymerase (50 units) was added, mixed by gentle pipetting and the mixture incubated for 10 minutes at 37° C. After 10 minutes, 90 µl of 50 mM Tris-HCl, 10 mM $MgCl_2$ (pH 7.5) was added, followed by 1 µl of DNAse I, diluted to 0.5 µg/ml in the same buffer. The mixture was again incubated for 10 minutes at 37° C. After the incubation period, DNAse activity was terminated with the addition of 10 µl Na_2 EDTA, pH 8.0. The entire reaction mixture was phenol extracted, aqueous phase removed to a new tube and re-extracted with phenol: chloroform: isoamyl alcohol (25: 24: 1). The aqueous phase was removed to a new tube and to it was added 0.5 volumes of 7.5 M ammonium acetate and 2.5 volumes of absolute ethanol. The mixture was centrifuged for 30 minutes at 15,000 x g at room temperature. The supernatant was removed, the pellet washed in 70 % ethanol (2 times) and the dried pellet resuspended in TE buffer, pH 7.5 (10 mM Tris-Cl, 1 mM EDTA).

2.2.1.3. Preparation of RNA and northern blotting

For RNA preparation, it was important that all solutions and equipment were RNAse free. All solutions were prepared using diethylpyrocarbonate (DEPC) treated water to deactivate ribonucleases. This was prepared by adding DEPC directly to water for a final concentration of 0.2 %, agitating until dissolved and autoclaving to deactivate remaining DEPC. Glassware and metal utensils were baked at 300° C for 4 hours to destroy RNAse. Plasticware was used directly out of sterile packages. All equipment was handled using gloves, changing frequently.

Chick embryonic tissue was dissected in sterile, DEPC treated saline at

4° C. Tissues were flash frozen in isopentane kept on dry ice and stored at −70° if not used immediately. Prior to extraction, frozen tissues were crushed in a mortar and pestle pre-cooled over dry ice. Because of the small size of the embryonic mandible and the limb buds, samples (approximately 10-15) of the same age were pooled to obtain sufficient quantities of RNA for blotting. RNA was isolated using a modification of the method of Chomczynski and Sacchi (1986). Briefly, the procedure relies upon the properties of guanidinium thyocyanate (GITC), a potent denaturing agent that is also a strong inhibitor of RNAse activity. After removal from the embryo, or immediately after removal from the freezer, the tissue was homogenized in 1 ml of 2 M sodium acetate, pH 4.0 and 10 ml of water saturated phenol using a ground glass hand homogenizer. The homogenate was transferred to a fresh tube, shaken vigorously, then incubated on ice for 15 minutes. After incubation, the solution was centrifuged for 20 minutes at 10,000 x g at 4° C. The aqueous phase was extracted and transferred to an eppendorf tube. It was important to avoid extracting the white interphase layer. To the tube was added 1 ml of isopropanol, the mixture inverted several times to mix components and placed at 20° C for 1 hour. RNA was then pelleted by centrifugation at 10,000 x g for 20 minutes at 4° C. Once centifugation was complete, the supernatant was removed and the remaining pellet washed in 75% ethanol and recentrifuged for 5 minutes. This procedure can be repeated. Once the final wash was complete, supernatant was discarded and the pellet allowed to air dry on ice. The pellet was resuspended in DEPC treated water and used directly for northern blots. For northern blot analysis, samples were loaded onto 1% denaturing agarose gels containing 1.1% formaldehyde and 1x MOPS (3-[N-Morpholino]propanesulfonic acid) buffer. Samples were diluted in buffer containing 1x MOPS, 6.5% formaldehyde and 50% formamide and incubated for 15 minutes at 55° prior to loading. Loading buffer containing bromphenol blue and xylene cyanol was added prior to sample application.

Gels were typically electrophoresed in 1x MOPS buffer for 2.5 hours at 100 V. In general, between 15 and 25 µg of total RNA were added to each well, as determined by absorption spectroscopy. After electrophoresis, gels were rinsed several times in DEPC treated water and washed in 10x SSC buffer (1.5 M NaCl, 0.15 M Na_3citrate, pH 7.0) for 30 minutes. The RNA was transferred to nitrocellulose membrane. Briefly, a glass plate was arranged to overhang a large glass tray containing 20x SSC. A wick cut from Whatman 3MM filter paper, slightly wider than the gel, was arranged so that it lay over the glass plate, with its two free ends submerged in buffer. After removing air bubbles, the gel was placed on top of the wick, bubbles were removed and a piece of nitrocellulose cut to the dimensions of the gel and pre-soaked in 20 x SSC was placed on top. It was important to wear gloves when handling the nitrocellulose membrane as it can easily become contaminated with extraneous nucleic acids and proteins. Several pieces of Whatman paper cut to the dimensions of the gel were moistened in SSC and placed on top of the nitrocellulose. These were in turn covered with a large (3-4 cm) stack of paper towels, another glass plate and finally a weight of 0.3-0.5 kg placed on top. The assembly was sealed around the edges with plastic wrap to prevent evaporation and the transfer allowed to take place overnight. After the transfer was complete, the gel was discarded and the nitrocellulose removed and baked in an oven for 2 hours at 80° C. The blot was then prehybridized in hybridization solution consisting of 50% formamide, 5x SSC, 1x Denhardt's solution (25 mM Tris-HCl, pH7.5, 0.5% Na pyrophosphate, 5% SDS, 1% polyvinylpyrolidine, 1% Ficoll, 25mM EDTA) and 150 µg/ml denatured salmon sperm DNA. Blots were prehybridized for 2 hours at 42° C in a hybridization oven. After prehybridization, the solution was discarded and fresh hybridization solution containing radiolabelled riboprobe was added and the blot allowed to hybridize overnight at 42° C.

After hybridization, the blot was washed twice in 1x SSC/0.1% SDS for 15 minutes at room temperature. It was then washed again twice in 0.25x SSC/0.1% SDS for 15 minutes at room temperature. The blot was then wrapped in plastic wrap and sealed with X-ray film in a cassette. The cassette was stored at –70 ° C until an appropriate exposure was produced. This time ranged from 4 hours to several days depending on the activity of the probe.

2.2.2. Western blot of BMP-2 and immunodetection

Chick mandibular and limb bud tissues were dissected in Ringer's solution, pooled, and homogenized in SDS sample buffer containing 0.1 M Tris-Cl (pH 6.8), 10% glycerol, 2% SDS, 1% β mercaptoethanol and 0.5 mg/ml bromophenol blue. Samples were loaded onto 15% SDS-polyacrylamide gels and electrophoresed at 100 V using a Mini Protean II electrophoresis apparatus (Bio-Rad). Running buffer consisted of 1 M Tris base, 1 M Tricine, and 1 % SDS, pH 8.2. Proteins were transfered to Zeta probe membrane (Bio-Rad Laboratories) using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) at 300 mA for approximately 2 hours in transfer buffer (191 mM glycine, 25 mM Tris, 20% methanol and 0.05% SDS). Membranes were washed overnight in 4% BSA in THS buffer (100 mM Tris, 500 mM NaCl, 10 mM glycine, pH 8.0) or 5% milk protein in 10 mM Tris-HCl pH 7.4, 140 mM NaCl and 0.1% Tween 20 to reduce non-specific binding to the membrane during probing of the blot.

After incubation, nitrocellulose membranes were incubated with BMP-2 antibody (h4b2) which had been diluted to 1.0 µg/ml in THHST buffer (100 mM Tris, 1.5 M NaCl, 10 mM glycine, 0.05 % Tween 20) pH 8.0 for two hours. Blots were washed in THHST at room temperature 3 X 10 minutes. Alkaline phosphatase-conjugated goat anti-mouse IgG diluted 1:1000 in

THHST was added and incubated at room temperature for 3 hours. Sheets were then well washed in THHST. Bands were visualized using the alkaline phosphatase reagent BCIP-NTB (Sigma #B5655). After bands appeared, blots were washed in distilled water and stored in plastic wrap for analysis.

Recombinant human BMP-2 used as a positive control in Western blotting was provided by Genetics Institute, Cambridge, Mass.

2.3. Results

Chick embryo *BMP-2* and *TGF-β4* mRNA was screened by hybridization to specific radiolabeled probes. Approximately 20 µg total RNA derived from pooled samples of chick embryonic tissue was used. The *BMP-2* specific probe hybridized to transcripts of approximately 3.2 and 2.6 kb (Fig. 4). In addition, a third band of approximately 6.1 kb was seen. These transcripts were seen in embryos ranging from stages 16 through 32 although bands were most intense after stage 18. Prior to stage 16, no transcripts were detectable. *TGF-β* probes hybridized to a single transcript of approximately 1.9 – 2.2 kb (Fig. 5) In addition, a second intense signal was setected at approximately 4.2 kb, co-migrating with 18S chick ribosomal RNA and may represent non-specific hybridization. Similarly to *BMP-2*, the intensity of the band was low at stage 14 and increased through stage 27.

Western blots show that h4b2 antibody recognized recombinant human BMP-2 (Fig. 6C). On denaturing gels, bands representing 2 monomers of 14 and 18 kDa were seen, as well as a triplet of 28, 32 and 38 kDa denoting the possible dimers. Controls (goat serum, ovalbumin) were negative. Blots of embryonic mandibular and limb bud protein showed banding around 40 kDa (Fig. 6D).

2.4. Discussion

BMP-2 transcripts of 3.2 and 2.6 kb were detectable in whole embryo total RNA. These values agree with those reported by Francis et al. (1994). In addition, a single band of approximately 6.1 kb was detected. The identity of this transcript is not known. A transcript of this size has not been reported and may represent non-specific hybridization of the probe with RNA not related to *BMP-2*.

TGF-β1 probes hybridized to a single band of approximately 1.9 kb, consistent with previously reported values (Fig.5) (Choy et al., 1991; Jakowlew et al.,1991). Five TGF-β isoforms were originally proposed, based on similarities to TGF-β1. All were derived from different species and named TGF-β1 through TGF-β5. It appears that TGF-β4, isolated from chick, is in fact the homologue of mammalian TGF-β1 (Burt and Paton, 1992). In the present study, a mammalian *TGF-β1* probe producing a single band of approximately 1.9 kb was therefore probably detecting chick *TGF-β4*. Despite the fact that at least 4 isoforms exist, all TGF-β proteins display comparable activities in different systems and may have similar developmental roles (Massague, 1990). The effects can be stage specific. Cells of one developmental age may respond differently to an isoform than the same cell types from a later or earlier stage (Chimal-Monroy and Díaz de León, 1997).

On Western blots, recombinant human BMP-2 is bound by h4b2 antibody. The size is consistent with that reported for human BMP-2 and Xenopus BMP-2. Controls (ovalbumin or goat serum) remain negative. Chicken embryonic protein exhibits bands at about 38 – 40 kDa, a size slightly larger than that reported for human BMP-2 dimers, which range from 28 – 36

kDa. The 40 kDa protein may represent a form of chick embryonic BMP-2 or BMP-2 dimerization with other members of the superfamily. The lack of bands representing monomers is puzzling but may reflect the low levels of protein in the samples. In addition, the extreme stability of the oligomers may make disociation of subunits difficult.

In summary, both *BMP-2* and *TGF-β* transcripts are present in the chick embryo during the times studied. In addition, Western blot results suggest that BMP-2 protein is expressed in embryonic mandible and limb bud.

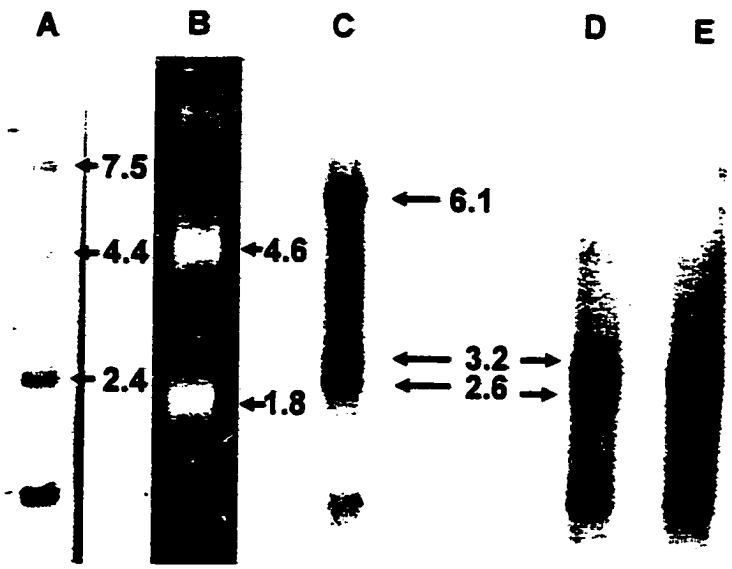


Figure 4: Northern blot of stage 21, 26, and 32 whole embryo total RNA probed for BMP-2 transcripts. Sizes are indicated by arrows and are given in kilobases (kb).

A) RNA ladder

B) Ethidium bromide stained gel of stage 26 whole embryo total RNA, indicating sizes of chick ribosomal RNA.

C) Northern blot of stage 26 whole embryo RNA transferred to membrane and probed with radiolabeled BMP-2 riboprobe. A doublet composed of transcripts of approximately 3.2 and 2.6 kb was seen, agreeing with those recorded in chick limb bud (Francis et al., 1994). In addition, a large transcript of approximately 6.1 kb was evident. Approximately 20 μg of RNA was loaded in (C), (D), and (E).

D) and E) Blots of stage 32 and 21 whole embryo total RNA respectively, probed for BMP-2. As in (C), two bands of 3.2 and 2.6 kb were evident.

Figure 5

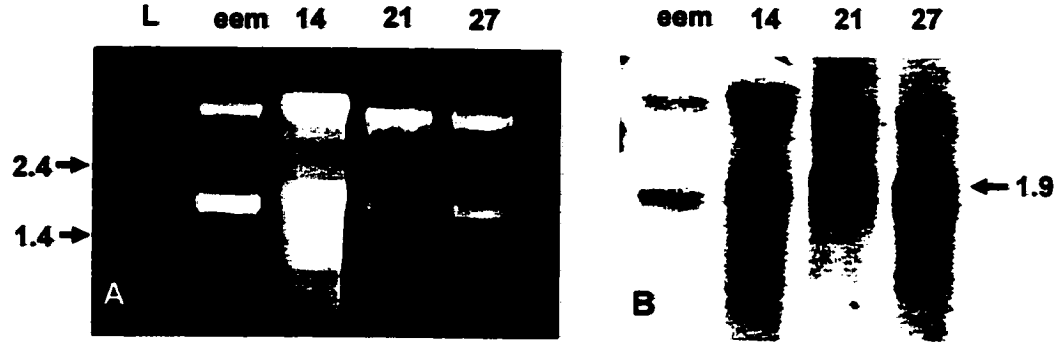


Figure 5: Northern blot of chick whole embryo RNA probed with radiolabeled TGF-β1 probe.

A) Ethidium bromide stained blot showing the ribosomal bands. RNA ladder (L) was in the first lane, with bands of 2.4 and 1.4 kilobases indicated by arrows. Extraembryonic membrane (eem) and embryo stages 14, 21, and 27 are indicated above lanes in both (A) and (B). Approximately 20 μg of RNA was loaded in each well.

B) Northern blot probed with radiolabeled TGF-β1 riboprobe. No signal was detected in either eem. A low level signal of approximately 1.9 kb was seen in stage 14 embryonic RNA as well as stronger signals in stages 21 and 27 embryos. The bands above appear to co-migrate with 18S ribosomal RNA and may indicate non-specific binding. This band was equal in size to chick TGF-β4 mRNA, the avian equivalent of mammalian TGF-β1.

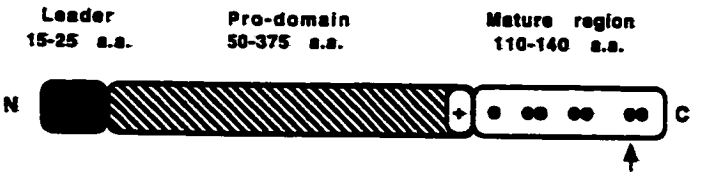
Figure 6: BMP superfamily protein structure and Western blots

A) General structure of TGF-β superfamily polypeptide chains. The precursor protein is divided into three regions of variable length, depending on the specific member. The leader sequence (black) precedes a prodomain (hatched). The region making up the mature protein (white) is cleaved from the precursor at a cluster of basic residues (+). Conserved cysteines are shown as black dots within the mature region.An arrow indicates the cysteine responsible for disulphide bond formation between monomers. N = amino terminal; C = carboxy terminal.

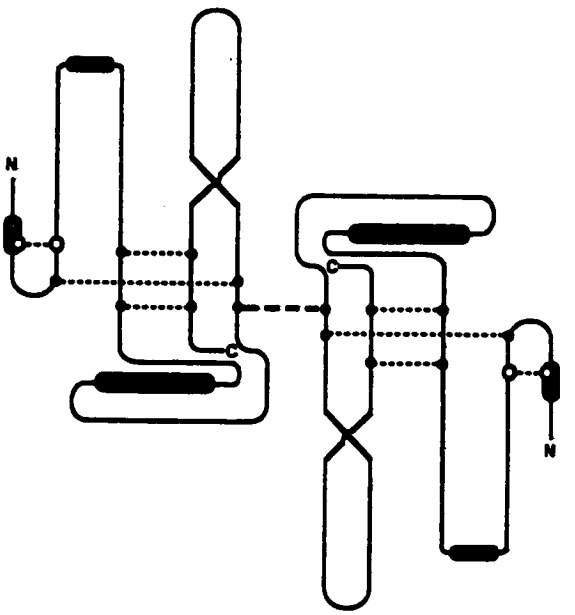
B) Schematic diagram of dimer formation resulting from the interaction of two mature proteins, held together by a disulphide bond (heavy dashed line). Disulphide bonds within the monomers are represented by light dashed lines. Cysteine residues are represented by circles. Hatched areas are α-helices.

C) Western blot of chick mandibular and limb bud protein probed withBMP-2 antibody. Lane 1 = stage 23 mandible; lane 2 = stage 23 limb bud; lane 3 = stage 26 mandible; lane 4 = stage 26 limb bud. Major band of approximately 40 kDa is indicated.

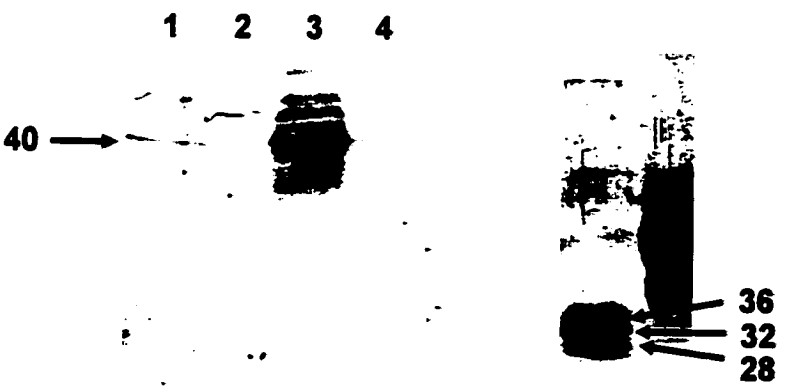
D) Recombinant human BMP-2 probed with BMP-2 antibody. Monomers (18 and 14 kDa) and dimers (36, 32, and 28 kDa) are indicated.



A



B



C

D

Figure 6

Chapter 3: Immunohistochemical detection of BMP-2 and TGF-β in developing chick embryos.

3.1. Introduction

Studies of gene expression have indicated the appearance of growth factor specific mRNAs at sites of epithelial/mesenchymal interactions. Immunohistochemical analysis of the occurrence of BMP and TGF-β during development of vertebrate embryos are however lacking. It is important to establish the presence of growth factor protein in the embryo,to localize it, then to compare this to gene expression at specific sites.

Because growth factor gene expression takes place in a specific area does not necessarily establish the location of the growth factor protein in the same region. Proteins may be produced in specific cells, secreted, and affect other sites distant from the source. Cells receptive to growth factor signals may respond to a gradient of diffusing cytokines but do not inevitably produce the same factor themselves. Similarly, growth factors may be exported and sequestered in a secondary site, for example the extracellular matrix, allowing migrating or otherwise interacting cells access to the proteins. In this way, a physical range of growth factor action is suggested.

Secondly, the specificity of antibodies allows blocking of specific growth factor *in vivo* and *in vitro*. Some other methods of repressing growth factor action rely upon inhibition of intracellular signal transduction components that may be shared by other growth factors. Guanine nucleotide-binding (G) proteins, for example, form a family of signal-transducing molecules linked to a variety of cell surface receptors which may use identical downstream effectors (Gilman, 1987; Bourne et al., 1990; Kolodziejczyk and Hall, 1996). G-protein signalling, for

example, may be inhibited with pertussis toxin, but since so many other molecules use the same intracellular cascade (or parts of it) such studies may not be as specific (Gilman, 1987). It has been suggested that TGF-β superfamily members utilise G-proteins as part of their signalling cascade but, given the variety of family members as well as other factors which may use the same system, inhibition with pertussis toxin (PT) could yield inconclusive results.

In situ localization of *BMP* transcripts during limb and mandibular development in the mouse and chick have been reported, but to date no immunohistochemical data has been presented. The immunochemical detection of TGF-β has been characterized in a number of tissues although these are generally limited to adult animals. Embryologically, much remains unresolved. In this chapter, regions of developing embryos expressing BMP-2 and TGF-β protein are determined. Mandibles and limb buds are the primary focus. In addition, other regions of interest, notably the vertebral column and somites are discussed.

3.2. Materials and methods

3.2.1 Preparation of embryos

Fertilized eggs of the domestic fowl, *Gallus domesticus* , were obtained from Cook's Hatchery (Truro, Nova Scotia) and incubated at 36° C and 95% humidity in a Favorite Incubator model 30E (Leahy Manufacturing Company, Higginsville, Mo.). Eggs were opened, embryos removed and transferred to disposable Petrie dishes (Fisher Scientific Company, Nepean, Ontario) containing chick Ringer's solution (New, 1966) and staged according to Hamburger and Hamilton (1951).

3.2.2. Immunohistochemistry

3.2.2.1. Antibodies

Murine monoclonal antibodies against human BMP-2 were obtained from Genetics Institute, Cambridge, Massachusetts. One antibody, designated h4b2 was found to return a more specific and intense signal than others tested and was used for subsequent studies. Specificity was determined by immunostaining sections through the mouse craniofacial region and looking for signals in the developing whisker follicles. In addition, chick sections treated with different BMP-2 antibodies were compared to determine the extent of background staining and signal intensity. Pan-specific TGF-β antibody raised in rabbits was obtained from R & D Systems (# AB-100-NA). TGF-β immunostaining showed that the distribution of the growth factor is widespread throughout many regions of the embryo, especially at the very earliest stages tested. In order to establish that this distribution was unique, some sections were also incubated with rabbit anti-skeletal myosin antibody (Sigma #M7523) to provide a comparison. After testing serial dilutions, it was determined that BMP-2 (h4b2) antibody was most effective at a final dilution of 1: 100 from an original stock of 1.3 mg/ml. TGF-β was applied at a dilution of 1:100 from an original concentration of 1 mg/ml. Anti-myosin was used at dilutions of 1:200 from an original stock concentration 1.0 mg/ml.

3.2.3. Preparation and sectioning of specimens

Embryos used for immunostaining were fixed in 4% paraformaldehyde or

70% methanol at 4^0C for at least 30 minutes. Specimens fixed in paraformaldehyde were not used for procedures using fluorescently labelled secondary antibodies to avoid autofluorescence which is sometimes associated with aldehyde fixation procedures. In some cases, embryos were dissected in sterile Ringer's prior to fixation. Embryos or tissues were dehydrated in an isopropanol series ending in Histoclear (National Diagnostics, Atlanta, Georgia). Vacuum embedding was carried out at 43^0C using low temperature paraplast. Sections were cut at 5μm.

Sections were mounted on poly-L-lysine coated slides and dried overnight at 37^0 C prior to immmunostaining. Slides were deparaffinized in Histoclear followed by a graded series of ethanol washes ending in distilled water. Anti-BMP-2 primary antibody was diluted in phosphate buffered saline (130 mM NaCl, 10 mM, 130 mM NaH_2PO_4, pH 7.4) containing 1% BSA (Calbiochem, La Jolla, California) or 10% non-immune goat serum (Vector Labs, Burlingame, California) to block non-specific staining. TGF-β antibody stock was reconstituted in PBS at pH 7.4 and diluted to working concentrations in the same buffer with the addition of 10% non-immune rabbit serum.

For BMP-2 immunostaining, it was found that subjecting the sections to proteolytic digestion prior to antibody addition provided a more intense signal, possibly by increasing accessibility to epitopes. Digestions were accomplished with 0.1% trypsin (BDH, Poole, England) and 0.1% $CaCl_2$ in PBS, pH 7.8 in a solution prewarmed to 37^0C prior to use. Digestion times varied from 5 to 45 minutes, depending on the degree of digestion required and were carried out in a humid chamber at 37^0C. In general, digestion times of 20 - 30 minutes were sufficient. Longer times often resulted in overdigestion of mesenchymal cells and/or their detachment from the slide. By controlling the digestion, it was possible to remove most of the epithelium and mesenchyme, leaving behind the

epithelial basement membrane alone. Sections were rinsed in cold PBS to remove enzyme and stop the digestion process.

Barriers were drawn around sections on the slides using a PAP pen (Daido Sangyo Co. Ltd., Japan) to minimize reagent volume and prevent sections flowing off the slide. For both BMP-2 and TGF-β antibodies, undiluted goat serum or 1% BSA in PBS, pH 7.4 was applied to sections to block non-specific binding and the slides were incubated at room temperature for 30 minutes. After blocking, slides were tipped and blotted to remove excess solution but not rinsed. Primary antibodies were diluted to final working concentration in PBS, pH 7.4, containing 10% goat serum, added to slides and sections incubated at 37 ^{0}C for 2 hours or overnight at 4 ^{0}C in a humidity chamber.

After incubation, sections were rinsed in PBS (4 x 5 minutes) and secondary antibodies were applied. All secondary antibodies were diluted to working concentrations in PBS, pH 7.4 containing 10 % goat serum. Initially, alkaline phosphatase secondary antibody was tested but endogenous activity was too great even when blocked with levamisole. For BMP-2 immunostaining, secondary antibodies used were conjugated to FITC, immunogold beads or biotin. For TGF-β immunostaining, biotinylated goat anti-rabbit secondary antibodies were used. Slides were incubated at room temperature for 30 minutes to 2 hours. FITC secondary antibody was applied at a concentration of 1 : 200 and incubated in the dark. After incubation, slides were rinsed in PBS (4 x 5 minutes). FITC stained sections were mounted and analysed under an fluorescence microscope. Sections stained with 10 nm immunogold bead-conjugated secondary antibody (Sigma Chemical Co., St. Louis, Missouri, # G7777) at a dilution of 1:50 in PBS. were enhanced with silver staining using the Silver Enhancer Kit (Sigma Chemical Co. #SE-100) prior to mounting.

Biotinylated sections were visualized with the avidin-biotin –peroxidase complex immunohistochemical method using the Vectastain Elite ABC kit (Vector Labs, Ca.). Briefly, after incubation with ABC reagents for 30 minutes at room temperature, sections were rinsed in PBS and treated with DAB (10 mg in 20 mls 0.05M Tris-HCL, pH 7.6, containing 4 µl 30% H_2O_2) for 2 – 5 minutes at room temperature to produce a brownish-red precipitate. Endogenous peroxidase activity was assessed by treating some sections with the ABC kit and DAB alone. Only what appeared to be kidney tubule precursors showed peroxidase activity. Biotinylated goat anti-mouse and goat anti-rabbit antibodies were used at dilutions of 1: 200.

Prior to mounting, sections were rinsed thoroughly in PBS and mounted with Crystal/Mount (Biomeda, Foster City, California) or with entolin overlain with glass cover slips. Slides were analyzed and photographed using a Leitz ARISTOPLAN microscope equipped with 3-1 Ploempak incident-light fluorescence illuminator and Wild MPS 46 Photoautomat. Photographs were taken with ASA 64 Kodak Ektachrome Tungsten film. Prints were captured on a UMAX Astra 1220 S flat bed scanner using Adobe Photoshop, version 4.0.

3.3. Results

3.3.1. BMP-2 localization

Localization of BMP-2 protein in the developing mouse vibrissae was confined to the epidermal components including the perimeter of the follicle shaft and the bulb of the follicular matrix surrounding the dermal papilla. The papilla itself remained free of signal, as did the central core of the follicular shaft. The staining in the shaft perimeter corresponded to the location of the root sheath (Fig. 7). In addition, staining was seen in the raised epidermal cap

directly above the follicle. This cap will ultimately extend down towards the growing follicle and produce a canal through which the hair can grow to the exterior.

TGF-β was readily detectable throughout much of the developing embryo as early as stage 11 (Fig. 8A). This was especially evident in the anterior of the embryo, including the brain and developing visceral tissues. Posteriorly, somites remained relatively free of TGF-β signal, as did the epidermal ectoderm of the embryo (Fig. 8A). In the chick mandible, immunostaining with h4b2 antibody did not produce an easily detectable signal until at least stage 14. Prior to this stage, mandibular primordia were devoid of staining, as was the entire embryo (Fig. 8B). Sections of the anterior neural tube and developing brain exhibited low levels of staining when detected using biotin conjugated secondary antibodies. No signal was detectable in the somites at this stage.

By stage 14, lateral sections through the center of the mandible revealed BMP-2 staining in the epithelium of the arch. This signal was evident in the epithelium of the junction between the mandibular and hyoid arches (Fig. 9A). By stage 18, much of the epithelium surrounding the growing mandibular arch stained positively for BMP-2 (Fig. 9B). Signal was beginning to appear in the mandibular mesenchyme as well, underneath the BMP-2 positive epithelium. Controls in which no primary antibody was added remain negative (Fig. 9C). Proteolytic digestion using pancreatin and trypsin separated the epithelium from the mesenchyme. Immunostaining and detection using FITC labeled secondary antibody revealed a very brightly staining epithelium around both the mandibular and hyoid arches (Fig. 9D).

The epithelium of stage 20 – 21 mandibles exhibited staining for BMP-2 as did more of the arch mesenchyme than seen in earlier embryos, especially in regions near the epithelium (Fig. 10). Epithelial basement membrane was

brightly stained at this stage, suggesting BMP-2 protein associated with the extracellular matrix. While epithelial staining was evident in earlier sections, it was only around stage 19 – 21 that intense staining was seen in association with the basal regions of the epithelial cells and indeed with the basement membrane. That BMP-2 was translocated to localize in the basement membrane matrix is better illustrated in frozen sections with silver grain-conjugated secondary antibodies. When enhanced with immunogold beads, a precise location at the basal side of the epithelium was seen (Fig. 11A). BMP-2 staining was evident within the hyoid epithelium and, to a lesser degree, within the hyoid mesenchymal cells. In addition, bead localization in the underlying mesenchyme was clearly shown, with concentrations falling off further away from the arch epithelium (Fig. 11B). Careful examination of the epithelium revealed regions where beads appeared to be on both the proximal and distal surfaces of the epithelium and others where staining was restricted to the proximal, or basal, surfaces.

BMP-2 signal was detectable in the epithelium up to at least stage 27. Mesenchymal signal increased reaching a peak around stage 21, although it appeared to become more diffuse towards the center of the mandibular process. Mesenchymal signal remained highest in proximity to epithelial cells expressing the protein. In addition, BMP-2 signals appeared within the mensenchyme of later stage mandibles deeper in the arch in regions far from the epithelium. These regions however appear to be in close association to blood vessels, the endothelium of which stained positively (Fig. 18A). In addition, sub-populations of chick embryonic blood cells showed positive BMP-2 staining (Fig. 18B).

3.3.2. TGF-β localization

TGF-β imunostaining was widespread in the embryo. To provide a

contrast against which TGF-β antibody localization can be compared, some sections were immunostained for myosin. In longitudinal sections of stage 15 embryos for example, regions of TGF-β and myosin staining differed substantially (Fig. 13). Overlap was seen in some regions, most notably the region of the vertebral column and somites, although even here the distribution is not identical. The myosin staining patterns within the somites of the stage 15 chick embryo, when viewed in lateral section, describe a semicircle confined to the most ventral portions of the myotome. TGF-β staining was, on the other hand, restricted to a crescent shaped domain in the somitic region just dorsal to the spinal cord (Fig. 12). This exclusivity of staining domains was seen with respect to BMP-2 localization.

TGF-β was first detected in the developing chick mandible at stage 13 - 14 when the mandibular arch was beginning to form as an outpocketing of tissue from the ventral head fold (Fig. 13A). Staining was seen at the apex of the expanding arch within the mandibular mesenchyme and especially in those cells near the epithelium, but not in the epithelial cells themselves (Fig 13B). As the arch begins to grow, much of the associated mesenchymal cells immunostain positive for TGF-β protein. By stage 19 the mandible has expanded greatly and the TGF-β signal is primarily restricted to the mesenchymal cells around the perimeter of the arch although some low level staining was detectable in more proximal cells as well (Fig. 14A). Again, there was no evidence of TGF-β staining in the mandibular epithelium. As the mandible increased in size, staining around the periphery of the arch continued but appeared to fade in intensity. By stage 22, the core was negative but the deep staining at the edges persisted. Throughout this period, TGF-β never appeared to be associated with epithelial cells (Fig 14).

Limb buds exhibited similar domains of TGF-β expression to those seen in mandibles. By stage 22, TGF-β expression was seen in limb mesenchyme,

especially dorsally. There was no evidence of TGF-β in the epithelium (Fig. 15). Mesenchymal expression of TGF-β eventually became restricted and disappeared from the core mesenchymal cells. By stage 25, the entire perimeter of the expanding limb stained positively to a depth of 5 – 10 cell diameters (Fig. 15). Epithelial expression of TGF-β was still negative at this stage. BMP-2 was present in the limb but was detectable in the epithelium as well as in the underlying mesenchyme. Unlike TGF-β, BMP-2 is expressed in the AER of the limb bud in regions that show little overlap with the domains of TGF-β protein expression (Fig 16A, B). The signal remained confined to this region until at least stage 25.

At stage 29, digit formation was well underway and condensations of cells destined to become skeletal elements were forming in the limb. BMP-2 was undetectable in the limb at this stage but TGF-β staining domains have shifted. Peripheral staining was still intense but staining was beginning to appear around areas of digital skeletal elements. Epithelial staining remains negative (Fig. 15). Stage 27 mandible weakly stained for BMP-2 and was most intense in the rostral epithelium and the mesenchyme directly underneath. In particular, the basement membrane of this region exhibited particularly intense BMP-2 staining. TGF-β in the stage 27 mandible was found in the mesenchymal perimeter but not in the epithelium. It also appeared around the areas where mandibular skeletal elements will form, for example, Meckel's cartilage (Fig 14).

3.3.3. Immunostaining in other regions of chick embryos

Although the majority of the analyses involved the localization and distribution of TGF-β and BMP-2 within the mandible and limb buds, other regions of the embryo exhibit immunostaining for these factors. Among areas of interest were the somites. TGF-β was localized to the somites as early as stage 11 when a faint signal was detected around the border (Fig. 8A). By stage 15,

longitudinal sections reveal that TGF-β was localized in what appears to be the ventral myotome, with a distribution markedly different from that seen for skeletal muscle-specific myosin (Fig. 16A, B). In cross sections through stage 25 embryos, the TGF-β domain encompassed the myotome and the region around the notochord. Distribution of BMP-2 signal was different from that seen for TGF-β. Myotomes lacked BMP-2 protein but the dermatome and the dorsal tips of the neural tube stained positive. (Fig. 17). Unlike TGF-β, no BMP-2 staining was associated with the notochord. The dorsal aorta (endothelium) appeared faintly positive for BMP-2.

As mentioned above, one of the most consistent sites of BMP-2 localization was the endothelium of the embryonic blood vessels, and mesenchymal cells surrounding the vessels. In some cases, a diffuse signal was noticeable at a distance of several cell diameters from the endothelium (Fig. 18A). In addition, some embryonic blood cells exhibited BMP-2 immunostaining (Fig. 18B). These cells looked like leukocytes based upon their resemblance to adult avian blood components. A specific characterization of these embryonic cells, which may differ substantially from adult forms, was not done. Both TGF-β and BMP-2 localized to the developing avian heart (Fig. 18 C, D). TGF-β was found in the endocardium of embryos and was detectable as early as stage 13. BMP-2 immunolocalized to the heart myocardium but not endo- or pericardium. BMP-2 first appeared in this region at around stage 14 and persisted through at least stage 19. By stage 22, BMP-2 wasnot detectable in the heart. BMP-2 was seen in stage 17 chick embryos at a site and time consistent with the appearance of the thyroid primordia (Fig. 19). The signal was strong but transient and not specifically notable in later embryos. In the stage 19 embryo, BMP-2 was detected in an 'inclusion' or dense cluster of cells residing in the epithelium forming the cleft between the hyoid and mandibular arches (Fig. 19). Its precise remains unknown.

3.4. Discussion

The facial vibrissae of the mouse are composed of epidermal hair matrix surrounded by an outer and inner root sheath, and the dermal papilla at the base (Davidson and Hardy, 1952). Initially, an epidermal thickening produces the placode that grows down from the surface. An aggregation of mesenchymal cells at the base of the elongating hair plug forms the dermal papilla. A molecular signal from these mesenchymal cells directly under the region, the 1st dermal message initiates placode formation. It has been suggested that BMP-4 is a candidate for this first initiation signal (Jones et al., 1991). The so-called epidermal message produced by the epithelial cells may organize and promote the elongation of the expanding follicle (Hardy, 1992). BMP-2 mRNA is localized in the early placode and subsequently throughout the migration of these cells to form the hair matrix, and inner root sheath (Jones et al., 1991; Lyons et al., 1990). It is precisely these regions where BMP-2 protein is detected with immunostaining confirming specificity of the h4b2 antibody. BMP-2 transcripts are not seen in the dermal placode or through the center of the follicular canal. A second dermal message then passes from the dermal papilla to the adjacent epithelial cells, the matrix, and is responsible for the differentiation of the sheath and formation of the keratinized hair.

BMP-2 has been implicated in bone and cartilage development and maintenance. The fact that BMP-2 and TGF-β are detectable well in advance of the first appearance of bone or cartilage in the limb or mandible and their localization in non-skeleton forming regions suggests an additional function for these factors. The detection of these factors along the borders of the growing limb buds and mandibular (and hyoid) arches suggests an involvement in the initial patterning of

these structures.

In situ studies with embryonic mice have indicated that BMP-2 transcripts appear not only in the developing face and head but in many other regions. In studies of *BMP-2* and *–4* expression in the mouse embryo, transcripts were found in many of the same regions and at comparable developmental times to those seen in the chick. In addition, the *in situ* data support the immunohistochemical localization of BMP and TGF-β presented here.

BMP-2 is found in murine mandibular processes at about day 10.5 post-coitum, associated with neural crest-derived mesenchyme and in the limb bud apical ridges as well as in heart primordia (Bennett et al., 1995). In later stages of development, transcripts are seen in the facial mesenchyme as well as mesenchymal condensations destined to form Meckel's cartilage (Lyons et al., 1990). Limb bud expression is seen in thickening ventral epithelium, prior to formation of the AER, at approximately embryonic day 9.5 (Lyons et al., 1990; Bennett et al., 1995). Eventually, BMP-2 expression becomes localized to the AER. Expression in the epithelium fades as mesenchymal expression increases and eventually is seen in the interdigital mesenchyme (12.5 d.p.c.). (Lyons, et al., 1990).

BMP-2 and *–4* mRNA expression during development of the chick face has been examined (Francis-West et al., 1994). During chick development, *BMP-2* expression is detectable in specific epithelial regions of facial primordia by at least stage 16/17. Expression was seen in sections through the centre of the mandible but not laterally. Mesenchymal expression was seen through to stage 24 as a diffuse signal in the mandibular mesenchyme but appeared more intense in the area bordering the hyoid arch. By stage 28, transcripts were restricted to lateral mandibular primordia. In general, *BMP-2* expression in the

mesenchyme was preceded by expression in the associated epithelium (Francis-West et al., 1994

Immunohistochemical localization of BMP-2 protein with h4b2 antibody is first seen at stage 14 and persists until at least stage 28. Distribution of *BMP-2* transcripts in the above studies appears to correlate with the localization of BMP-2 protein, occurring first in the epithelium and subsequently in the mesenchyme of the mandible. Limb bud expression of *BMP-2* is similar in that low levels of BMP-2 protein are seen in the epithelium

In situ localization of BMP-4 transcripts in some ways more closely parallels the immunolocalization for BMP-2 protein seen in the chick. In the mouse, BMP-4 transcripts are detectable in the epithelium at approximately day 9 p.c. and precedes expression in the mesenchyme (Bennett et al., 1995). Mesenchymal expression of BMP-4 increases slowly until day 10.5 p.c. in conjunction with a decrease and eventual cessation of mandibular epithelial expression. This corresponds to the time of osteogenic induction in the mouse embryo.

TGF-β 1, 2, 3, and 4 are expressed in chick embryonic myocytes and chondrocytes cultured from day 7 or later animals. (Jakowlew et al., 1991). This may in part explain the visualization of TGF-β in somites, specifically the myotome. In the late stage mandible and limb bud, TGF-β localizes in regions destined to form cartilage. This may be due to a direct expression of TGF-β in chondrocytes. It appears from immunostaining data that TGF-β staining is confined to the perimeter of the developing skeletal element, perhaps indicating that chondrocyte expression of the factor is limited to the perimeter cells of the condensations (presumptive perichondrium).

TGF-β1 has been immunolocalized in the developing chick heart beginning

at stage 11 where it is appears in the endo- and epicardial cushions (Choy et al., 1991; Nakajima, 1997). Subsequently, staining is seen in the endocardial cushions (stages 18 – 26) and finally in heart valves (stage 36). Our study supports this, with staining noted in the developing endocardial tissue at stage 15. BMP-2 localization in the myocardium, as presented here, has not been reported to date. Heart development and formation then appears to involveme at least two members of the TGF-β superfamily.

Early in vertebrate development, segmental blocks of paraxial mesoderm lying on either side of the neural tube form somites, which will differentiate into specialized regions (Christ and Ordahl, 1995; Cossu et al., 1996). Dorsally, the somites will give rise to the dermomyotome, which will be the source of muscle precursor cells, including those of the limbs, hypaxial muscles and connective tissues and dermis of the back (Cossu, et al., 1996, 1997). Ventrally, somitogenesis produces the sclerotome. Sclerotome cells migrate to specific anatomical sites, form condensations and differentiate into chondroblast precursors. Cartilagenous components are replaced through endochondral bone formation to form the ribs, scapulae and vertebrae (Nifuji et al., 1997). Located under the dermomyotome is a specialized region derived from the dorsomedial somite adjacent to the neural tube called the myotome, which produces the first skeletal muscle in the embryo.

Immunostaining of stage 25 embryo cross sections indicate the presence of BMP-2 protein in the dermatome and the dorsal tip of the neural tube. In addition a weaker signal is seen in what appears to be the ventral myotome. TGF-β on the other hand, is undetectable in the neural tube, dermatome, or sclerotome but present throughout the myotome.

In general, BMP-2 expression is seen in the epithelium and mesenchyme

during development of the mandible and limb buds although expression appears to be greater and more widespread in the mandible. Epithelial expression precedes mesenchymal expression. Furthermore, mesenchymal cells expressing BMP-2 protein tend to be adjacent to epithelial cells which are or were BMP-2 positive. A similar effect is seen in mesenchyme adjacent to BMP-2 positive blood vessel endothelium. This may indicate that BMP-2 is diffusing out from the epi/endothelium and into the surrounding tissue. Direct diffusion over a large distance (i.e., more than a few cell diameters) may be unlikely. In *Xenopus* for example, the TGF-β superfamily members activin and Vg1 are inducers of mesoderm in early development. While both factors have been shown to act on adjacent cells, they do not appear to travel through the extracellular spaces to induce distant cells (Reilly and Melton, 1996). Induction of distant mesoderm then may require the participation of a secondary signal, relaying information to remote cells. Similarly, *shh*, which affects anteroposterior patterning in the vertebrate limb and is localized in the AER, does not act via diffusion. Digit patterning is closely linked to shh expression and is concentration dependant, posterior digit formation correlating to high *shh* expression. This effect does not appear to be diffusion dependant as two forms of the shh protein having different diffusion distances produce identical effects in bead implantation assays in the chick wing bud (Yang et al., 1997). Patterning of distant cells may rely upon a secondary messenger, possibly a BMP. *Shh* upregulates BMP-2 expression in the vertebrate limb (Laufer et al., 1994).

TGF-β immunostaining indicates that this cytokine is rarely if ever associated with epithelium. In early limb bud and mandible, TGF-β is usually found in the perimeter mesenchymal cells. This may reflect its role in modelling developing structures by creating a 'border' or region of differential cell division at the edges. TGF-β may in this capacity, inhibit or control mitosis and thus prevent uncontrolled growth. TGF-β is expressed in regions of the embryo undergoing rapid

morphogenesis, including sites where there is extensive myogenesis and osteogenesis (Ellingsworth et al., 1986).

Localization of BMP-2 with immunogold labelled secondary antibodies indicates that the epithelial basement membrane may be an important storage area for the protein. That extracellular matrix components can influence development and differentiation is known (Adams and Watt, 1993). During heart development for example, TGF-β is sequestered by an extracellular protein LTBP-1 which appears to act as a storage site for unactivated TGF-β protein (Nakajima et al., 1997). TGF-β3 has been localized to the developing chick myocardium. Furthermore, the distribution of active TGF-β3 is limited but the latent form is found distributed throughout cardiac extracellular matrix (Ghosh and Brauer, 1996). Incubating tissue in an acidic medium results in TGF-β activation and suggests that a similar process *in vivo* allows the utilization of the protein from ECM stores as required. Reconstituted extracellular matrix is able to stimulate chondrogenesis in chick limb bud mesenchymal cells, although the effect is not blocked by TGF-β or FGF antibodies (Bradham et al., 1995). Whether or not this is related to BMP sequestered in the matrix remains to be seen.

In general, TGF-β never appears in cells of epithelial origin in either the developing chick mandible or limb bud. Mesenchyme expresses TGF-β protein in both mandibular and limb outgrowths. Initially much of the mesenchyme in the early primordia immunostains positively for TGF-β but this signal is eventually restricted to the peripheral regions of the growing mandibular arch or limb bud. The domains of expression within both the embryonic mandible and limb are strikingly similar and may reflect similar developmental processes. In the mandible, TGF-β is detectable by around stage 13, just as the arch is beginning to form. It is detectable in other regions of the embryo as early as stage 11.

BMP-2, in contrast to TGF-β, is detected in the epithelium prior to expression in the mesenchyme in both the developing mandible and limb bud. In the limb, expression of the BMP-2 protein is initially observed at the apical ectodermal ridge and subsequently expands around much of the epithelium, although concentrated near the AER. It appears that in the limb bud, BMP-2 continues to be expressed in the epithelium at low levels and in the mesenchyme below to a depth of about 5 cell diameters. In the mandible, a similar pattern of expression is seen except that more widespread mesenchymal expression is evident. Expression in the mandible is most intense between stages 19 and 24, then decreases steadily but persists to at least stage 27. Mesenchymal signal appears to increase in domain but concurrently decrease in strength.

Figure 7: Section through 14 d.p.c. mouse embryo lateral muzzle showing localization of BMP-2 in developing vibrissae. Secondary antibody was conjugated to immunogold beads and the signal amplified with silver grain enhancement.

A) Longitudinal section through mouse vibrissae showing the presence of BMP-2 around the perimeter of the follicle shaft (s) and localization within the overlying epithelium (E).

B) Section showing longitudinal sections through vibrissae. The vibrissa toward the top of the figure was sectioned through the outer root sheath whereas the lower vibrissa was sectioned directly through the bulb itself. Staining is evident in the bulb perimeter but absent in the dermal papilla. BMP-2 staining is evident in the shaft (s) but is primarily confined to the region of the bulb exterior and the shaft perimeter.

C) Detail of staining in the vibrissa bulb. BMP-2 was confined to the bulb matrix (m) and was absent in the dermal papilla (dp).

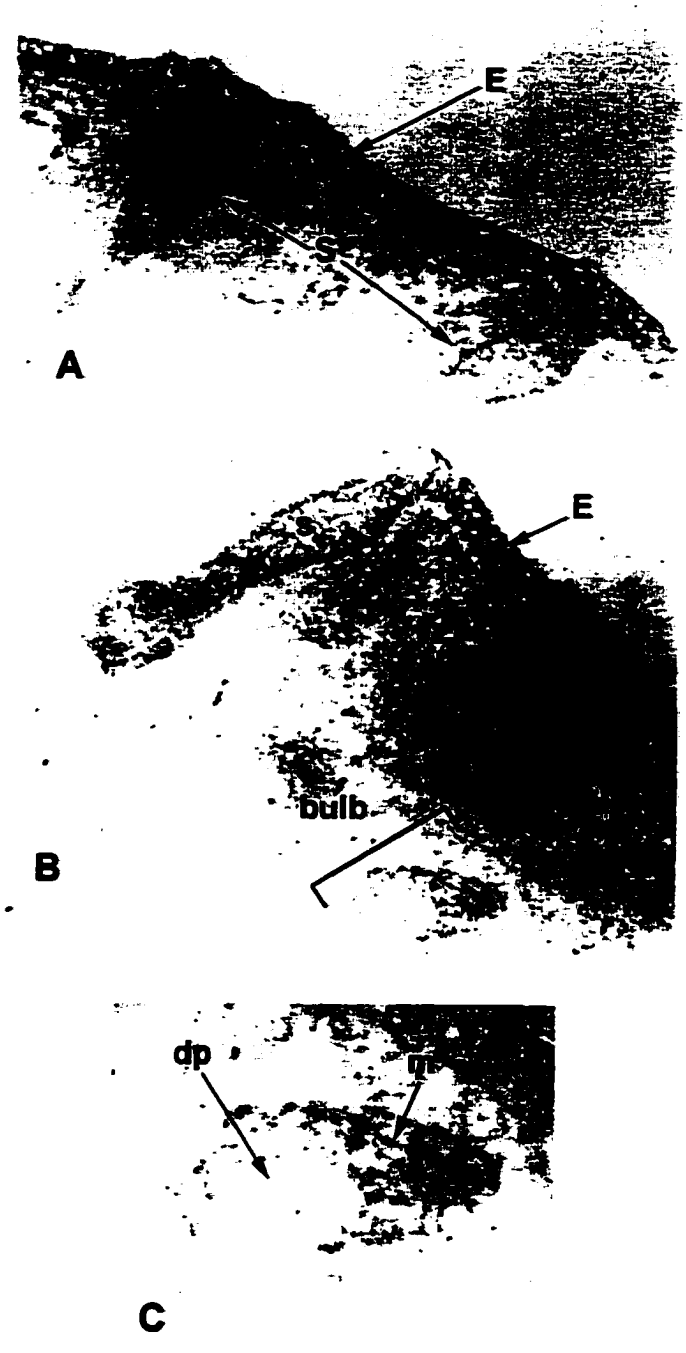


Figure 7

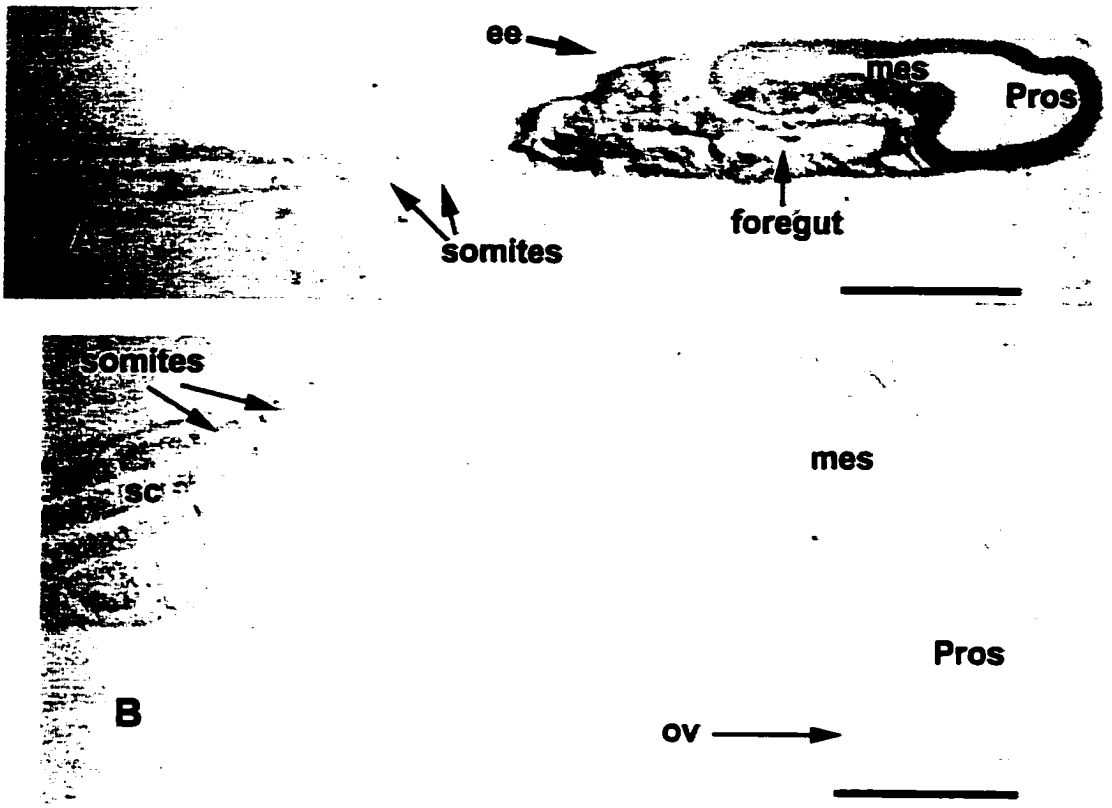


Figure 8: Longitudinal sections through stage 11 and 12/13 chick embryos stained for BMP-2 or TGF-β.

A) Section through midline of stage 11 embryo stained for TGF-β. Note staining throughout the developing mes/prosencephalon and foregut. Somites and epidermal ectoderm (ee) appear negative for TGF-β. mes = mesencephalon; pros = prosencephalon

B) Section through midline of stage 12/13 embryo midline immunostained for BMP-2. Staining was negative with exception of rostral neural tube, which exhibited very slight positive staining, indicated by white arrowhead. ov = optic vesicle; sc = spinal cord. Scale bars = 150 μm.

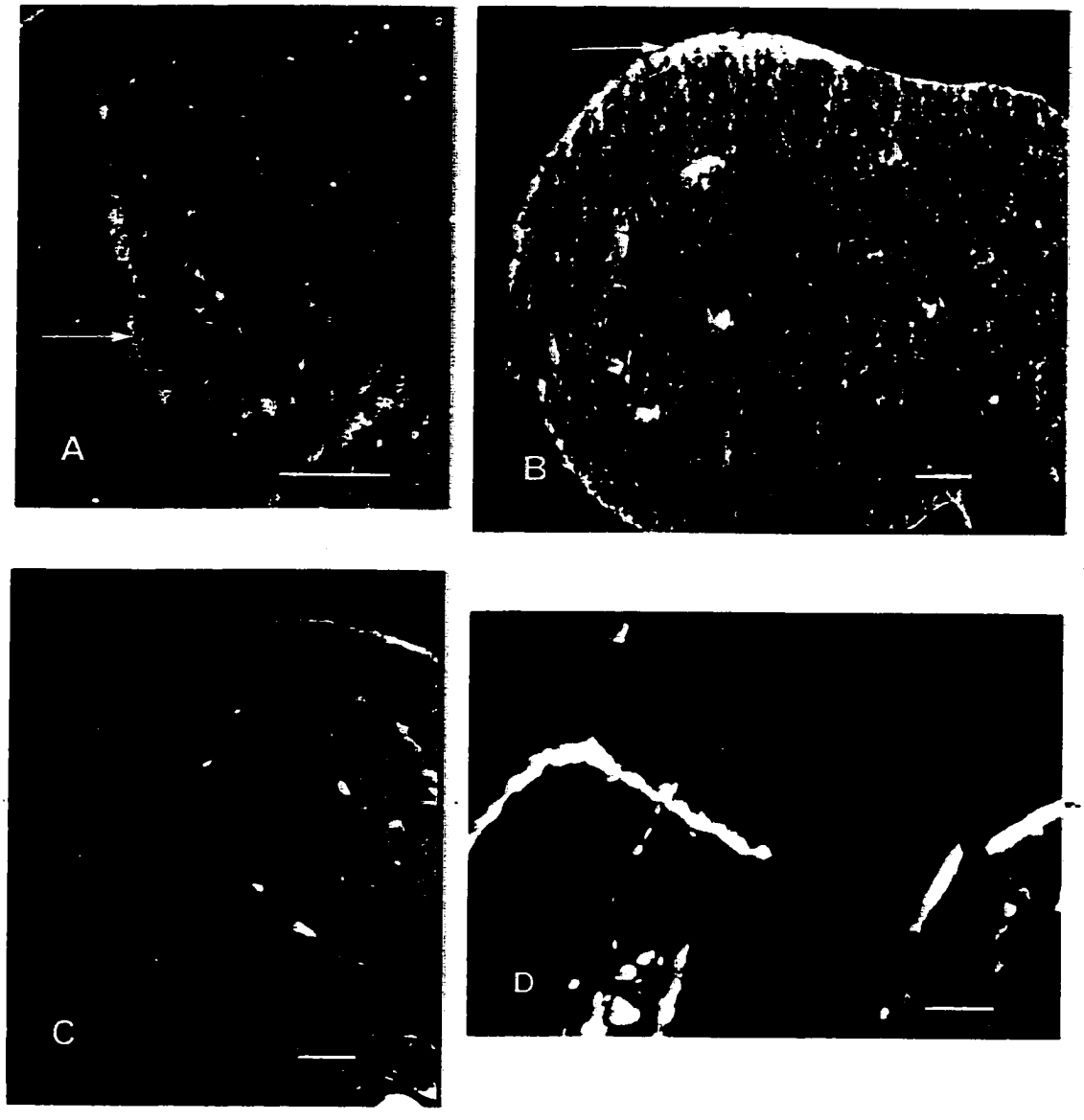


Figure 9: BMP-2 staining in sections through the middle of chick mandibular arches detected with FITC conjugated secondary antibody.

A) Stage 14 mandibular arch. Arrow indicates epithelium where initial BMP-2 signal was detected.
B) Stage 18 mandibular arch stained in apical region of epithelium (arrow).
C) Stage 19 mandibular arch control. No primary antibody was added prior to secondary antibody addition.
D) Epithelial layer from stage 19 mandibular arch immunostained for BMP-2. Epithelium was separated from mesenchymal cells with trypsin/pancreatin digestion. Scale bars =100 μm.

Figure 10: Chick mandible sections immunostained for BMP-2.

A) Longitudinal section through mandible of stage 17 chick embryo immunostained for BMP-2 using a FITC labelled secondary antibody. Epithelium was positive as was blood vessel (bv) endothelium, adjacent cells and blood cells within the vessels. Md = mandible; Hy = hyoid. Scale bar = 100 μm.

B) Section through stage 18/19 mandible showing staining in epithelium (E) and adjacent mesenchyme (MM) as well as low level staining in core mesenchyme. Note BMP-2 staining in mesenchyme around region of blood vessel and in blood cells on far right. Scale bar = 100 μm.

C) Section through stage 21 mandible. Note regions of intense staining in the apical region of the epithelium (E) and epithelial basement membrane (BM). Endothelium of blood vessels (BV) also exhibited positive staining. MM = mandibular mesenchyme. Scale bar = 250 μm.

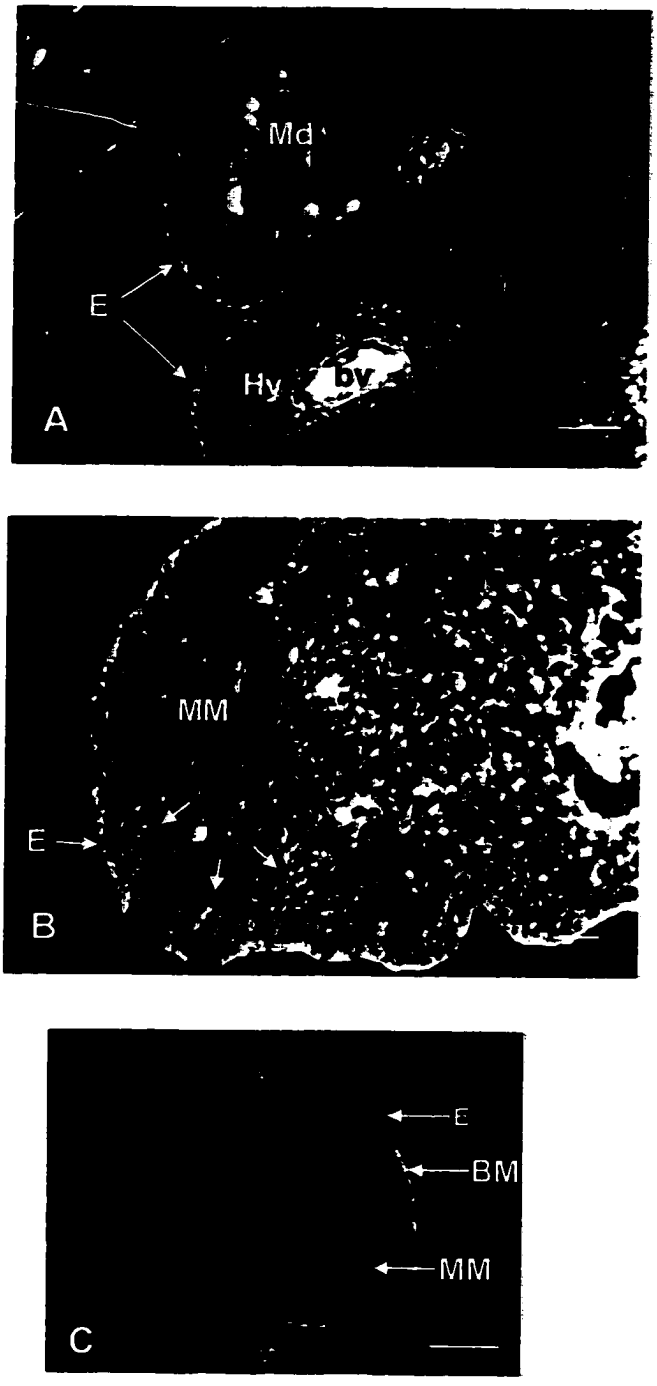


Figure 10

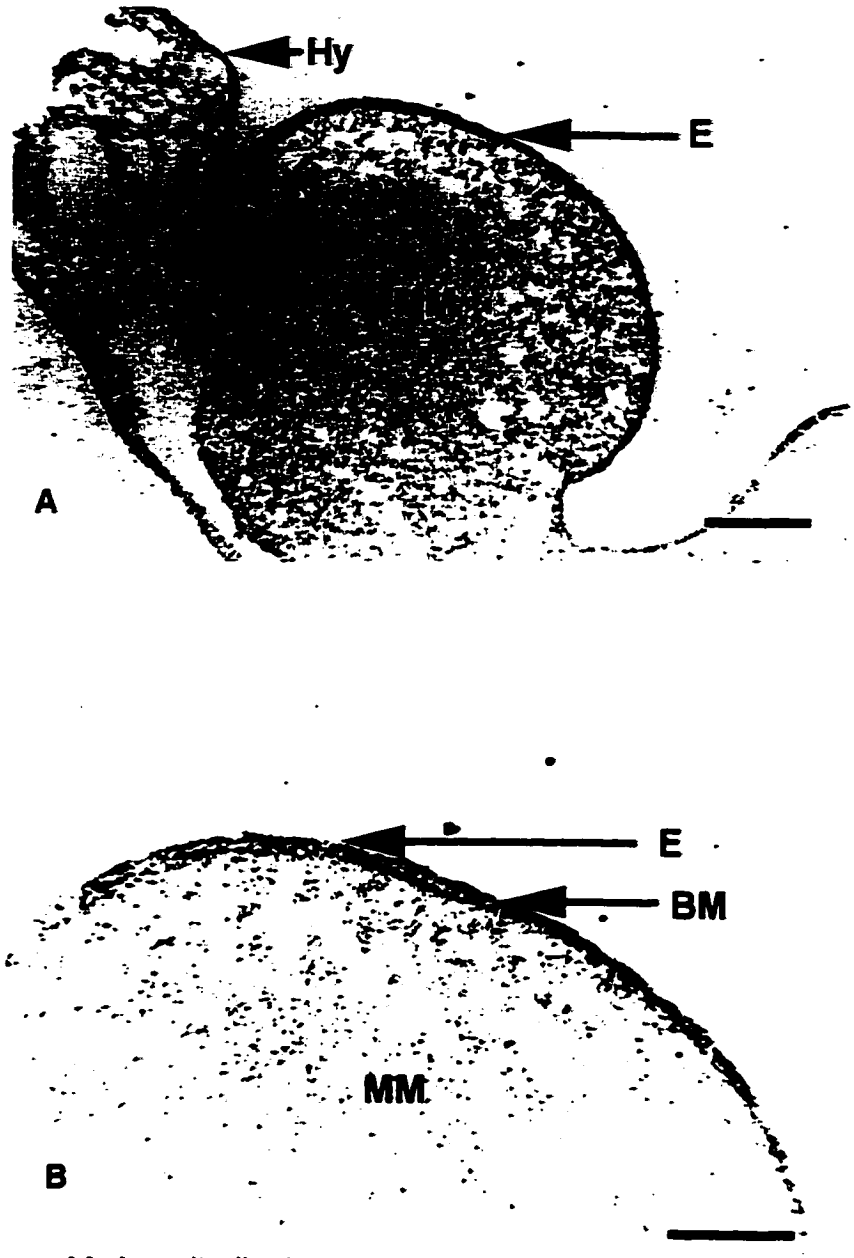


Figure 11: Longitudinal section through middle of mandibular arch of stage 19 chick embryo immunostained for BMP-2. Secondary antibody was conjugated to immunogold beads enhanced with silver staining.

A) Low magnification view of region of mandible showing dark staining in the epithelium (E) indicating positive staining. MM = mandibular mesenchyme; Hy = hyoid arch. Scale bar = 150 μm.

B) High magnification view of mandible. Note silver grains concentrating in the epithelium (E) and especially at the epithelial basement membrane (BM). Some silver grains indicating BMP-2 can be seen in the mesenchyme as well. Scale bar = 100 μm.

Figure 12: Composite images of longitudinal sections through stage 15 chick embryos.

A) Embryo stained for myosin exhibited intense staining throughout. Differences in staining intensity was seen in somites, and craniofacial region. Secondary antibody was conjugated to peroxidase. Note lack of staining in neural tube.

B) Embryo stained for presence of TGF-β. Highest intensity signal is seen in portions of somites although signal is apparent in regions of the head including mandibular and hyoid arches, as well as in neural tube and brain. Scale bar = 100 μm.

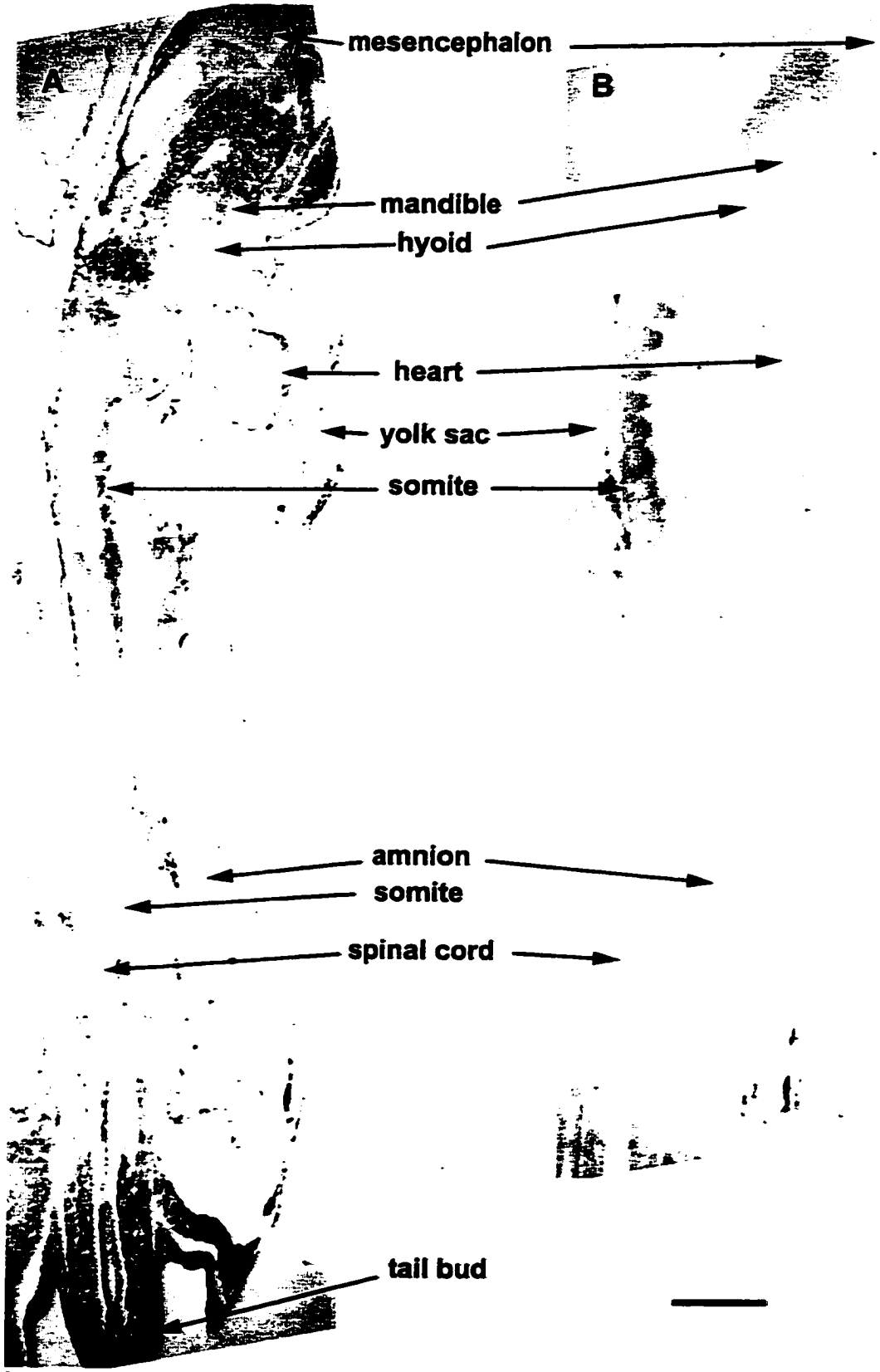

A
B
mesencephalon
mandible
hyoid
heart
yolk sac
somite
amnion
somite
spinal cord
tail bud

Figure 12

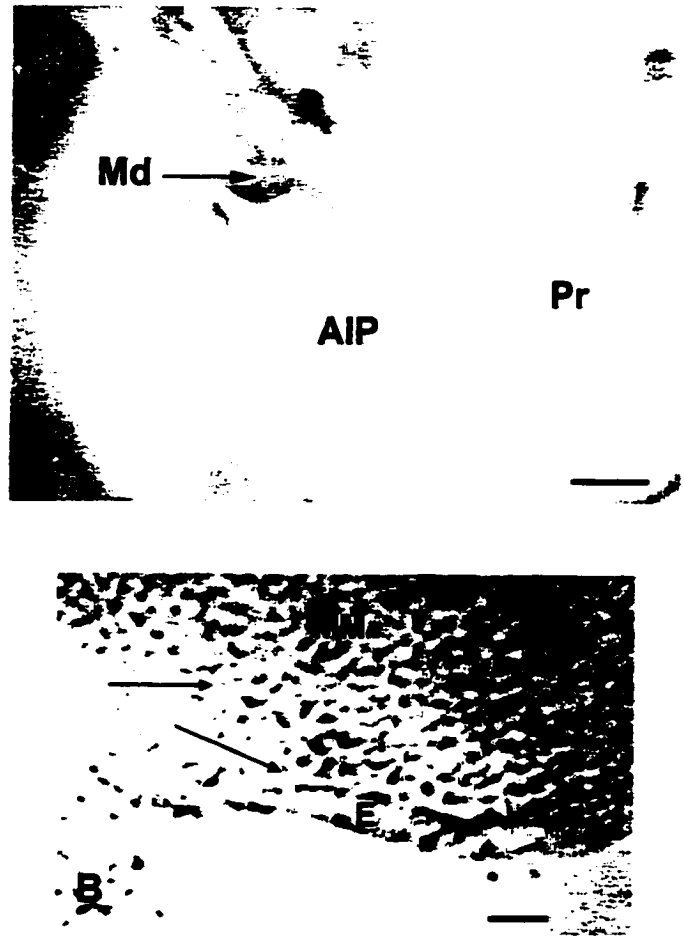


Figure 13: Longitudinal section through anterior of stage 13 -14 chick embryo showing regions of TGF-β localization using avidin-biotin-peroxidase complex (ABC) immunostaining method.

A) In general, low level positive staining was evident in many regions. Mandible (Md) begins to express TGF-β toward the apex of the growing arch, indicated by arrow. AIP = anterior intestinal portal; Pr = prosencephalon. Scale bar = 500 μm.

B) Section of stage 14 mandible showing TGF-β in mesenchyme (arrow) and absence of signal in epithelium. MM = mandibular mesenchyme; E = epithelium. Scale bar = 50 μm.

Figure 14: Longitudinal section through middle of stage 19 - 27 chick mandible showing regions positive for TGF-β and BMP-2 using avidin-biotin-peroxidase complex immunostaining.

A) Section through stage 19 mandible. TGF-β signal was most intense in mesenchyme immediately beneath the distal epithelium around the periphery of the arch (arrows). Epithelium (E) remained free of staining. Scale bar = 100 μm.
B) Stage 20 mandible showing expansion of the signal around the edge of the arch as well as staining in the mandibular mesenchyme, especially distally (MM). Scale bar = 100 μm.
C) Phase contrast image of stage 23 mandible shown in (C).
D) Stage 23 mandible TGF-β signal surrounds the periphery and also appears in much of the mandibular mesenchyme. The central core remains relatively free of signal. Note the lack of staining in the epithelium (E) which has become detached from the mandible towards the top of the photograph. Spaces in section are preparation artifacts. Scale bar for C) and D) = 300 μm.
E) Stage 27 mandible section stained for BMP-2. Staining is diffuse and is primarily seen in the rostral epithelium and adjacent cells. e = epithelium; bm = basement membrane.
F) Stage 27 mandible stained for TGF-β. Epithelium remains negative, but staining is seen around periphery of mandible as well as around what appear to be coalescing skeletal elements forming Meckel's cartilage (c) and muscle (m). Scale bar for E) and F) = 200 μm.

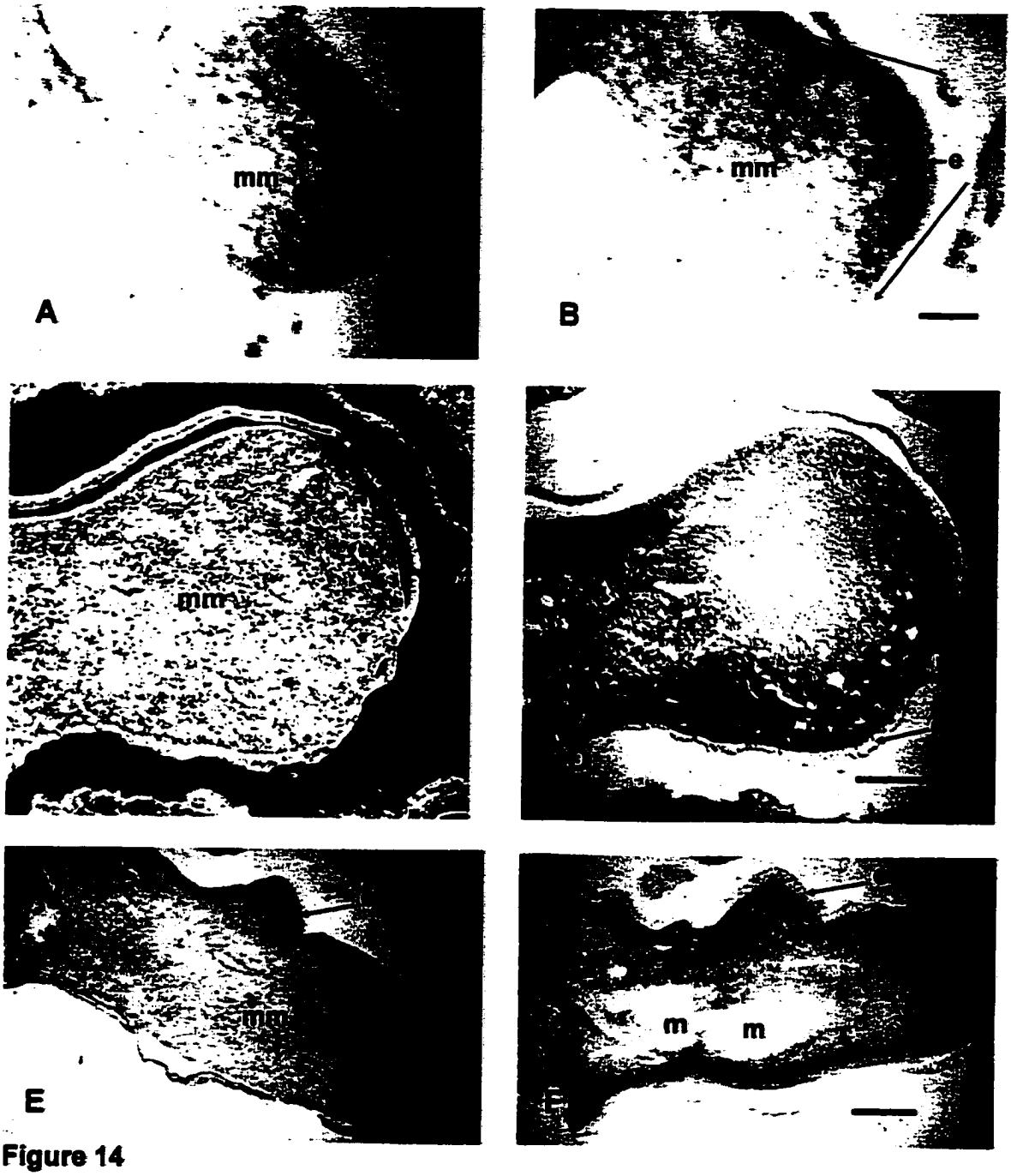


Figure 14

Figure 15: Sections through stage 21 - 29 limb buds stained for BMP-2 and TGF-β.

A) Stage 21 limb bud stained for BMP-2 showing low level signal at the apex of the growing bud, including the epithelium and mesenchyme immediately underneath. lbm = limb bud mesenchyme. Scale bar = 100μm.

B) Stage 21 limb bud stained for TGF-β. Staining was evident around the edge of the bud and infiltrating into the limb bud mesenchyme proximally. Epithelium remained devoid of TGF-β signal. lbm = limb bud mesenchyme. Scale bar = 100 μm.

C) Phase contrast view of stage 25 limb section showing lack of TGF-β staining in epithelium. e = epithelium; lbm = limb bud mesenchyme. Scale bar = 200μm.

D) Limb bud section in (C) stained for TGF-β showing presence of growth factor around the periphery of the growing limb bud. Central core of limb was totally devoid of TGF-β activity. e = epithelium; lbm = limb bud mesenchyme. Scale bar as in (C).

E) Stage 29 limb bud stained with HBQ showing development of digits. Thin arrows indicate cartilage (c) forming digits. Thick arrows indicate interdigital spaces. Scale bar = 500μm.

F) Section of same limb as in (E) stained for TGF-β. Note epithelium is not stained while cells around the periphery of the limb were stained. Note staining in interdigital spaces (large arrows) and around cartilagenous components of digits. Scale bar as in (E).

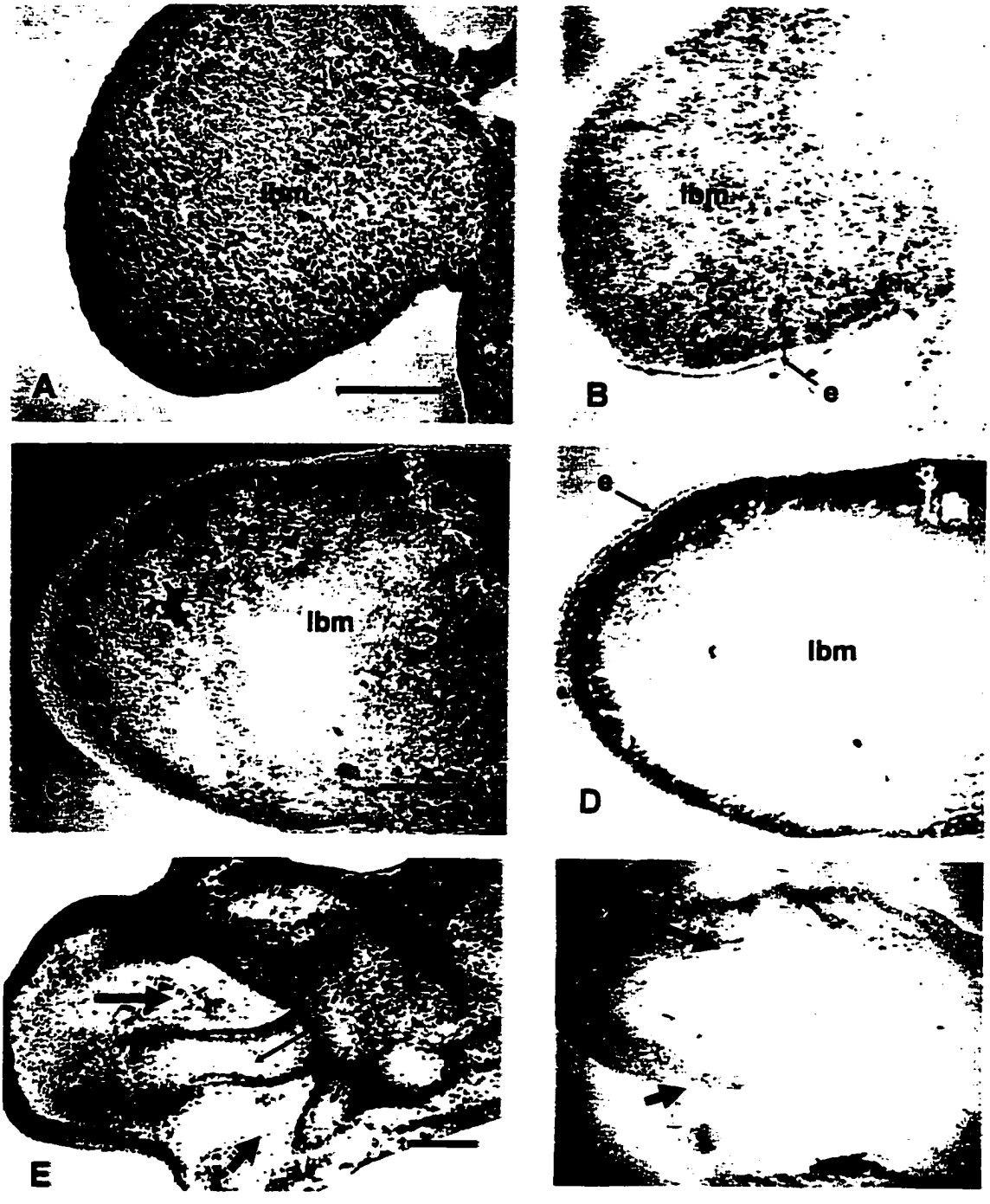


Figure 15

Figure 16: Sections through stage 15 chick embryo spine stained for TGF-β.

A) Lateral section through spinal chord immunostained for the presence of myosin. Signal was confined to the dorsal and ventral regions of the somites and intersomitic areas (arrows). Major portion of the center of the somites remained clear of signal. Scale bar = 100μm.
B) Lateral section stained for TGF-β. Note intense staining (arrows) through middle and dorsal regions of somite and lack of signal ventrally. Periphery of more rostral somite halves stained but center remained clear. Scale as in A).
C) Dorsal view of somites stained with myosin. Signal was distributed throughout somite and spinal cord. Scale as in A).
D) Dorsal view of somites stained for TGF-β. Spinal cord and somite periphery stain, with low level signals apparent in the center of somites as well arrows). Scale as in A).

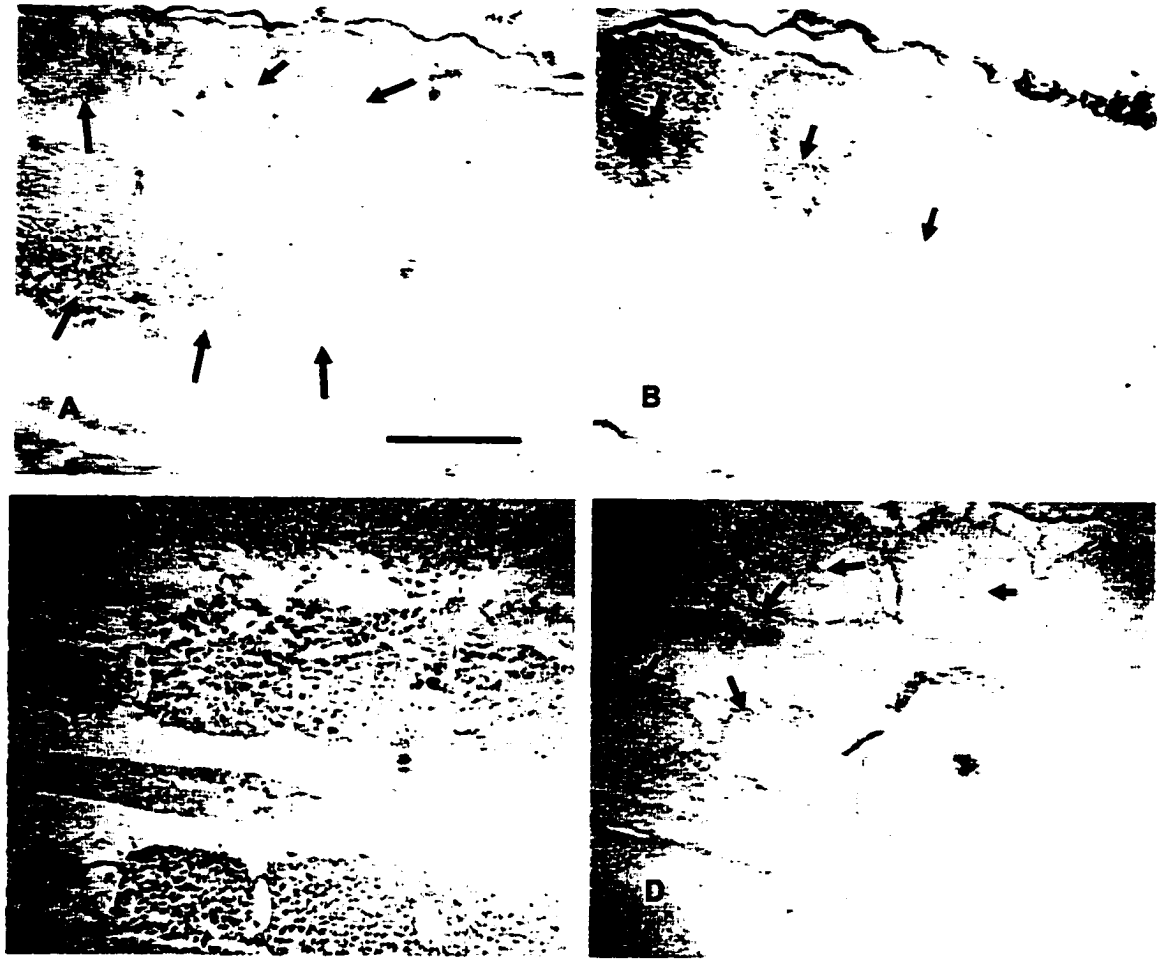


Figure 16

Figure 17: Cross sections through spine of stage 25 chick embryo stained with HBQ, BMP-2, and TGF-β.

A) Section through spine stained with HBQ. Section was taken from mid region of embryo, near the region of wing bud initiation. Blue indicates cartilage or connective tissues. DA = dorsal aorta. SG = spinal ganglia. Scale bar = 200 μm.
B) Section stained for presence of BMP-2. Dermatome stained intensely while additional low level staining was noticeable in spinal cord (SC), especially at the dorsal apices of the neural tube. dt = dermatome. nc = notochord. DA = dorsal aorta. Scale bar = 200μm.
C) Section stained for presence of TGF-β. TGF-β appeared in the myotome (mt) and around the notochord (nt). Unlike BMP-2, TGF-β was not present in either the dermatomes or the spinal cord. Scale bar = 200 μm.

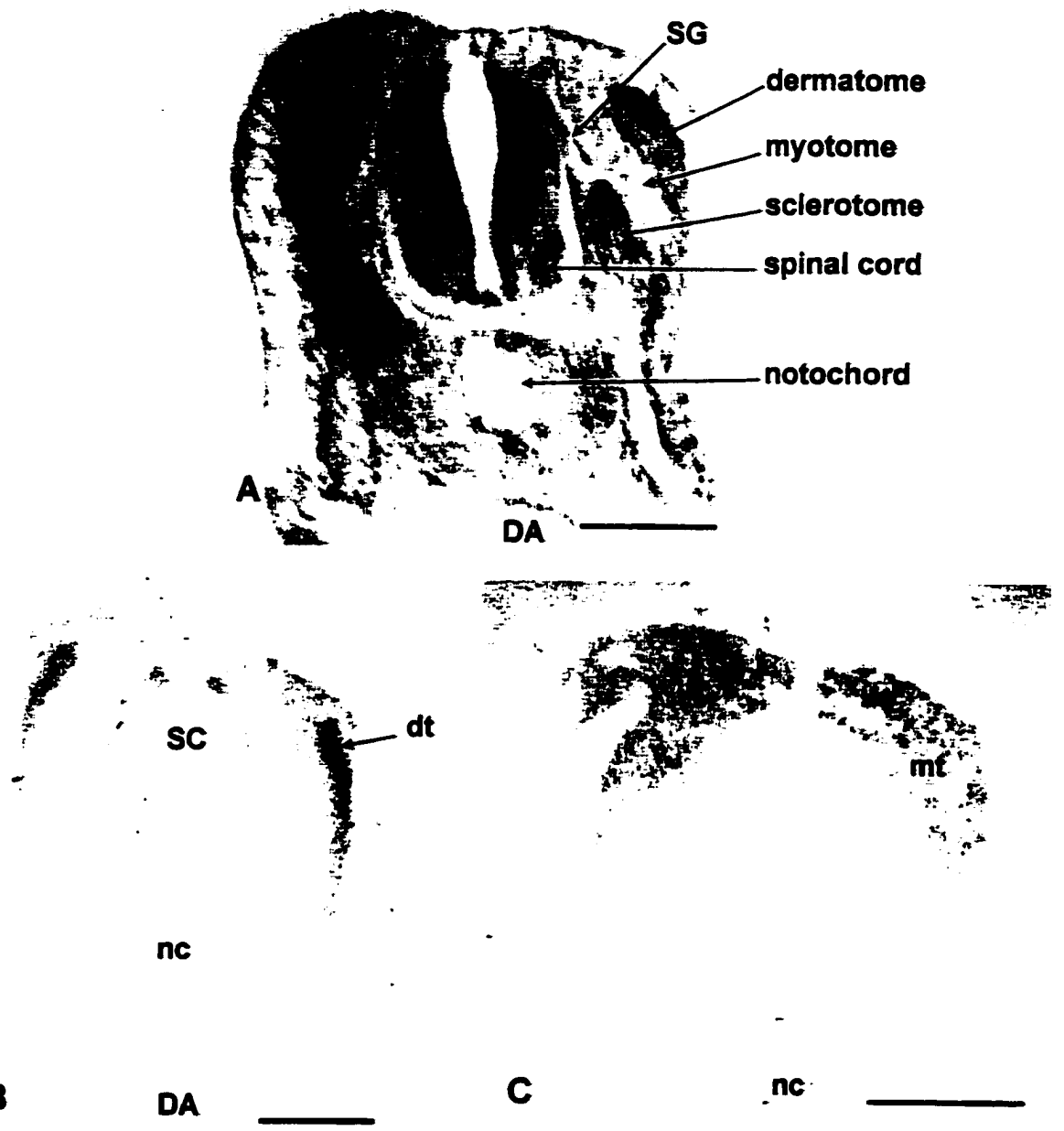

SG
dermatome
myotome
sclerotome
spinal cord
notochord
A
DA
SC
dt
mt
nc
B
DA
C
nc

Figure 17

Figure 18: Other regions of embryonic chick immunostaining for growth factors.

A) Endothelium (et) of stage 19 chick blood vessel (bv) probed for BMP-2. Mandibular mesenchyme (MM) around vessel or near periphery of arch also exhibited positive staining, though not as intensely. Scale bar = 100μm.
B) Embryonic chick blood cell smear showing sub-populations staining positive for BMP-2. Oval cells (leukocytes?) appear to stain intensely whereas round cells, possibly lymphocytes, do not appear to express BMP-2. Scale bar = 75 μm.
C) Section through heart of stage 15 chick embryo heart. Endothelium (endo) expressed TGF-β whereas myocardium (myo) and pericardium (peri) remained free of growth factor signal. Scale bar = 75 μm.
D) Section through stage 19 chick embryo heart immunostained for BMP-2. Unlike TGF-β, BMP-2 was expressed in the myocardium but not in the endocardium. Scale bar = 100 μm.

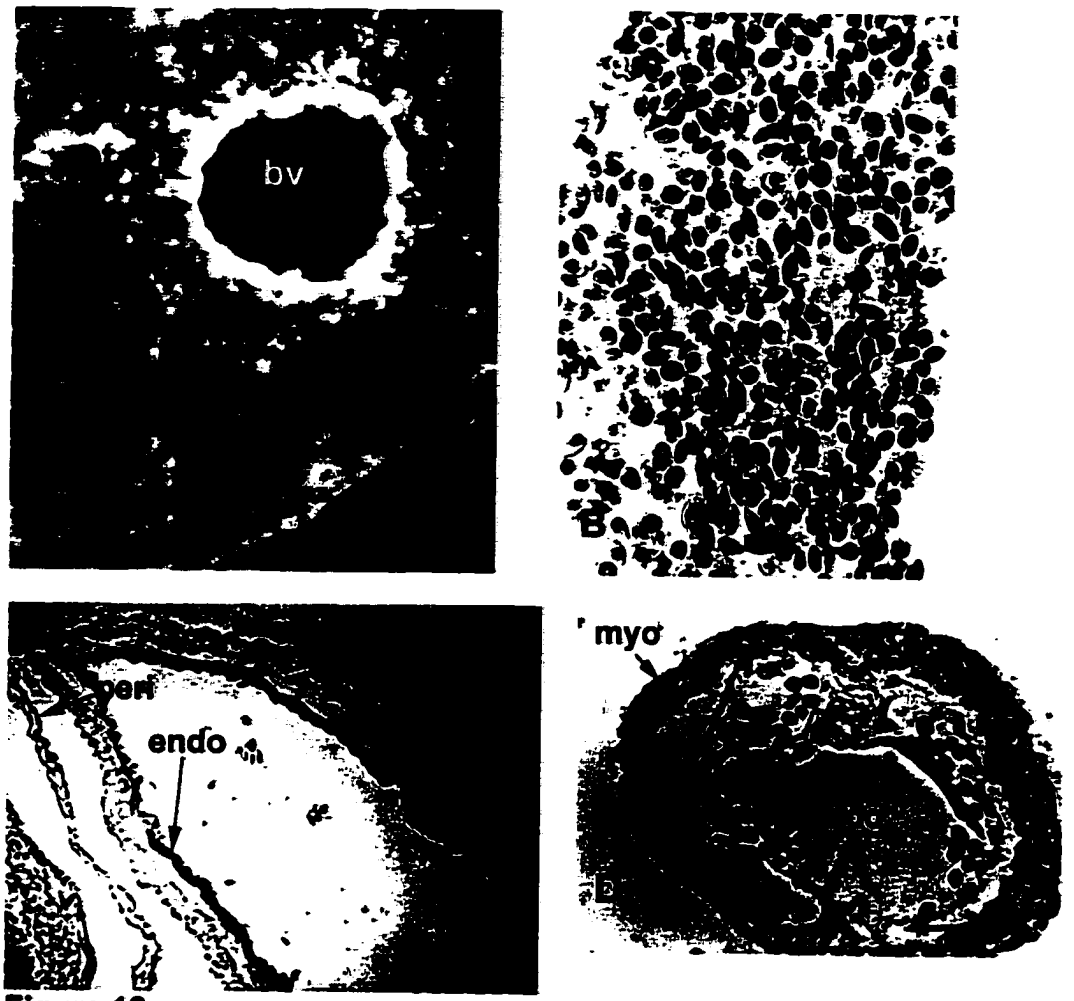
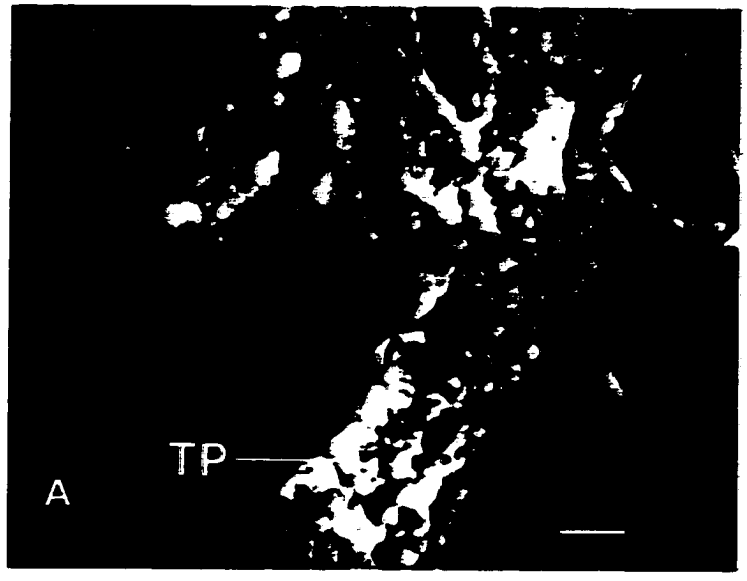


Figure 18

Figure 19: Other regions of embryonic chick immunostaining for growth factors.

A) FITC immunostaining of section through stage 17 chick embryo showing BMP-2 staining from region caudal to mandibular and hyoid arches, possibly a portion of the thyroid primordium (TP). MM = mandibular mesenchyme. Scale bar = 100 μm.
B) Inclusion, indicated by white arrow, appearing in epithelium of cleft between mandibular (Md) and hyoid (Hy) arches of stage 19 embryo. Secondary antibody was conjugated to immunogold grains and the signal was amplified by silver enhancement. and E = epithelium. Scale bar = 100 μm.
C) Enlarged view of (B), showing staining of the inclusion and the epithelium in which it resides. Scale bar = 200 μm.



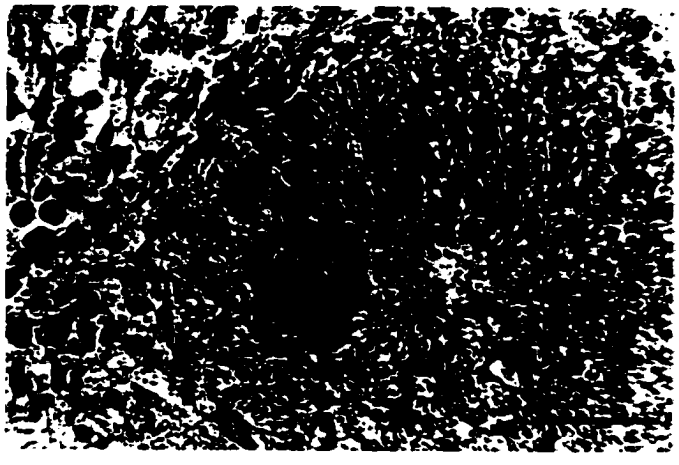


Figure 19

CHAPTER 4: Effect of injection of BMP-2 and TGF-β antibodies on limb and craniofacial development of chick embryos.

4.1. Introduction

Experiments outlined in this chapter were done to investigate the effects of antibody inhibition of the growth and differentiation factors TGF-β and BMP-2 on the development of chick embryos *in ovo*.

The *in vivo* role of growth factors during embryonic development can be approached by one of two methods. The first is to increase the endogenous levels of cytokine. In general this can be accomplished in two ways, either by the direct addition of exogenous growth factor to the embryo or by microinjection of the appropriate mRNA. A second method is to block growth factor signaling. Growth factor action can be down regulated or eliminated in several ways, including the use of dominant negative receptor constructs, the addition of antisense oligonucleotides, or with dominant-negative forms of cytokine. By so doing, the resultant phenotypes can be examined for clues as to the role of the factors during normal development. One method of inhibiting growth factor activity has relied upon the targeted disruption of specific genes, usually those specifying receptors or ligands, to create so-called knockouts or null mutants. TGF-β1 null mice, for example, express a variety of vascular defects including heamorrhagic telangiectasia (Dickson et al., 1995). Mice that are heterozygous for theTGF-β2 null allele are born essentially normal. Animals that are homozygous for the null allele, however, are seldom delivered live. Those that survive gestation are born cyanotic and die shortly after birth, probably because of collapsed airways (Sanford et al., 1997). TGF-β2 is expressed in the developing mouse lung (Pelton, et al., 1991a, b). In the neonatal mouse lung, TGF-β levels are up to 4.5 times higher than in adult mice (McGowan et al., 1997). The high level may function to maintain elastin levels

in the young mouse. Elastin is an extracellular matrix component that confers elastic properties on alveolar tissues. In cultures of mouse lung fibroblasts, TGF-β1 antibodies or antisense oligonucleotides, were shown to downregulate elastin production (McGowan, 1997).

Histological analysis of homozygous TGF-β2 null mice shows that a single allele, and by implication a single growth factor, affects the development of a variety of systems. Among the developmental processes altered are those affecting the normal genesis and growth of the heart, lungs, face and skull, spine, ears and eyes (Sanford, 1997).

BMP-7 null mice show specific developmental defects constrained to the kidney and eye. Interestingly, in regions where colocalization with other BMPs is seen, defects are largely absent, the exception being in the eye, where overlap with BMP-4 is incomplete (Dudley et al., 1997). The suggestion is that, in some cases at least, deficiencies in particular BMPs can be overcome by the action of colocalizing members of the same family.

GDF8, a relatively new member of the TGF-β superfamily appears to be necessary for the restriction of muscle growth. GDF8 null mice exhibit extreme increases in skeletal muscle mass compared to normal mice (McPherron, 1997). In fact, GDF8 -/- mice can weigh up to 3 times more than either GDF8 -/+ or +/+ mice. Mouse fanciers have for decades propagated a strain known as the se or *short ear*. Much prized for its short ears and nose, and wider skull, the mouse also has a number of skeletogenic defects. These involve the loss or reduction of skeletal and cartilagenous elements, and include both those formed early and late in development. Recently, the defect has been traced back to a deficiency in BMP-5 production (Kingsley et al., 1992; Kingsley, 1994).

In order to initiate the intracellular signaling cascade, TGF-β superfamily members must interact with cell surface receptors. Receptors come in two types, type I and type II. A third receptor, type III, is not directly involved in signaling but rather is responsible for presenting ligand to the other receptors. Oligomeric complexes formed from the association of type I and II receptors are required to propagate signals downstream. Ligand binding initiates phosphorylation of the type I receptors' intracellular glycine-serine rich domain by the action of type II receptor kinase (Kolodziejczyk and Hall, 1996). *Xenopus* embryos expressing a truncated BMP-4 type I receptor, produce ventral mesoderm in regions normally exhibiting dorsal mesoderm (Graff et al., 1994). The overexpression of truncated activin type IIA or type IIB receptors in *Xenopus* result in specific developmental abnormalities (New et al., 1997). Both receptors bind activin with high efficiency but truncation of the kinase domain in each mutant receptor inactivates downstream signaling by its inability to phosphorylate the type I receptor. *Xenopus* embryos expressing dominant negative type IIB receptors exhibit a lack of mesoderm induction. Truncated type II receptors do not limit mesoderm induction but rather disrupt the ventral patterning of the embryo. Mouse constructs expressing a dominant negative TGF-βII receptor die by day 10.5 of gestation from abnormal yolk sac haematopoeisis and vascularization, a situation not observed in either -/+ or +/+ animals (Oshima et al., 1996). The study supports the finding that mice that are deficient in TGF-β1 protein express identical defects (Dickson, 1995). Mice lacking TGF-β3 and exhibiting aberrant palatogenesis usually die within 20 hours of birth from pulmonary defects (Kaartinen, 1995). Homozygous BMP-7 -/- null mice express a variety of developmental defects and die soon after birth. Examination of the animals shows that abnormalities are present throughout the skeleton, kidney and eye. Among specific defects encountered are retarded ossification of bones, tail malformations, hind limb polydactyly and lack of retinal pigment (Jena et al., 1997).

The action of growth factors can also be inhibited using antisense oligonucleotides that prevent the translation of mRNA into protein. Isolated embryonic mouse palatal shelves will undergo fusion *in vitro* without the addition of any exogenous factors. When growth medium is supplemented with antisense TGF-β3 oligonucleotides, shelves are unable to fuse (Brunet et al., 1995). This work supports that of Kaartinen (1995), who found that mice which were dominant negative for TGF-β3 exhibit the same features. That antisense nucleotides can indeed be used to block the action of growth factors is demonstrated by direct topical application to mouse dermal wounds. In young mice, dermal wounds heal readily and without much scarring or inflammation. In adult animals however, epidermal damage repair takes a longer time and is associated with much more intense scarring. The introduction of antisense TGF-β1 to the wound site results in decreased scarring and down regulation of TGF-β mRNA expression (Choi et al., 1996). Antisense oligonucleotides are often added to cell culture systems in an attempt to study growth factor action with respect to tumourogenesis and other disease states. While this approach does not precisely mimic the situation *in vivo*, it can implicate specific factors as potentially important for tumour maintenance and growth. Malignant mouse or human mesothelioma cell lines treated with TGF-β1 or TGF-β2 antisense oligonucleotides exhibit down regulation of both TGF-β mRNAs and an associated reduction in tumour growth (Marzo et al., 1997).

Since both TGF-β and BMP-2 are implicated in several different developmental events, it was decided to interfere with their signaling in order to establish whether or not these factors are required for limb and craniofacial development in the chick. As the construction of null mutant chicks has yet to be done, inhibition via antibody loading was used in an attempt to perturb the development of systems suspected to be dependent upon these factors. Similar approaches have been used to investigate the role of lectin in *Xenopus* craniofacial development (Evanson and Milos, 1996). In addition, signaling by the

osteoinductive protein Vgr-1 (BMP-6) has been blocked *in vitro* in the ROB-C26 mesenchymal cell line using antibodies (Gitelman et al., 1995).

4.2.Materials and methods

As TGF-β and BMP-2 are likely to be of major importance to the proper formation of chick embryos, removing them or limiting their availability to embryos could have serious consequences. In order to inhibit the action of TGF-β and BMP-2, the air spaces of developing chicken eggs were injected with antibodies specific for these growth factors. Fertilized *Gallus domesticus* eggs were obtained from Cook's Hatchery, Truro Nova Scotia and incubated at 36.5 °C in a forced draught incubator (Humidaire Model No. 50, Humidaire Incubator Company, New Madison, Ohio).

Since both growth factors are first detected at or near stage 14 and are very much more prevalent and intense by stage 25, eggs likely to have embryos falling within this 'window' were chosen for study. It is difficult to precisely determine the developmental stage of an embryo without removing it from the egg. Fertilization in chickens is internal and the eggs may have been retained in the oviduct for differing times prior to being laid. This means that a single chicken can lay eggs covering a series of developmental stages. In order to minimize this range, eggs were candled upon arrival for the presence and approximate size of the embryo. A representative number of eggs was then opened and precisely staged under a dissecting microscope to ascertain the most common stage present in a given batch of embryos. In general, embryos opened at any one time interval were found to be at approximately the same stage of development, + or – 1 stage. Injections were done at two representative stages, namely stage 14 and stage 26. At stage 14, embryos have just begun to form visceral arches and limb primordia. By stage 26, both mandible and limb bud development are very well established.

Upon arrival, selected eggs were first candled for the presence of embryos and location of the perivitelline space. Confirming the presence of an embryo was easier in the older stage eggs than in the younger due to the larger size. In general, eggs lacking embryos constituted less than 5% of the total surveyed. After locating the perivitelline (air) space, a pencil was used to indicate the point of injection. The location was such that the insertion of needles would not damage or directly contact the embryo within. Needle positioning was approximately at right angles to the long axis of the egg. Prior to injection, eggs were wiped with 70% ethanol. All solutions used were sterile and all procedures were carried out aseptically. Holes wer sealed with cellophane tape to prevent the eggs from drying out.

Antibodies used included murine anti-BMP-2 monoclonal antibody, (designated h4b2, obtained from Genetics Institute, Cambridge, Mass.) and rabbit pan-specific TGF-β neutralizing antibody (R & D Systems, Minneapolis, Minn.). Lyophilized antibodies were reconstituted in sterile PBS, pH 7.4 to a final concentration of 1mg/ml. Antibodies were prepared for injection by dilution in sterile TBS, Ph 7.6 (50 mM Tris-HCL, 150mM NaCl). BMP-2 antibodies were administered at final concentrations of 0.1, 1.0, 2.0, and 5.0 µg/ml. TGF-β antibodies were used at final concentrations of 0.01, 0.1, 1.0, 2.0, 1.0, and 5.0 µg/ml. Injections were carried out using a 27 gauge needle attached to a 1.0 ml. tuberculin syringe (Becton-Dickinson). Injection volumes were 0.5 ml. in all cases. Controls were injected with 0.5 mls TBS alone. As an additional control, additional eggs were injected with purified rabbit IgG (Sigma # I5006). Immediately after injection, eggs were returned to the incubator and allowed to develop.

Incubation times after perivitelline injection varied from 48 - 72 hours for stage 26 embryos to up to 4.5 days for stage 14 embryos. Animals were thus allowed to mature to a maximum of approximately stage 30 (approximately 6.5 days total incubation) before analysis. A number of stage 25 – 27 embryos were

allowed to incubate for 4.5 days to ensure that any effects of antibody injection that were observed were not due to incubation time alone. Embryos were analyzed for obvious gross developmental abnormalities such as misshapen limbs or craniofacial aberrations, detectable under a dissecting microscope. Unusual features were noted with emphasis placed on limb, tail, or craniofacial defects. Embryos were fixed and sectioned or stored for staining and analysis. The number of embryos injected at any one antibody concentration ranged from 7 to 24.

4.3. Results

Stage 13 - 15 or 25 - 27 sham injected eggs (TBS alone) exhibited high survival rates after 48 hours of incubation with a slightly higher mortality rate seen in the older embryos. (Fig. 20; Table 1). Over 90% of stage 14 (16 out of 17) and 90% of stage 25 – 27 (9 out of 10) embryos survived sham injection and were morphologically normal under both macroscopic and microscopic analysis (Fig. 21). The slight difference in survival may have been due to the fact that older embryos were larger and more vascularized, making injection into the space without puncturing blood vessels more difficult.

4.3.1. BMP-2 antibody injection

Most early stage embryos injected with low concentrations of antibody (0.1 µg/ml), had no obvious defects or developmental abnormalities. Embryonic limbs, tail and craniofacial features were developing normally at the time of sacrifice. The size of the embryo and its orientation in the egg were identical to sham injected or uninjected controls of the same developmental age. Only two embryos out of 20 (10%) had any visible developmental defects, irregularly shaped fore limbs and slight limb bifurcation. No craniofacial or other problems were noted. Sixteen of 20 embryos (80%) appeared to be unaffected by the injection of h4b2 and were essentially morphologically normal while two embryos did not survive treatment

(Fig.22; Table 2).

As h4b2 concentrations were increased, the number of morphologically normal embryos decreased and the number of craniofacial and limb abnormalities both increased, as did the mortality (Table 2). At a concentration of 1.0 µg/ml, embryo mortality is approximately 35%, while 25% remain relatively unaffected and appeared morphologically normal. Craniofacial (3 of 16) or limb (3 of 16) defects were noted in approximately 40% of treated embryos. Aberrant development at this dose was minor and included digit abnormalities and perturbations of hyoid arch and mandibular development. Obvious limb bud defects consisted primarily of differences in overall size and shape as well as of the development of normal numbers and arrangement of digits. Craniofacial defects were more severe, consisting mainly of irregular growth of mandible and maxilla. The mandible in at least two embryos appeared to be growing ventrally and rostrally (toward the maxilla) farther than normally, creating a 'hooked' appearance. Heads were generally very large and appeared highly vascularized.

The toxicity of h4b2 increased at 2.0 µg/ml and 5.0 µg/ml with approximately 65 % and 85% lethality respectively. At 2.0 µg/ml, no obvious limb bud defects in isolation were noted but over 30% of embryos exhibited craniofacial development abnormalities (Fig. 22; Table2). At this dose, virtually no normal embryos were recovered. Head and face malformations included severe brain enlargement, extreme outgrowth of mandibular processes and eye reduction. Brains were in some cases abnormally swollen and haemorragic and appeared to be exposed directly to the exterior. Craniofacial defects were sometimes seen in conjunction with limb or tail abnormalities (10%). In the most severe cases, head growth was severely affected, with the embryo exhibiting a lack of eye development, atypical mandible and maxillary formation and extreme brain overgrowth. In one extreme case, the embryo showed an almost complete lack of caudal development,

including a lack of hind limbs, with the tail forming directly below the region of the forelimbs (Fig. 23). Fore limb development was apparently normal. A large quantity of blood and fluid had accumulated in the head region as well as throughout the visceral cavity. At 5.0 µg/ml, lethality of h4b2 was almost total (12 of 14), with only two embryos surviving, although appearing normal.

Injection of h4b2 at later stages of development did not appear to cause as severe effects as those seen in embryos injected at earlier stages. Stage 25 - 27 embryos injected with either 0.01 or 0.1 µg/ml usually developed normally, with 20% or fewer fatalities (Fig. 24; Table 3). Even at higher doses of up to 5.0 µg/ml, deaths never exceeded 30%. At all doses administered, a percentage of embryos were morphologically normal, unlike the situation in younger embryos. Even at the highest antibody concentration used, close to 30% of embryos were indistinguishable from sham injected or uninjected controls. Deaths overall were within the range of 20 to 30%. At a concentration of 1.0 µg/ml, approximately 30% of embryos died, while the rest appeared normal. Stage 23 – 25 embryos injected with 2.0 µg/ml BMP-2 antibody were generally normal in appearance 30% of the time (4 of 11), while 45% (5 of 11) developed abnormal craniofacial features. At 5.0 µg/ml, both limb and craniofacial developmental problems were seen. Most severe were irregularities of the face, brain and eye. The eye irregularities were interesting in that the eye on the side of the embryo lying closest to the surface (that is facing away from the yolk), was usually the one which exhibited the most aberrant features, being extremely reduced or altogether absent. This eye was on the side of the embryo closest to the perivitelline space and thus the point of injection. This may reflect a more concentrated antibody exposure for this side of the embryo.

Overall, some trends were evident for BMP-2 antibody injected eggs. It was apparent that older embryos (stage 25 to 27) were less susceptible to developmental disruption arising from exposure to BMP-2 antibodies. Stage 14

embryos were sensitive to very low doses of antibody, exhibiting abnormal development even at 0.1 µg/ml antibody. Mortality for stage 14 injected embryos ranged from 14% at 0.01 µg/ml antibody to almost complete mortality (85%) at a dose of 5.0 µg/ml. Mortality for stage 25 embryos remained relatively constant at 20% to 30%. In stage 14 embryos, BMP-2 antibody injection disrupted development of both hind and forelimbs, altering both size and shape. Limbs were reduced and showed obvious patterning problems. Craniofacial development was altered even more drastically. Affected mandibles and maxillae were usually overly large and grew at odd angles, resulting in major defects likely detrimental to survival. In addition, the brain and skull appeared very large and swollen with large amounts of blood and fluid. This defect appeared to preclude survival.

Embryos injected at stage 25 were less likely to exhibit major alterations in development as a result of BMP-2 antibody exposure at the concentrations administered. A small number of animals showed some developmental changes, primarily in the craniofacial region. At doses of 0.01 and 0.1 µg/ml antibody all surviving embryos were morphologically normal and indistinguishable from controls.

4.3.2 TGF-β antibody injection

Stage 13-15 and stage 25-27 embryos were used for perivitelline injection of TGF-β antibody. The volume of antibody plus TBS carrier injected was 0.5 ml, with final concentrations of 0.01, 0.1, 1.0, 2.0, 5.0 and 10.0 µg/ml. Stage 14 embryos treated with different concentrations of TGF-β antibody and incubated *in ovo* for up to 72 hours exhibited a range of responses that were dose-related (Fig. 25; Table 4). Eggs injected with 0.01 µg/ml of TGF-β antibody were, morphologically, not significantly different from sham injected controls. Survival was 100%. At a 10-fold higher concentration (0.1 µg/ml), embryos again appeared

identical to sham injected controls, however there is a slight increase in mortality (14% or 2/14) over both sham injected or 0.01 µg/ml treated embryos.

An increase of antibody concentration to 1.0 µg/ml resulted in a wide range of effects including both craniofacial and limb bud development problems (Fig 25; Table 4). Developmental abnormalities of the limb buds appeared in approximately 36 % of embryos while those of the craniofacial region were noted in almost 14 %. A single embryo (< 5% of total) clearly expressed defects of both limb and craniofacial regions, as well as tail bud truncation.

At 2.0 µg/ml, the proportion of embryos exhibiting limb abnormalities increased slightly (44%) over that seen at an antibody concentration of 1.0 µg/ml. The severity of the malformations however increased over those seen at lower doses and included polydactyly and extreme limb patterning anomalies (Fig. 26). Limbs in these animals grew out of the body at odd angles and often took on a jagged appearance. Some examples of these limbs as seen in longitudinal section are presented in figure 27. In some sections, what appeared to be a second AER or grown axis was evident.

The frequency of embryos having more widespread anomalies, including those of both craniofacial regions and limbs, rose to almost 25% (Fig. 25; Table 4). In these extreme cases, limb growth was altered as described above. Mandibular development was essentially normal but other aspects of head development were changed. Eyes were often reduced or absent. Inhibition of TGF-β by antibodies affected caudal growth and can render these regions underdeveloped. Hind limbs were usually formed but may exhibit polydactyly or altered shape (Fig. 26, 27). In some embryos, brain development was incomplete and large openings to the exterior were evident. One embryo analyzed showed signs of incomplete posterior neural tube fusion as well as large openings in the

mesencephalon (Fig. 28).

An increase of antibody to 5.0 µg/ml produced no substantial developmental changes from those seen at 2.0 µg/ml. Mortality remained about the same at 20%. Developmental perturbations were again primarily seen in the limbs and tail buds, head (eyes and brain), and spine. These defects were slightly more frequent than seen at the previous antibody concentration. Atypical craniofacial development occured in conjunction with abnormal development of the limb buds in approximately 10% of embryos screened, although mandibular growth was not notably changed from sham controls. A comparison of the appearance of mandibles in both TGF-β and BMP-2 challenged embryos is shown (Fig. 29). Embryos with obvious craniofacial growth abnormalities were not found in isolation from limb bud aberrations.

Injection of TGF-β antibodies into older (stage 25-27) embryos produced similar results to those seen in younger embryos (Fig. 30; Table 5). As with stage 13-15 embryos, little of note occured with antibody concentrations of less than 1.0 µg/ml. At or above 1.0 µg/ml however, developmental differences, including those of the craniofacial and limb regions were seen, although the percentage of embryos displaying these effects was lower than seen in those injected at earlier stages (Fig. 30; Table 5). At 1.0 µg/ml, the proportion of embryos exhibiting limb bud growth aberration was approximately 15% (4/26). This level remained constant at antibody concentrations of up to 5.0 µg/ml, ranging between 14% and 16%. Craniofacial development was abnormal in approximately 8% and 14% of embryos injected at concentrations of 2.0 and 5.0 µg/ml respectively. At 1.0 µg/ml, 8% of embryos (2/26) displayed obvious signs of growth problems in both face and limbs. As with younger embryos injected with TGF-β antibodies, mortality did not increase with increasing antibody levels. Mortality remained at an almost constant 15%. Unlike younger embryos, more than 50% showed no sign of obvious developmental

problems, even at the highest antibody concentration tested. At stage 13-15, there was a steady decrease in morphologically normal embryos with increasing antibody concentration.

At low concentrations of either TGF-β or BMP-2 antibody, there was little effect upon mortality or normal development. As concentration was increased to 1.0 µg/ml, normally developing embryos were much less likely to be found, ranging around 25 to 30% of total embryos treated. Developmental processes in the limb buds and craniofacial areas were unusually affected by both antibodies. BMP-2 antibody primarily affected craniofacial development at concentrations of between 1.0 and 5.0 µg/ml. Anti-TGF-β, on the other hand, affected both craniofacial and limb development. At higher doses (> 1.0 µg/ml), mortality increased steadily for BMP-2 antibody treated embryos, until almost total lethality was achieved. Mortality associated with TGF-β antibodies remained constant throughout the dose range used.

Similarly, older embryos exposed to increasing antibody concentrations showed differential responses. In general, older embryos were more robust and resistant to developmental perturbation induced by anti TGF-β or BMP-2 antibodies than younger embryos. One of the more obvious differences seen in these embryos was the decrease in mortality seen even at higher concentrations of neutralizing antibodies. In fact, even at 5.0 µg/ml, mortality was never higher than 25% for either antibody. Inhibition of BMP-2, as in younger embryos, affected primarily craniofacial development. Some limb bud effects were seen at higher dose levels. Conversely, anti-TGF-β affected primarily limb patterning, but also altered normal craniofacial development at higher concentrations.

Mortality trends for perivitelline injection of h4b2 antibody into stage 13 –15 and stage 25 – 27 embryos are shown in figures 31A. There was a steady

decrease in normal embryos with increasing antibody load in stage 13 – 15 animals. This was almost mirrored by the steady increase in embryos mortality with increasing antibody concentration, reaching an LD_{50} at 1.5 µg/ml. Limb and craniofacial developmental irregularities increased between 0.1 and 2.0 µg/ml, dropping off at antibody levels approaching 5.0 µg/ml, although this may reflect the almost total mortality observed at this dose.

The response of later stage embryos over the dose range tested differed in some respects. Mortality, unlike the situation in young embryos, remained steady at between 20 and 30% over the entire range of antibody concentrations. LD_{50} was not approached over the course of treatments (Fig. 31A). The occurrence of unaffected animals did decrease with increasing h4b2 concentration but never fell below 30%. Limb and craniofacial development remained normal until concentrations of between 1.0 and 2.0 µg/ml were reached. Developmental irregularities associated with these regions increased sharply at 2.0 µg/ml antibody concentration, a level 20 times greater than that required to produce similar irregularities in younger embryos.

In stage 13 –15 embryos treated with TGF-β antibody, there was a decrease in morphologically normal embryos with increasing concentration, as seen in embryos treated with h4b2 antibody. Unlike the situation for h4b2 treatment however, lethality remained low, peaking at 20%. LD_{50} is unknown over the range administered (Fig. 31B). Developmental anomalies of the limb and head initially appeared between 0.1 and 1.0 µg/ml. Limb effects continued to increase with concentration until 50% of animals were affected. Craniofacial development on the other hand was relatively unaffected, peaking at 20% at a concentration of 2.0 µg/ml. Animals expressing aberrant development of both areas remained below 10%.

Although not specifically a focus of this study, TGF-β antibody affected caudal development of the embryo. Approximately 30% of embryos treated with 2.0 – 5.0 μg/ml anti-TGF-β showed severe aberration of the spine, including uncharacteristic torsion of the body (Fig.32). Instead of lying in a curled position with head and tail in close proximity, the embryos often bent in the opposite direction. In addition, most embryos thus affected showed either constrictions or other anomalies in the region of the spine caudal to the forelimbs or an absence of caudal development altogether.

Older embryos appeared to be very resilient to TGF-β inhibition. Even at levels of 5.0 μg/ml, more than 50% of treated animals remained morphologically and developmentally normal. Mortality remained less than 20%. As seen for younger embryos, limb development was affected more than craniofacial development, although incidence of defects was relatively low. In essence, older animals appeared less reliant upon TGF-β for normal growth because inhibition of its action had less of an effect than that seen in younger animals. BMP-2 inhibition on the other hand critically affected development of animals at both stages chosen for study, although mortality was less of a consequence of treatment in older embryos.

4.4. Discussion

This does not change the observation that, in general, TGF-β antibodies affected primarily the development of limb and tail while BMP-2 antibodies primarily influenced craniofacial growth.

The high incidence of aberrant craniofacial development in embryos treated with anti-BMP-2 suggests that the factor may be required to direct normal head growth and patterning. Implantation of BMP-2 saturated agarose beads directly into

the developing chick mandible at stage 22 resulted in retardation of mandibular growth and had little direct effect upon either chondro- or osteogenesis (Ekanayake and Hall, 1997). The frequency of affected embryos was dose dependent, and areas of localized cell death were found in proximity to the bead.

During development of the mouse forebrain, *BMP-2, -4, -5, -6,* and *-7* transcripts are coexpressed in the dorsomedial telencephalon, correlating with regions of apoptosis and inhibition of cell proliferation. Furthermore, cultured lateral telencephalon explants treated with exogenous BMP-2 and -4 soaked beads exhibit an increase in cell death and decrease in mitotic activity (Furuta, 1997). The brains of chick embryos injected with BMP-2 antibody are often greatly enlarged, as are other regions of the head, for example mandible and maxillary. Since BMP-2 addition to cultured brain tissue results in the down-regulation of cell division and an increase in apoptosis, the inhibition of its action through antibody treatment may have the opposite effect. In other words, the removal of all or some of the active BMP-2 protein through antibody adsorption should increase mitotic activity and decrease the level of apoptosis in brain tissue. This action would lead to an increase in overall brain size as cell division would be largely unchecked by the endogenous BMP-2 growth limiting mechanism.

That some growth factors may function as regulators of mitotic activity and as instruments of pattern formation has been proposed (Cohn and Tickle, 1996; Graff, 1997; Yamaguchi, 1997; Niswander, 1997). During limb development in many vertebrates, programmed cell death in the interdigital spaces is responsible for the regression of tissue and the ultimate freeing of the digits. (Gañan et al., 1996; Zou and Niswander, 1996). BMP-2, -5, and –7 have been implicated as possible mediators of apoptosis in the developing limb (Lyons et al., 1990, 1991, 1995; Jones et al., 1991; Francis et al., 1994). The programmed depletion of specific cell populations plays an important role in the establishment of tissue

pattern in the developing head (Graham et al., 1994, 1996; Noden, 1983).

Neural crest cells migrate into the branchial arches early in development in response to positional information acquired while resident in the neural primordium. Groups of neural crest cells destined to populate specific arches are separated by apoptotic foci which are linked to the expression of *BMP-4* and *Msx*. Interestingly, patterning, and more specifically, digit formation, in the developing limb appears to rely upon activation of BMP-4 and Msx genes (Zou and Niswander, 1996). The involvement of TGF-β superfamily members in establishing body plan may be widespread throughout the embryo. The perturbation of development resulting from cytokine inhibition may reflect in part an interference with apoptotic mechanisms. Antibodies are unlikely to affect neural crest directly. Neural crest cells begin migrating at about stage 8.5 to 10 and start to populate the branchial arches at stage 15 (Le Douarin, 1974; 1982). The earliest stage injected with antibodies in this study was stage 13, well after the cells have been specified as neural crest (but not bone) and begun to migrate into the arches. Immunohistochemical analysis suggests that BMP-2 appears first in the epithelium of the mandibular and hyoid arches. Neither TGF-β nor BMP-2 were detected in embryos younger than stage 13.

The addition of either antibody to early stage embryos alters limb development. Anti-TGF-β exerted a stronger effect upon limb development than did BMP-2 antibody, showing a trend that is dose dependent. BMP-2 antibody produces aberrant growth of limb buds at low dose levels (< 2.0 µg/ml). At higher levels fewer limb abnormalities are seen but there is a concurrent increase in craniofacial effects and mortality. It is unclear why limb abnormalities are rare at high concentrations of BMP-2 antibody. It is possible that, as mortality increases to almost 85% at 5.0 µg/ml, limb malformations are less likely to appear. Embryos that die early after perivitelline injection are often found to be partially or almost

completely resorbed or otherwise broken down, making accurate determination of morphology difficult. It is of note that at 2.0 µg/ml, the incidence of craniofacial and limb aberration in the same animal increases. This indicates a dose-response effect on limb growth. The types of limb abnormalities were similar for both antibodies tested, however it appears that the severity of pattern disruption and certainly the incidence of polydactyly is higher in those embryos stressed with anti-TGF-β. The incidence of limb malformation resulting from antibody inhibition of growth factor action is lower overall in those animals injected at the later stages of development. The limb bud growth perturbation which does occur is generally less severe than that seen in younger embryos, with no polydactylous limbs noted.

BMPs, including the closely related BMP-2, –4 and -7, are expressed in the limb bud during development and are involved in correct limb patterning. These form part of a signaling cascade which includes activation by upstream *shh* (Niswander, 1997; Hogan, 1996; Francis-West et al., 1995). All three are intimately involved in the patterning of limb skeleton (Duprez et al., 1996a; Kawakami et al., 1996). Furthermore, BMP-2 can regulate the expression of FGF-4, one of the primary cytokines produced by the apical ectodermal ridge (Duprez, et al,1996b).

The chick limb originates from the embryonic flank. More specifically, the wing mesoderm arises from medial somatopleure while the apical ectodermal ridge is derived from the overlying ectoderm; dorsal and ventral limb ectoderm arise from the paraxial and lateral somatopleure ectoderm, respectively (Zeller and Duboule, 1997; Michaud et al., 1997). Signals arising from the somites are responsible for dorsalization of the limb, while signals from the lateral somatopleure provide ventralizing information (Michaud et al.,1997). The nature of these signals is not known. Limb growth alteration by antibody inhibition, especially by TGF-β, may result from action on two fronts. In the early embryo, TGF-β and BMP-2 may be inhibited at the level of the somite as growth of the limb from the embryonic flank

progresses. At later stages, the antibodies may act at the limb periphery, TGF-β being inhibited in the mesenchymal cells while BMP-2 is inhibited in the epithelium.

As mentioned above, embryos treated with TGF-β antibodies often exhibit malformation of the limbs and sometimes the head and associated structures, including either reduction or absence of eyes. In addition to these regions however, a high percentage of embryos have spinal deformities and severely altered caudal development. In one case, it appeared that neurulation was affected and fusion of the neural folds was incomplete.

The torsion and flexion normally exhibited by the embryo during development brings the body's rostral-caudal axis into a single plane and causes the bending of the embryo so that the head and tail curl towards each other. This is the opposite of what is seen in many of the TGF-β antibody treated embryos where torsion appears to be affected and the rostral caudal axis not aligned within a single plane. Flexion of the body is often reversed, with the head and tail moving apart rather than together (Fig.32). Embryo orientation is also affected by BMP-2 antibody exposure however far less severely and more likely to affect positioning of the body anterior to the forelimbs. (Fig.32)The inhibition of TGF-β action with antibodies grossly affects the development of the posterior of the embryo. Defects observed include constrictions in the body along the spine, altered growth and development of caudal structures including the tail and hind limbs and in the most extreme cases, the absence of posterior structures. Often associated with this is the presence of wisps of membranous tissue where the posterior of the body would normally develop. While embryonic lethality was rare in TGF-β challenged animals, it is doubtful whether or not these severely affected embryos would survive to hatching.

Patterning and differentiation during vertebral development in the mouse

requires BMP-4, specifically for the development of ventrolateral cartilage (Monsoro-Burq et al., 1996). In addition to *BMP-4* expression, *Msx-1* and *-2* genes are activated in the process, possibly stimulated by BMP-4 protein. Both BMP-2 and -4 have been implicated in the development and organization of elements of vertebral and axial skeleton formation (Nifuji et al., 1997). Exogenous BMP-2 or -4 perturbed development in a temporal specific manner, probably related to the stage of somitic development. BMP-2, for example, altered vertebral growth in mice if administered at embryonic day 2 but not 3. BMP-4 is involved in mediolateral somite patterning and specification of hypaxial muscle lineage in the chick embryo (Pourquie et al., 1996).

The above effects were usually only seen in embryos injected at stage 13 – 15. Development proceeds, in general, from head to tail. The process of neurulation, for example, involves the fusion and closure of the neural tube from rostral to caudal. The failure of posterior structures to develop properly may reflect the developmental stage of the embryo. This may explain why older embryos treated with the same antibodies fail to exhibit the major anomalies seen in younger animals. Animals at around stage 25 – 27 have completed torsion, flexion and neurulation. Limbs are well formed and digit formation underway. The continued growth of established structures, rather than the development of new ones, may render the older embryos less sensitive to antibody inhibition, at least at the doses tested. It would appear unlikely that a structure such as a limb could drastically alter its developmental course at this stage. Younger animals at the 13 – 15 stage on the other hand have just begun to undergo the processes of torsion and flexion. Limb primordia are just becoming established and little limb growth has occurred. Neurulation is far from complete, although fusion of the neural tube may extend beyond the region of the forelimbs by stage 15. Tail formation is not yet underway. In addition, the fact that the embryo is not yet completely enclosed by ectoderm may allow the

antibody greater access to the interior. It should be noted that BMPs can form heterodimers. This may mean that in addition to inhibition of BMP-2 dimers alone, some BMP combinations may be rendered partially or completely inactive. Since colocalization of different BMPs has been noted in many instances, and since it has been speculated that different BMPs can substitute for one another, it is not unreasonable to assume that heterodimer formation may in fact occur in the developing chick embryo. The addition of an antibody against a particular factor may interfer with the action of other, closely related factors as well.

Fig. 20: Results of injection of sham injection of TBS carrier into perivitelline spaces of stage 13-15 and stage 25-27 chick embryos.

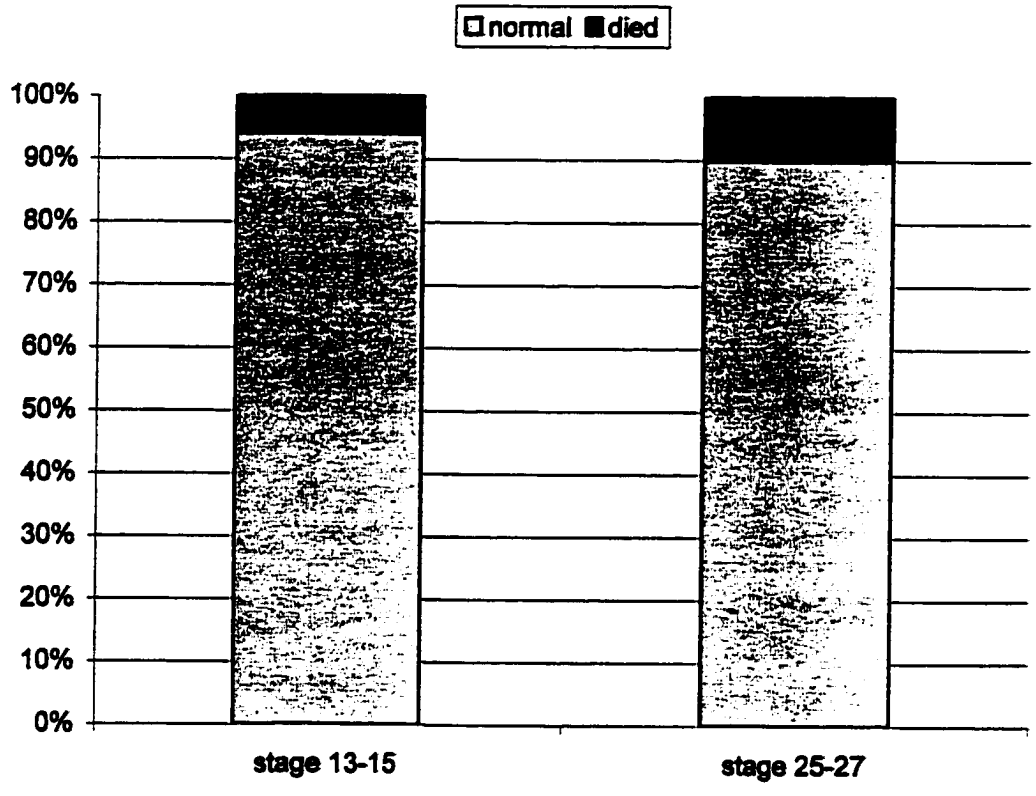


Table 1: Perivitelline injection controls: Stage 13-15 or stage 25-27 chick embryos sham injected with 0.5 mls TBS carrier. The number of morphologically normal and dead embryos are indicated.

	normal	dead	limb def.	c.f. def.	total
stage 13-15	16	1	0	0	17
stage 25-27	9	1	0	0	10

Figure 21: Results of sham injection into perivitelline spaces of stage 13 – 15 chick embryos incubated 72 and 120 hours.

A) Embryo sham injected with TBS carrier at approximately stage 13 – 15 and incubated for 72 hours. The embryo had developed to stage 25 and exhibited essentially normal morphology. The development of the limbs had progressed to the point where digit demarcation was evident. Md = mandibular arch; fl = fore limb; hl = hind limb. Scale bar = 1 mm.

B) Embryo sham injected with TBS carrier at approximately stage 13 – 15 and incubated for 120 hours. Embryo was near stage 30 and appeared morphologically normal. Limbs were lengthened and elbows/knees were clearly visible in fore and hind limbs. Webbing between digits was present. Mandibular development is greatly advanced and becoming beaklike. Neck had increased in length. Scale bar = 2 mm.

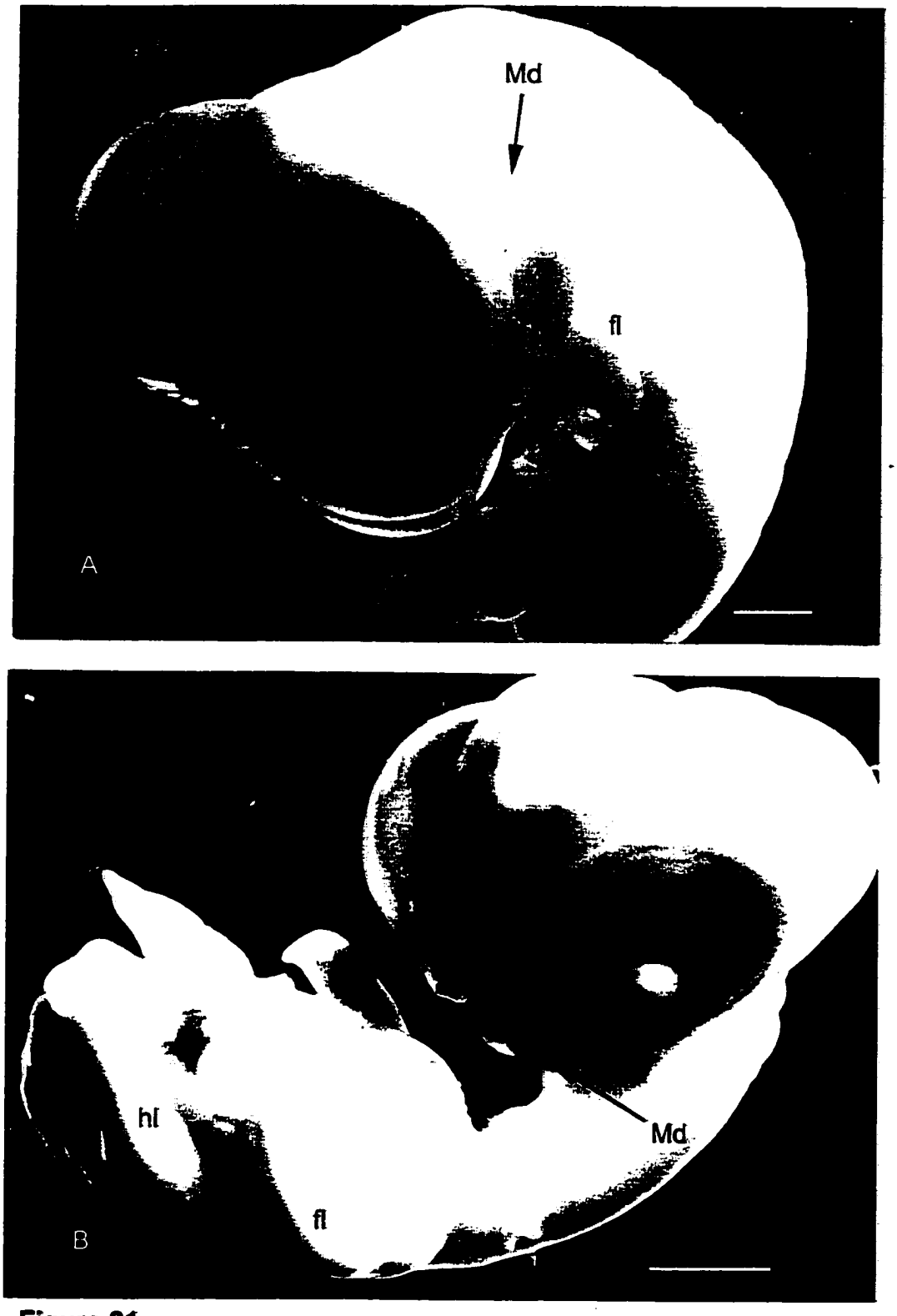


Figure 21

Fig. 22: Results of BMP-2 antibody injection into perivitelline spaces of stage 13 - 15 chick embryos.

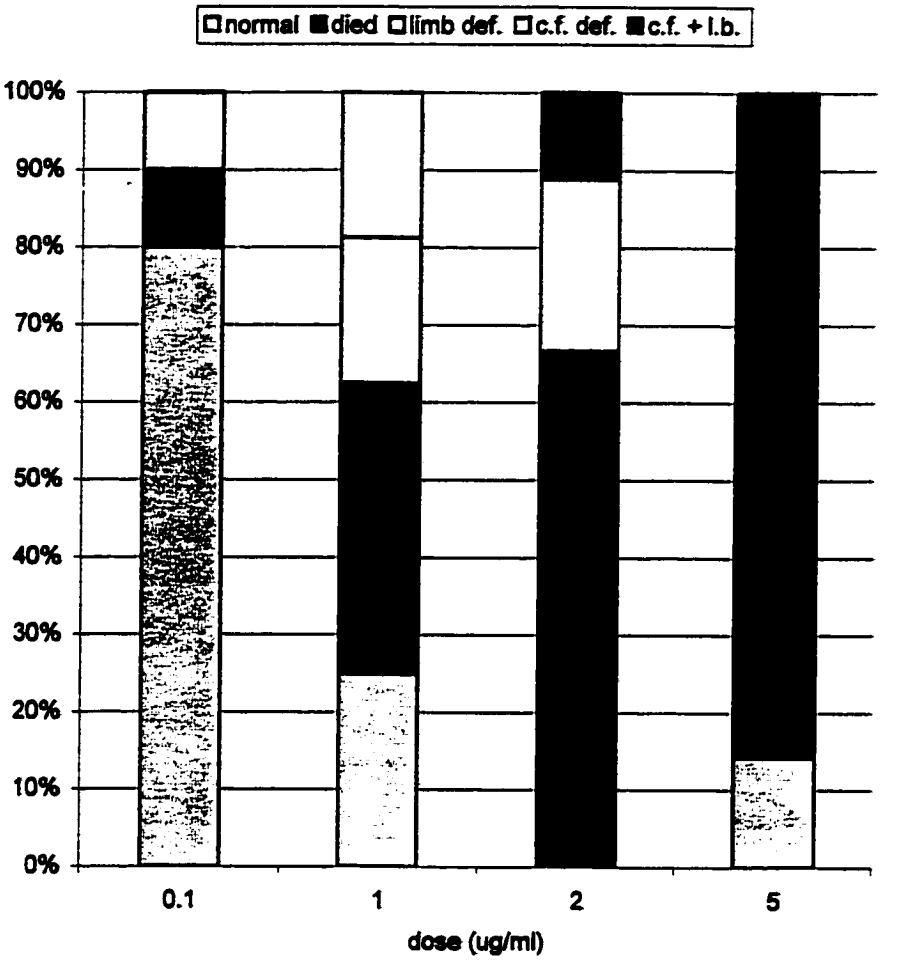


Table 2: Results of BMP-2 antibody injection into perivitelline spaces of stage 13-15 chick embryos. Concentration of antibody injected (dose) in a final volume of 0.5 mls TBS carrier is given in μg/ml. The numbers of morphologically normal and dead embryos are given, as well as the number of embryos exhibiting gross limb (l.b. def.), craniofacial (c.f. def.) or craniofacial and limb (c.f + l.b.) developmental abnormalities.

dose (μg/ml)	normal	dead	l.b. def.	c.f. def.	c.f. + l.b.	total
0.1	16	2	2	0	0	20
1	4	6	3	3	0	16
2	0	12	0	4	2	18
5	2	12	0	0	0	14

Figure 23: Examples of growth aberration seen in chick embryos after perivitelline injection of BMP-2 antibody.

A) Head of normal control sham injected at stage 13 – 15 and incubated for approximately 4 days. Mandible (Md) and maxillary (Mx) are indicated. Scale bar = 1 mm.

B) and C) Example of embryo injected at stage 13 – 15 with 2 μg/ml BMP-2 antibody and incubated for 4.5 days. Both the mandible (Md) and maxillary (Mx) were excessively developed and atypically shaped. Note the extreme enlargement seen in the brain and the lack of visible eyes. The brain appeared exposed and not completely covered by epidermis. There was truncated development of the caudal region of the body. Fore limbs (fl) appeared normal but hind limbs were absent and caudal development had abruptly ceased. The body terminated prematurely in what appeared to be a truncated tail bud (*). Scale bar = 2 mm.

Figure 23

Fig. 24: Results of BMP-2 antibody injection into perivitelline spaces of stage 25-27 chick embryos.

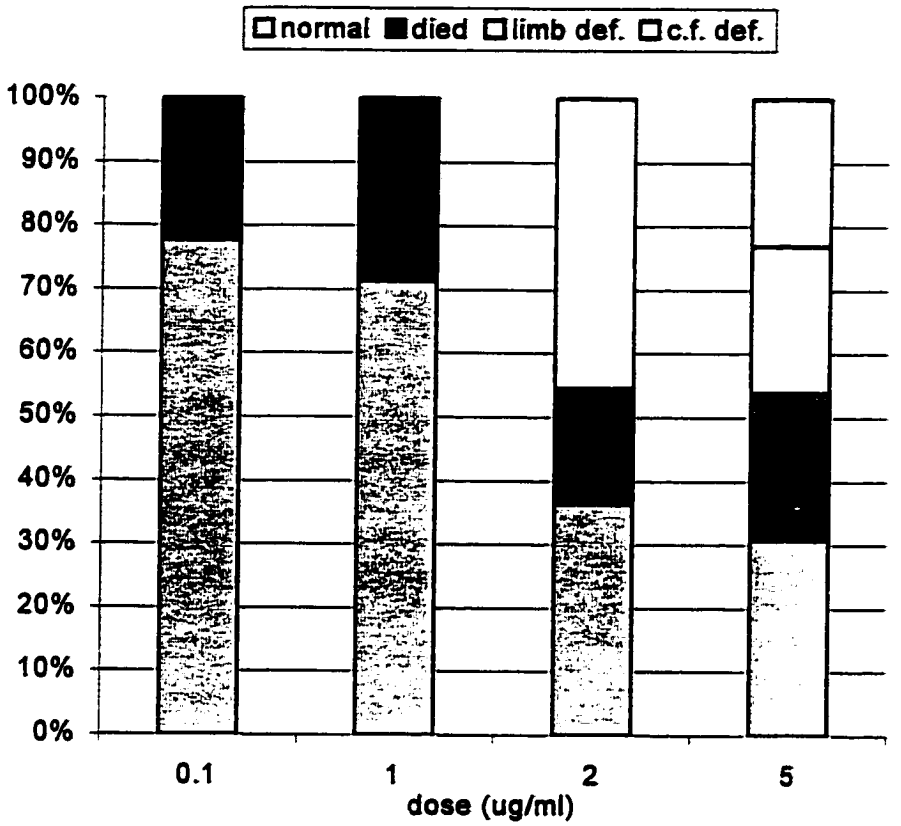


Table 3: Results of BMP-2 antibody injection into perivitelline spaces of stage 25-27 chick embryos. Concentration of antibody injected (dose) in a final volume of 0.5 mls TBS carrier is given in μg/ml. The number of morphologically normal and dead embryos are given, as well as the number of embryos exhibiting gross developmental abnormalities of the limbs (l.b. def.) or craniofacial region (c.f. def.).

dose (μg/ml)	normal	dead	l.b. def.	c.f. def.	total
0.1	7	2	0	0	9
1	10	4	0	0	14
2	4	2	0	5	11
5	4	3	3	3	13

Fig. 25: Results of TGF-beta antibody injection into perivitelline spaces of stage 13-15 chick embryos.

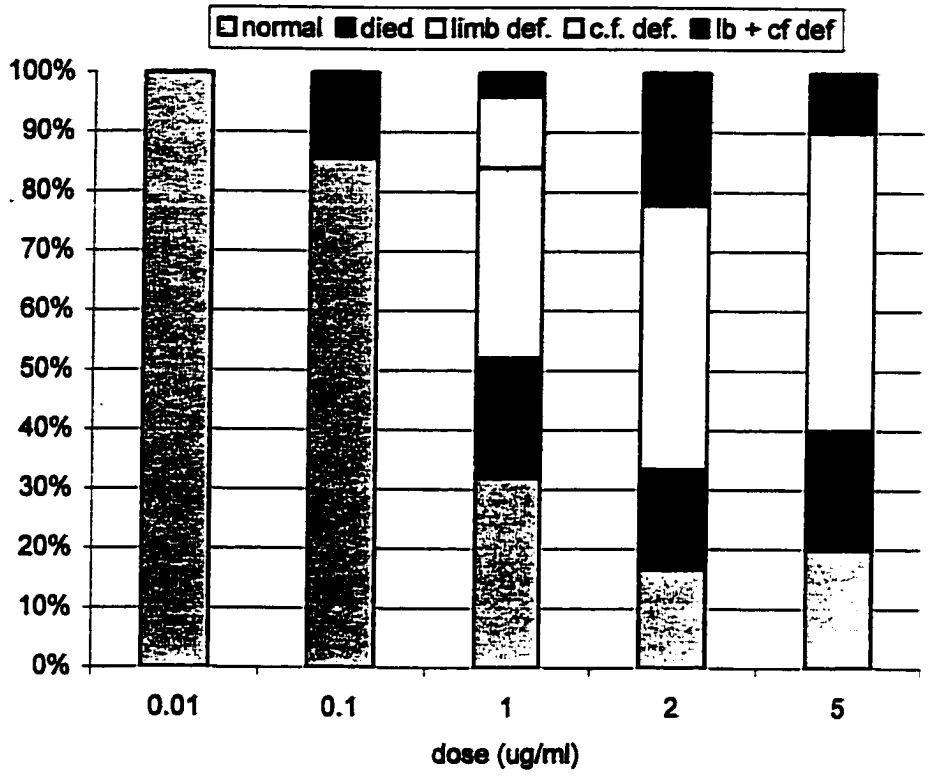


Table 4: Results of TGF-β antibody injection into perivitelline spaces of stage 13-15 chick embryos. Concentration of antibody injected (dose) in a final volume of 0.5 mls TBS carrier is given in μg/ml. The number of morphologically normal and dead embryos are given, as well as the number of embryos exhibiting gross developmental abnormalities of the limbs (l.b. def.) or craniofacial region (c.f. def.) or both (l.b. + c.f. def.).

dose (μg/ml)	normal	dead	l.b. def.	c.f. def.	l.b. + c.f. def.	total
0.01	10	0	0	0	0	10
0.1	12	2	0	0	0	14
1	8	5	8	3	1	25
2	3	3	8	0	4	18
5	2	2	5	0	1	10

Figure 26: Stage 13 - 15 embryos exposed to TGF-β antibodies and incubated for 72 hours.

A) Embryo treated with 2.0 μg/ml TGF-β antibodies showed severe developmental defects. These include reduced eyes, retarded head growth and misshapen fore (fl) and hind (hl) limbs. Mandibular growth (Md) did not appear unusual. White arrow indicates extraneous membranous tissue associated with growth constriction midway along the rostral-caudal axis. (*) = tail bud.
B) Embryo treated with 2.0 μg/ml TGF-β antibodies and exhibiting misshapen limbs and polydactyly. White arrow indicates termination of caudal development as a mass of membranous tissues located posterior to the hind limbs (hl). Note the lack of eye development.
C) Close up view of polydactylous hind limb (hl) and possible bifurcation of fore limb (fl).

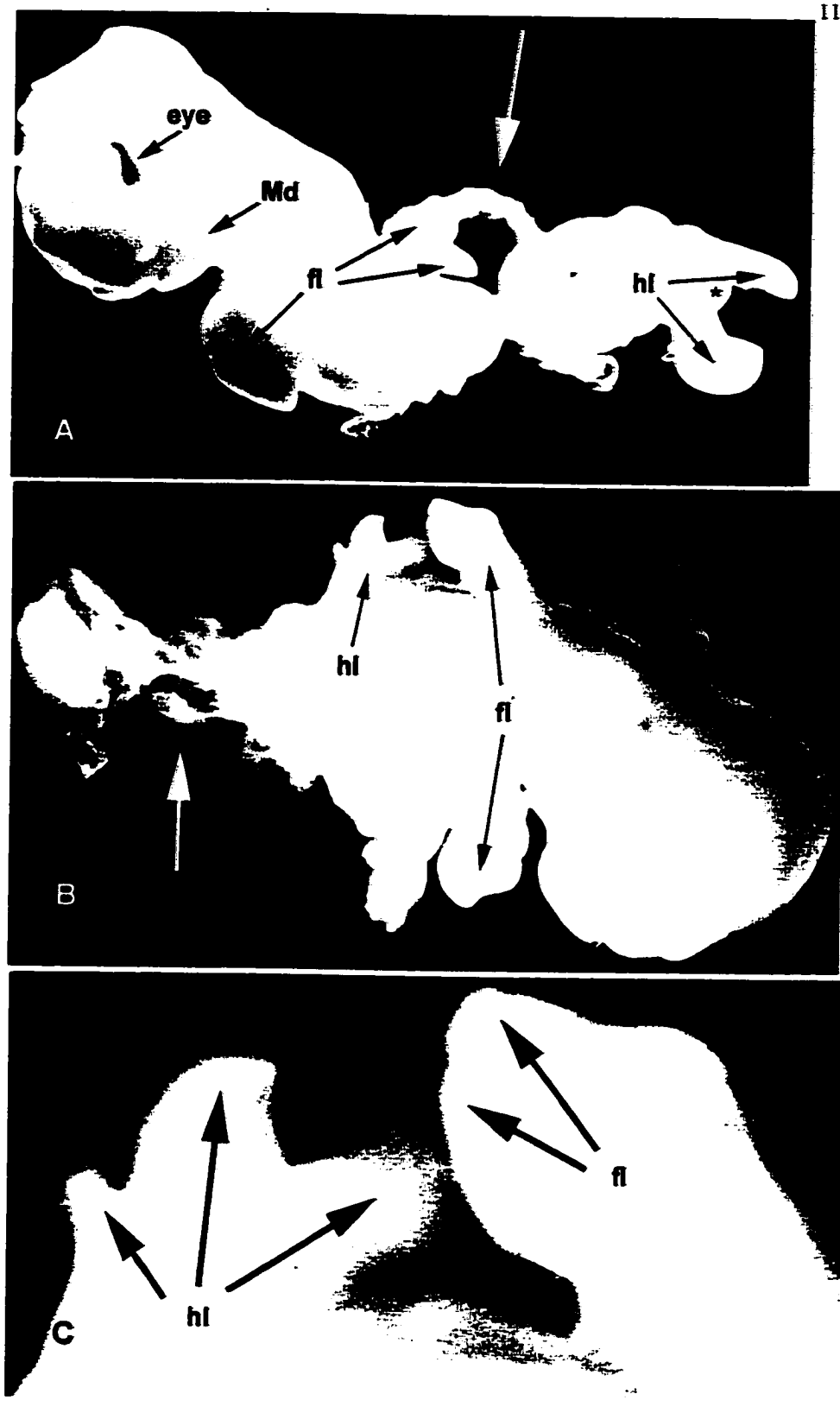


Figure 26

Figure 27: Examples of limb growth in embryos challenged with eitherTGF-β or BMP-2 antibodies.

A) Section of normal limb bud from sham injected embryo. Note digits (arrows) are delineated and there is 'webbing' between digits, forming ridges. Cartilage precursors to limb long bones are indicated (c). Scale bar = 1 mm and is applicable in all cases illustrated.
B) Section through limb of embryo treated with 1.0 μg/ml BMP-2 antibody at stage 13 - 15. Two digits are clearly visible. Cartilage of long bones (C) can be seen. Note lack of indentation between digits and thickened apical ectodermal ridge (AER).
C) Chick embryonic limb bud section after perivitelline injection of 1.0 μg/ml BMP-2 antibody at stage 13 - 15. As in (B), only two digits are clearly visible, and AER is much thicker than in control. There may in fact be 2 AERs (arrows).
D) Chick embryo limb bud section after perivitelline injection with 2.0 μg/ml TGF-β antibody. Development of limb bud was severely altered, with radically hooked tip.
E) Chick embryo limb bud section after perivitelline injection with 1.0 μg/ml TGF-β antibody. As in (D), limb has hooked downward, although not as severely.

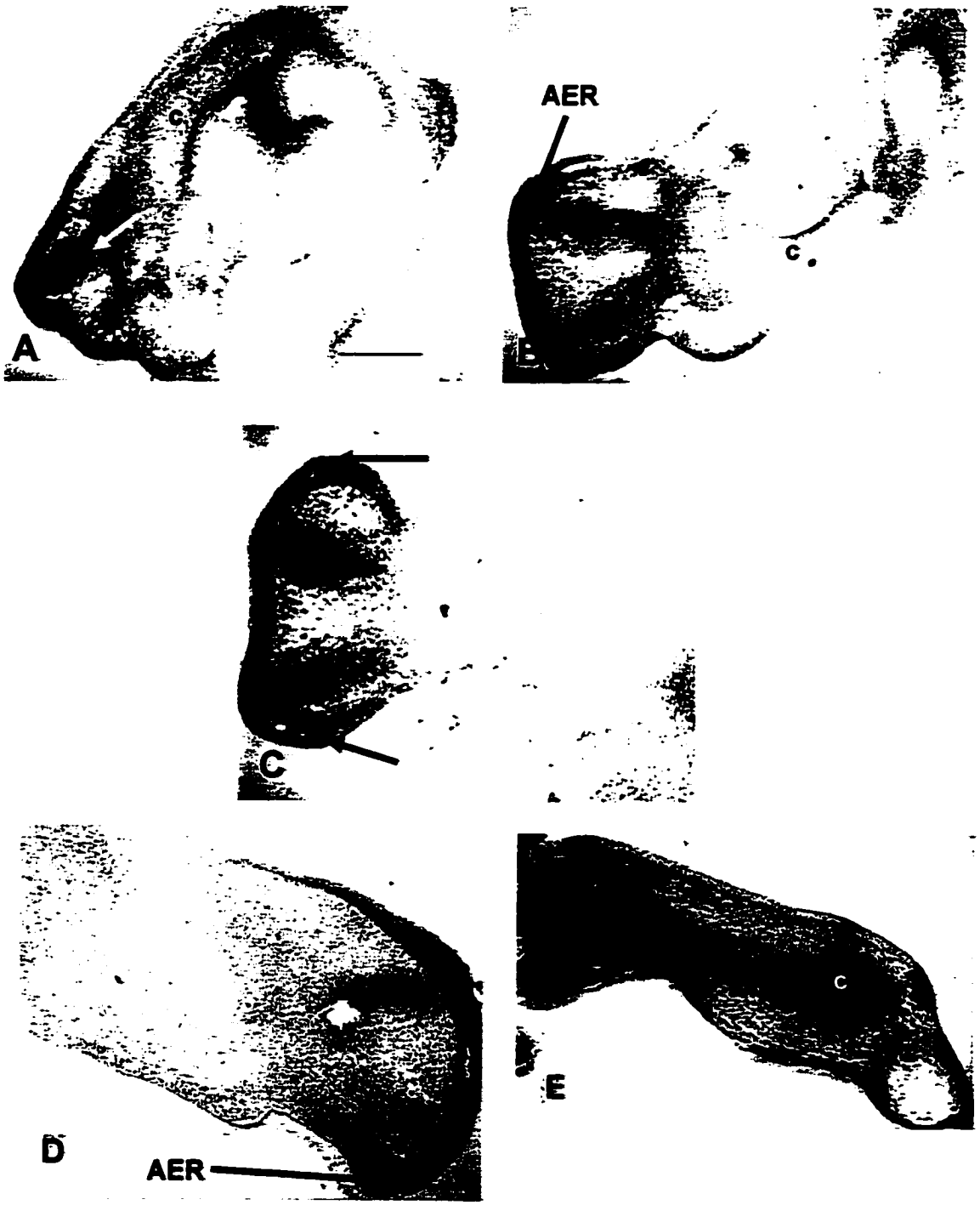


Figure 27

Figure 28: TGF-β- antibody injected embryo with neural tube defects.

A) Dorsolateral view of stage 14 embryo injected with 2.0 μg/ml TGF-β antibody. Note lack of tail and extreme angle of limbs. White arrow indicates region of abnormal mesencephalic development. Caudal neural tube did not fuse (thick black arrow).Eyes were reduced or absent. Scale bar = 2 mm.
B) Ventrolateral view of the same embryo oriented to show hole in brain (white arrow). Scale bar = 2 mm.

Figure 28

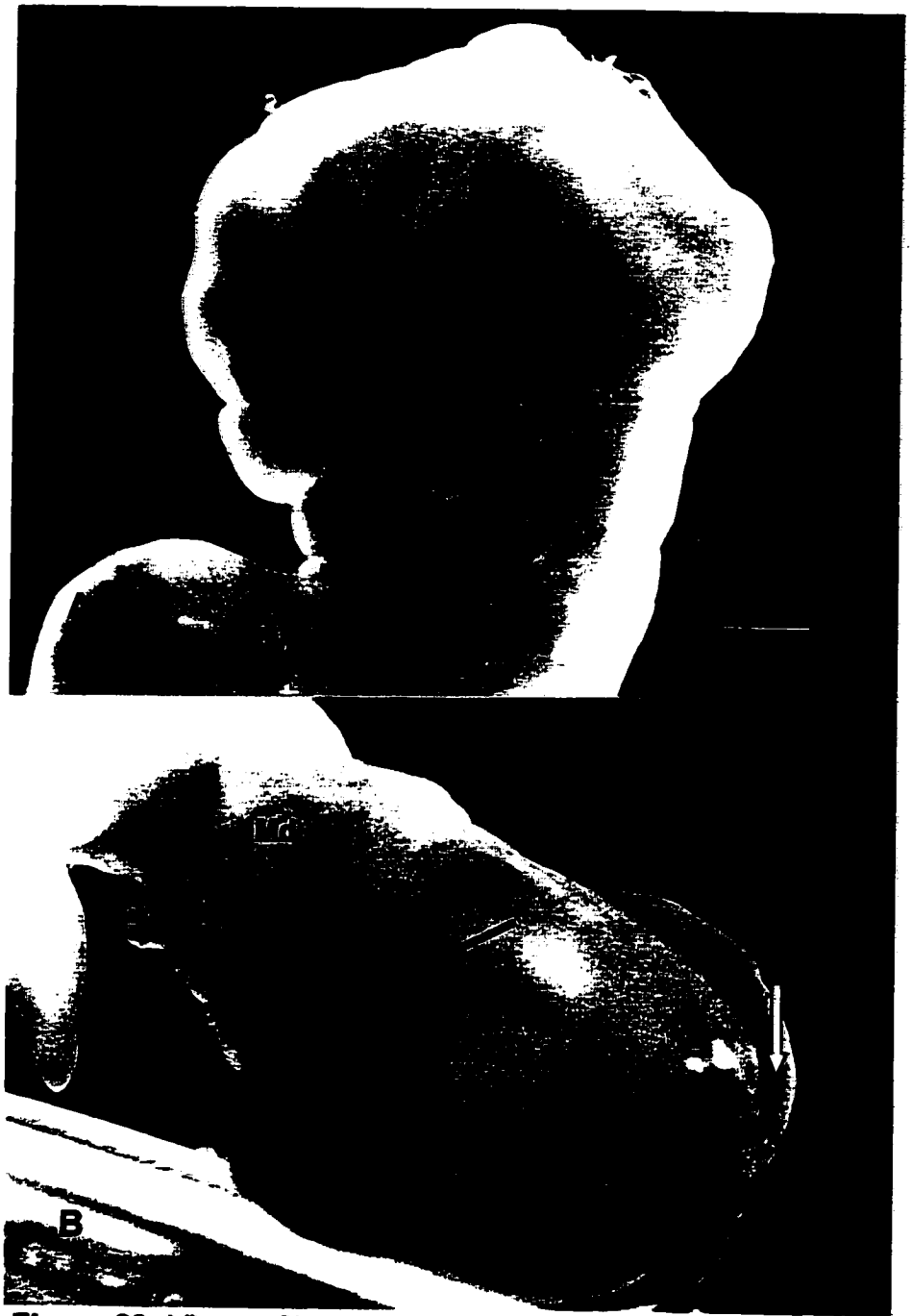


Figure 29: Views of heads of chicks injected in perivitelline space with TGF-β or BMP-2 antibodies.

A) Stage 13 – 15 embryo treated with 2.0 μg/ml anti-TGF-β showing normal mandible development. The development of the eye may indicate micro anopthalmy or reduction in size. Scale bar = 1.5 mm.

B) Stage 13 – 15 embryo treated with BMP-2 antibody showing abnormally large ventral oronasal cavity (Arrow). Note only one side of embryo has eye. Region of missing eye is indicated by white arrow.

Fig. 30: Results of TGF-beta antibody injection into perivitelline spaces of stage 25-27 chick embryos.

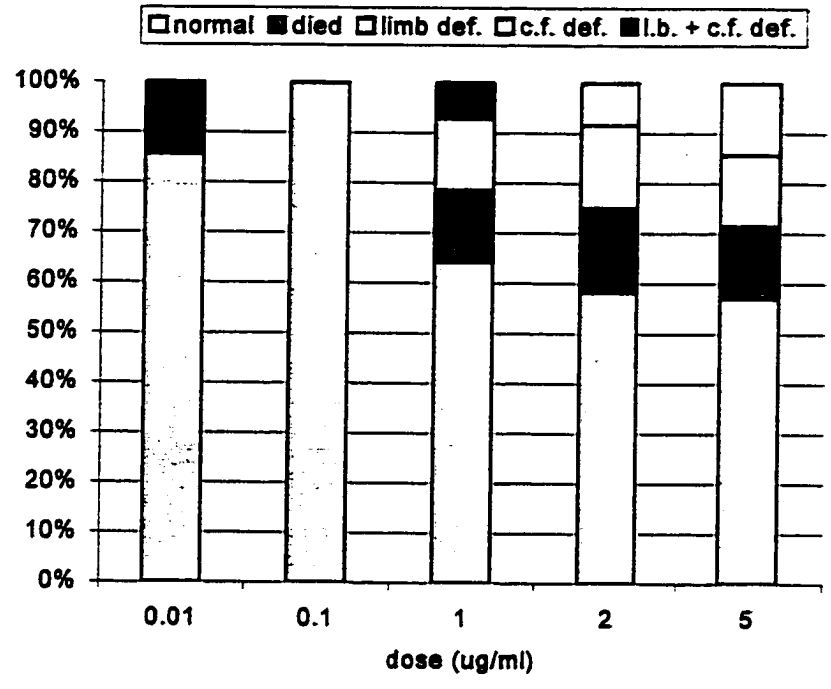
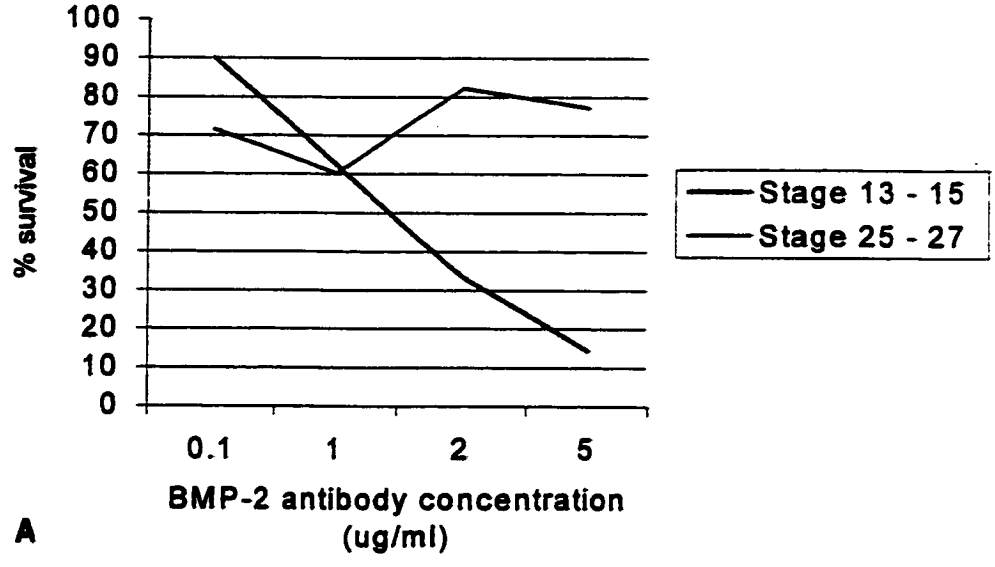


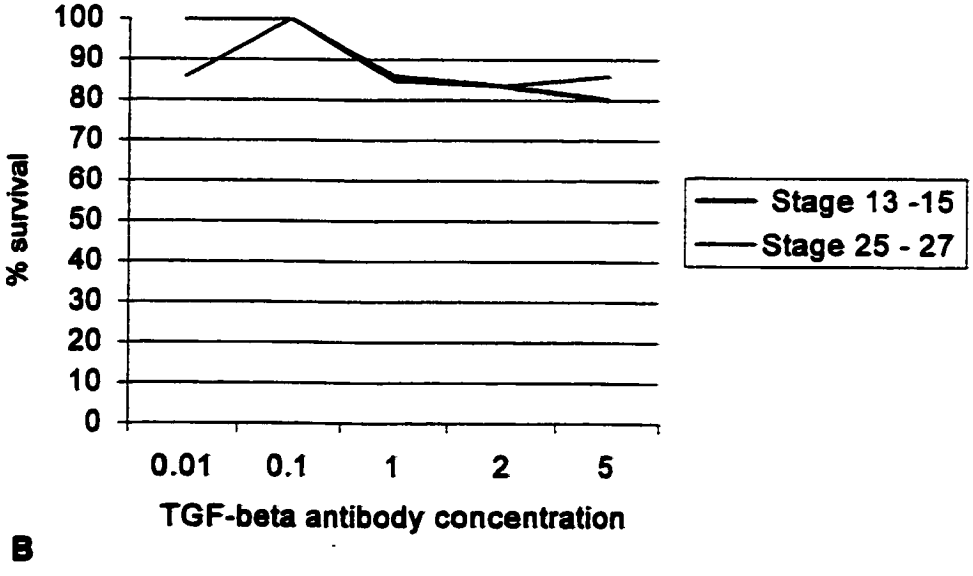
Table 5: Results of TGF-β antibody injection into perivitelline spaces of stage 25-27 chick embryos. Concentration of antibody injected (dose) in a final volume of 0.5 mls TBS carrier is given in μg/ml. The number of morphologically normal and dead embryos are given, as well as the number of embryos exhibiting gross developmental abnormalities of the limbs (l.b. def.) or craniofacial region (c.f. def.) or both (l.b. + c.f. def.).

dose (μg/ml)	normal	dead	l.b. def.	c.f. def.	l.b. + c.f. def.	total
0.01	6	1	0	0	0	7
0.1	14	0	0	0	0	14
1	18	4	4	0	2	26
2	14	4	4	2	0	24
5	8	2	2	2	0	14

Figure 31: Survival of embryos injected with BMP-2 or TGF-beta antibodies.



A



B

Figure 31: Survival of embryos after perivitelline injection of increasing concentrations of BMP-2 or TGF-β antibodies. In (A), LD_{50} for stage 13 – 15 embryos is approximately 1.5 μg/ml of BMP-2 antibody. For stage 25 – 27 embryos, BMP-2 antibody concentrations tested do not approach an LD_{50} value. For TGF-β antibody, toxicity is consistently low at all concentrations tested and at all embryonic ages.

Figure 32: Effect of antibody treatment upon embryonic orientation.

A) Stage 13-15 embryo treated with 1.0 μg/ml anti-TGF-β. While the head and neck regions were normal, the spine arched in the opposite direction to that normally taken by unchallenged embryos, as indicated by the thick arrow. Caudal development was retarded and hind limbs (thin arrow) appear stunted. Md = mandible. Scale bar = 1mm

B) Embryo treated with 2.0 μg/ml BMP-2 antibody. Both fore and hind limb and tail development were normal. In general, torsion and flexion of the embryo were not unusual except for the neck region which appeared elongated slightly over controls. Md = mandible; tb = tail bud. Scale bar = 1 mm.

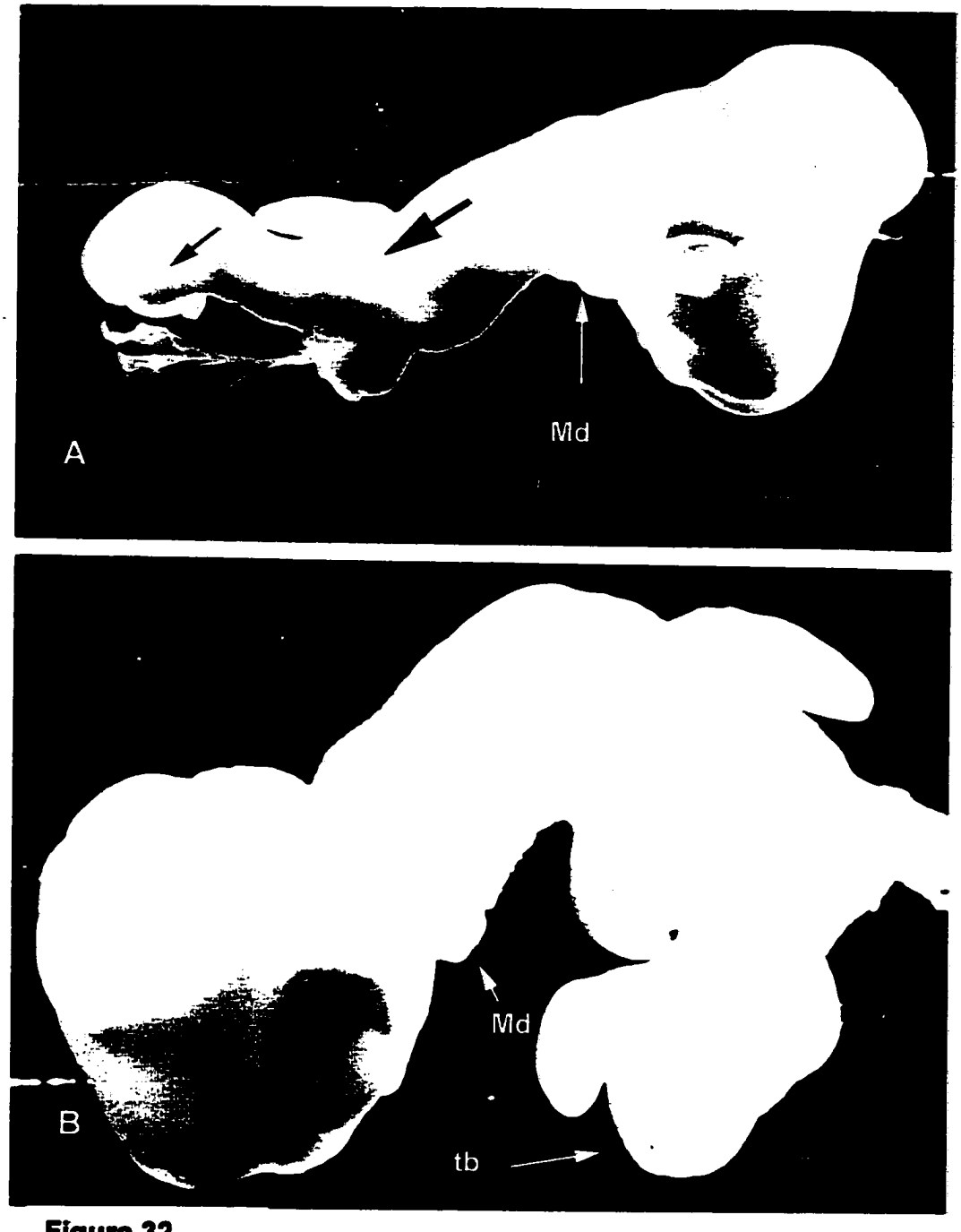


Figure 32

Chapter 5: Effects of TGF-β and BMP-2 antibodies and okadaic acid on the morphology and growth of chick mandibular and limb bud explants and the development of bone and cartilage.

5.1 Introduction

In the chick embryo, both the mandible and limb begin as epithelial pockets which encase mesenchymal cells that have migrated into the region. Both ultimately develop into complex structures that arise from their mesenchymal components' interacting with the epithelium. The origins of the mesenchyme of each structure differ however.

Limb bud mesenchyme derives from lateral plate mesoderm, more specifically from somatopleural cells in the embryonic flank (Haack and Kessel, 1994). The limb field first forms in the flank of the embryo at about stage 15, followed by thickening of the apical ectodermal ridge, with budding beginning at stage 17 (Richman, 1994). A phase of elongation and growth of the limb bud follows, from stage 18 to 24, with differentiation of cartilage and bone from stage 28 until hatching. In the case of the mandible, neural crest cells enter the primordial arch from the region of the midbrain at about stage 16, requiring interaction with the epithelium from stage 18 to 24 in order to develop normally (Tyler and Hall, 1977; Tyler, 1978). Both the mandible and limb bud, if of the right developmental age, are specified and able to develop *in vivo* from mesenchyme and epithelium alone, independent of any input from other parts of the embryo. These structures then are ideal for studying pattern formation and development in isolation. Their ability to grow for days or even weeks in culture enables the addition to the medium of a variety of factors and the subsequent analysis of the responses.

The process leading to the development of skeletal elements in the vertebrate embryo can be divided into three parts (for review see Hall and Miyake,

1995). In the first stage, epithelial-mesenchymal interactions precede the formation of cell condensations. Condensations are aggregates of cells critical for skeletogenesis and from which skeletal components are initiated (Hall and Miyake, 1992; Langille, 1994). The second phase is the formation of condensations. The appearance of condensations is accompanied by a concurrent increase in specific gene transcription, including type II and type IV collagen and core protein proteoglycan, all products associated with chondrogenesis (Hall and Miyake, 1992). The third phase is cell differentiation and proliferation. The TGF-β superfamily members BMP-2 and TGF-β are expressed during the first two phases, namely the precondensation and condensation phases (Hall and Miyake, 1995). The present study also indicates that BMP-2 and TGF-β are detectable during the third phase associated with cells forming skeletal elements.

In this series of experiments, all explants were derived from stage 21 chick embryos. By stage 21, cellular condensations leading to mandible skeletal element formation are established. Cartilage first appears at stage 25 and bone at stage 32 (Hall, 1990; Hall and Coffin-Collins,1990). In the limb, events are temporally similar, with condensations appearing by stage 22 (Cancedda et al., 1995). Chondrogensis begins at stage 25 and endochondral bone formation at stage 28 and continues until hatching occurs (Richman, 1994). Stage 21 explants are in the early stages of condensation, with no cartilage or bone present. During this stage, BMP-2 and TGF-β are expressed in both regions. This stage is ideal for the addition of appropriate factors to study their effects on development of pattern and skeletal elements in limb and mandible.

As shown in chapter 4, older embryos have developed to the point where pattern formation appears to be set and essentially unresponsive to antibody inhibition. While immunostaining of chick sections indicates that TGF-β and, to a

lesser degree BMP-2, are present in the embryo at later developmental stages, the change in distribution in the mandible and limb suggests that TGF-β related factors may have multiple developmental roles (See chapter 3). Pattern formation is an early event and requires the participation of these factors whereas later in development, TGF-β and, possibly BMP-2 are involved in skeletal element formation, including bone and cartilage.

The effects of exogenous antibodies on cell growth and differentiation can be readily studied in explant culture. The substance of interest is added directly to the medium. Concentrations are easily controlled and any gross morphological effect is determined by analysis with a microscope. TGF-β and/or BMP-2 antibodies were used to investigate the effect of the sequestration and removal of these growth factors in an attempt to understand their role in development of the limb bud and mandible.

One way of elucidating the role of specific peptide growth factors in living systems is to supply exogenous amounts directly *in vitro* or *in vivo* and measure the resulting responses. This approach can involve the addition of purified growth factor to the system of interest, upregulating the expression of the factor in the cell via the introduction of regulatory molecules or even through the insertion of specific gene constructs designed to produce the cytokine in the host cell. While these approaches can provide information about the effects of exogenous amounts of a factor, much useful information can be derived from the inhibition of growth factor action. The transfection of cell lines with dominant negative growth factor receptors allows the binding of ligand but effectively blocks downstream signal transduction. Truncated or mutant TGF-β superfamily receptors transfected into different cell lines, for example, are used to study receptor interactions and cell responses (Ebner et al., 1993; Wrana et al., 1994; Franzén et al., 1995). The expression of dominant negative receptors in transgenic animals is another approach that allows

the study of the effects of growth factor inhibition. The expression of a dominant negative TGF-β type II receptor in the epithelium of transgenic mice, for example, results in excessive and uncontrolled growth of the skin, indicating a possible role for TGF-β as an inhibitor of cell proliferation (Wang et al., 1997). Another novel method of inhibiting or interfering with the action of TGF-β involves its sequestration by the type III receptor. The type III receptor (betaglycan) is a membrane anchored protein which is not directly involved in the intracellular signaling cascade initiated by TGF-β, but rather binds ligand and makes it available to the type II receptors (López-Casillas et al., 1993, 1994). Transfection of a human breast cancer cell line, designated MDA-MB-23, with tetracycline-repressible human RIII expression vector, resulted in the binding and removal of TGF-β 1 and –2. MDA-MB-23 cells expressing the construct exhibited significantly lower tumor forming ability when compared to unaltered controls (Sun and Chen, 1997).

The use of antibodies to block specific proteins *in vivo* and *in vitro* provides a powerful and relatively fast alternative to other methods to inhibiting cytokine signaling. Monoclonal antibodies against lectin perturb or even prevent *Xenopus* lower jaw development *in vivo*, probably by interfering with cell adhesion and migration (Evanson and Milos, 1996). Anti-lectin antibody affects *Xenopus* neural crest migration *in vivo* (Milos et al., 1993). Latent TGF-β binding protein (LTBP) is thought to bind the latent (inactive) form of TGF-β and then associate with extracellular matrix components where TGF-β is activated. A polyclonal antibody against LTBP was used to block its binding of to extracellular matrix in mesangial cells *in vitro* (Hori et al., 1998). The expression of mRNA for fibronectin and collagen I was consequently down regulated in cells exposed to antibody.

Embryonic chick mandibles and forelimb buds were maintained in explant culture on a millipore filter substrate for up to 24 days. Untreated cultures did not

form monolayers but grew in three dimensions and, upon sectioning and analysis, were found to develop into several distinct tissue types, including chondrogenic and osteogenic cells. Explants grown in medium supplemented with antibodies against either BMP-2 or TGF-β showed increased growth rates and achieved significantly greater maximum heights than did controls. The addition of antibodies appears to accelerate the differentiation of bone in the explants as well as increase the amount of extracellular matrix molecules associated with both cartilage and bone. This is especially pronounced in explants treated with anti-TGF-β. In addition, the growth of limb bud explants in medium containing high concentrations of TGF-β antibody results in the formation of what appears to be membrane bone; the genesis of limb bones is the result of endochondral ossification, i.e., through the replacement of cartilage rather than through the direct formation of bone.

Explants had a tendency to remain in a compact unit and seldom grew beyond the limits of the filter which they were placed upon. However, explants treated with okadaic acid, a powerful and specific inhibitor of protein phosphatase, flattened and spread over the filter support and lost most of their initial shape rather than remaining compact and growing vertically. Okadaic acid is a polyether fatty acid produced by dinoflagellates and accumulates in the digestive glands of shellfish and certain marine sponges including *Halichondria okadaii* and *Halichondria melanodocia* (Cohen and Cohen, 1989; Cohen *et al.*, 1990). Okadaic acid is one of the agents which causes diarrhetic shellfish poisoning, often reported after ingestion of contaminated bivalves. It is a potent *in vitro* specific inhibitor of protein phospatases-1 and 2 and a tumor promotor (Cohen and Cohen, 1989). In human keratinocyte culture, exogenous TGF-β inhibits DNA synthesis and induces a state of growth arrest. This was accompanied by an increase in protein phosphatase 1 activity (Gruppuso et al., 1991). Okadaic acid enhances expression of the rat interstitial collagenase gene,

the homologue of the human collagenase-3 gene, in rat growth plate chondrocyte cultures (Grumbles et al., 1996).

5.2. Materials and Methods

5.2.1. Establishment of explant cultures

Fertilized eggs of the domestic fowl, *Gallus domesticus* , were obtained from Cook's Hatchery (Truro, Nova Scotia) and incubated at 36° C and 95% humidity in a Favorite Incubator model 30E (Leahy Manufacturing Company, Higginsville, Mo.) for approximately 96 hours. Eggs were opened under sterile conditions, the embryos removed and transferred to sterile 15 x 100mm disposable Petrie dishes (Fisher Scientific Company, Nepean, Ontario) containing chick Ringer's solution (New, 1966) where they were staged according to Hamburger and Hamilton (1951). Only stage 21 embryos were selected for explant culture.

Mandibular arches or forelimb buds were cultured as explants in order to examine their development and growth as well as their responses to exogenous growth factor antibodies and inhibitors as compared to untreated controls. The procedure is a modification of that adapted by Hall (1978) and more recently used for chick cochlear organ culture (Stone and Cotanche, 1991). Mandibular processes and forelimb buds were removed under a dissecting microscope and rinsed in fresh Ringer's cooled to 4° C. Explants were cultured on Millipore filters cut into squares 3 mm on a side having a pore size of 0.45 µm (Millipore Corporation, Bedford, MA.). These filters were supported by stainless steel grids placed into 35 mm diameter sterile Falcon plastic culture dishes (Becton Dickinson Labware, Lincoln Park, N.J.). Prior to use, grids were cut to size (approximately 25 x 25 mm), rinsed in several changes of 80% ethanol, rinsed in

Ringer's and autoclaved. Grids were bent slightly in the middle to produce a bridge onto which were placed the filters holding the tissues to be cultured. Grids were likewise soaked in 80% ethanol and rinsed in Ringer's solution prior to use, but not autoclaved. Culture medium was added to the dishes so that the grid and overlying filter maintained contact with the medium but the tissue remained exposed to the air. Culture medium consisted of Ham's F12 and BGJb in a ratio of 1:1, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin mixture (Gibco BRL). Collagen synthesis and mineralization of cultures were aided by the addition, at day 4, of ascorbic acid (75 μg/ml) and β-glycerophosphate(10 mM) (Tenenbaum and Heersche, 1982). Antibodies, diluted into fresh medium, were added only once on the first day of culture. Subsequently, medium was changed every 3-5 days or as indicated by phenol red staining. Explants were maintained in culture for up to 24 days in a humidified incubator at 37° C and 5% CO_2 before removal for fixation and subsequent analysis.

5.2.2. Treatment of explant cultures

Single explants were grown on individual 3 mm^2 filters, with up to four limb buds or mandibles per culture dish. Limb buds were placed in the same orientation, with the cut end in contact with the filter and medium and the distal surface of the limb bud facing up. Mandibles were also placed with the cut surface in contact with the filter and the rostral surface facing up. This assembly, when placed in a Petrie dish containing a defined nutrient culture medium, allowed the growth of the explant to take place at the air-liquid interface. Medium was free to diffuse into the explant which, when covered, remained hydrated in the high humidity environment of the incubator.

Explants were allowed to stabilize and attach to the filters for 18 - 24 hours. The medium was then removed by aspiration and replaced with fresh

medium containing differing concentrations of antibodies or okadaic acid. Cultures were grown with initial concentrations of 0.1, 1.0, and 10 µg/ml for either TGF-β or BMP-2 antibodies. Okadaic acid was added to a final concentration of 0.01, 0.1 or 1.0 µg/ml. Controls were grown in medium alone. Explants were examined periodically under a dissecting microscope and gross morphological effects were noted. Explant cultures were grown in an incubator maintained at 37° C and 5% CO_2. Media were changed initially after 1 day, prior to the addition of fresh medium containing antibody, and subsequently with fresh medium alone every 3-6 days. Explants were fixed and embedded in paraplast and sectioned. Selected sections were stained for the presence of cartilage and bone using Hall-Brunt Quadruple stain (HBQ) (Hall, 1986). Immunostaining was done according to the procedures outlined in chapter 3.

The peak explant heights, measured from the surface of the filter, achieved over time provided a measure of overall growth of the explant. In order to determine heights, explants and their attached filters were fixed, paraffin embedded, and sectioned. Sections of individual explants were analysed under a compound microscope. Individual explants were examined several times to determine the greatest height reached and the results pooled to provide a mean peak height.

5.3. Results

5.3.1. External appearance of explants

The explant culture system used allowed growth of explanted tissues and maintained their morphological integrity. Growth tends to be confined to the surface of the filter, which provides a physical boundary within which the explant

remains. Explants grown submerged in medium have a tendency to spread out and form a monolayer of mixed cell types on the bottom of the dish. Growing tissues in explant culture allows at least some of the cell and tissue organization found in the explant to be sustained. This arrangement may more closely mimic the actual situation found in the developing chick. Explants grown in this manner ultimately do not resemble their counterparts in the intact animal morphologically, however, and do not develop to the same extent. This is especially true of explants maintained in culture for long periods. On the other hand, distinct tissue types continue to develop including cartilage, bone and connective tissues.

In general, mandibular and limb bud explants grew well, although during the first few days of culture, a small area of cell death around the periphery may appear. Dead areas appeared as opaque areas initially found at the periphery of the explants. Under microscopic analysis, cells appeared shrunken with pycnotic nuclei. Both untreated controls and those grown in the presence of 0.1 and 1.0 µg/ml IgG did not differ. Figure 33 illustrates the appearance of mandibular and limb bud controls after 1 day of incubation. By this time, explants had anchored to the filter substrate but retained much of their original morphology. Left and right mandibular arches were recognizable by their position on either side of an opaque midline (Fig. 33A). The view in the figure is from above, with the epithelium of the distal end of the explant projecting upward. The mesenchyme of the cut end is in contact with the filter. Similarly, after 1 day of culture, the contours and overall appearance of limb bud explants were relatively unchanged from initial preparation (Fig.33B). The curve of the distal portion of the developing wing is apparent. Limb buds are likewise placed with the mesenchyme (proximal end) in contact with the filter. By day 2, mesenchyme continued to migrate out of the proximal end of the explant and began to cover the filter. In general, explants in both the control and treatment groups exhibited similar growth characteristics during the first few days of culture and were indistinguishable. After about five

days, some growth differences became apparent.

By day 15, explants have more than doubled their original sizes and may cover much of their filters (Fig. 33C). Mesenchymal cells from some explants began to attach themselves to the stainless steel grid supporting the filter. The majority of explants were confined to the area of the membrane, more often growing to the edge and then stopping. In a few cases, explants grew what appeared to be prominent processes or extensions. This appeared to be most pronounced and widespread in the explants exposed to TGF-β antibody, but occurred with less frequency in both control and BMP-2 antibody treatment groups. The growth of such processes seemed to be confined to limb bud explants, possibly a reflection of explant origin. The explant on the rght in figure 33C, a control limb bud after 15 days of culture, displayed prominent cartilages. The mandibular explant seen on the left was smoother and retained more of its original appearance. Limb bud explants, on the other hand, were much less constrained in their development and bore little resemblance to 1-day-old explants.

The most obvious signs of explant growth were the overall increase in area along the plane of the filter and the increase in explant height perpendicular to the filter. Explants had a tendency to grow up from the plane of the filter in a direction away from the medium. This growth was apparent in all explants except those treated with okadaic acid. This may be due in part to the restriction on growth imposed by the filter itself. In other words, there may be no other direction for growth to take place as explants cannot extend through the filter into the medium, nor do they usually grow beyond filter boundaries. Once mesenchymal cells have spread out of the cut end of the explant and attached to the filter, a process usually complete within a few days, expansion upward increased. Generally, one or two prominent growth peaks developed on each explant, and rarely more.

Limb buds explants cultured with medium containing antibodies against either TGF-β or BMP-2 reached greater heights and were generally larger than control explants. After 2 weeks of culture, explants treated with 1 µg/ml of antibody were noticeably larger than controls (Fig. 34). Similarly, mandibular explants exhibited growth differences when treated with BMP-2 or TGF-β antibody (Fig. 35). This was most evident in explants grown in medium containing TGF-β antibody (Fig. 35A). BMP-2 antibody addition noticeably increased explant height but not to the same degree.

Within 24 hours of exposure to okadaic acid in culture media, both limb bud and mandibular explants lost much of their three-dimensional structure and appeared flattened and diffuse. This occured at all doses tested. At concentrations of 0.01, 0.1 or 1.0 µg/ml, the explants maintain a translucent disc-like appearance while spreading to cover much of the filter by day 8 (Fig. 36). Analysis of sections showed that the height of the disc did not exceed 100µm. HBQ staining suggests that the cell mass was almost purely mesenchymal in nature and lacked any epithelium. This was in contrast to control explants that by day 8 were composed of a number of tissues including cartilage, bone, mesenchymal cells and epithelium.

At higher doses of okadaic acid (1.0 µg/ml), limb bud cultures dissociated and split into two distinct and seemingly pure cell types with very little overlap (Fig 36B). The morphology of the translucent cell type (seen in figure 36A and the right hand mass in 36B) suggested an undifferentiated mesenchymal cell line. The second opaque population of cells stained with alcian blue, indicating a chondrogenic line. These cells were small in comparison to chondrocytes and very loosely associated with each other, if at all. The identity of these cells remains to be determined, but they may be chondrocytes.

5.3.2. Growth of explants

5.3.2.1. Control explants

Mandibular and limb bud controls showed an increase in mean peak height over time. At day 1, mandibular and limb bud explants are approximately the same height, with mandibles averaging 294 µm compared to 280 µm for limb buds (Table 6; Fig. 37). After 8 days in culture, control limb buds were taller than mandibles, a trend that continues until the end of culture at day 24. After 20 days of incubation, most explants look healthy and exhibit few areas of senescence. By day 24 however, many explants began to show varying degrees of cell death, apparently progressing from outside toward the interior. Despite the increase in necrotic area, explants continued to grow. In the case of limb buds, growth began to slow slightly after day 20. This was not apparent in mandibular explants although the standard error is large at day 24 and may have hidden a similar decline in growth.

Linear regression analysis of control explants cultured in medium alone strongly indicated a linear increase in height over time (Fig. 37). The interpolated mean initial heights for both explants were almost identical at approximately 275 µm. Calculated R^2 values for mandibular and limb bud explants were 0.997 and 0.982 respectively, strongly supporting a linear growth profile. The average growth rate (slope) for mandibular explants was 11.6 µm/day and for limb buds 18.9 µm/day. This means that, under control explant culture conditions, limb bud had a growth rate almost 60% higher than mandibles. Log transformation of data yields curves that do not fit as well as a simple linear regression.

5.3.2.2. TGF-β antibody treatment

Mandibular explants grown in medium supplemented with 0.1 µg/ml of TGF-β antibody showed an almost immediate increase in size compared to control explants (Table 6; Fig.38). By day 8 of culture, treated explants averaged 487 µm in height while controls averaged 367 µm, a difference of 33%. Mandibles exposed to this level of antibody were consistently taller than controls until day 20. By day 24, treated explants exhibited a leveling of growth and approached the height of untreated controls. Control explants showed little evidence of reaching a growth plateau between day 20 and 24.

Regression analysis indicated that a linear growth profile provided an acceptable fit to the mandibular explant data with a slope of 15.4 versus 11.6 for controls ($R^2 = 0.88$). Log transformed data however appeared to provide an even closer fit ($R^2 = 0.95$) (Fig 38B). This curve suggested a profile in which initial growth was rapid but slowed after day 20 and approached a plateau. No data were available for higher concentrations of antibody so it is unknown whether or not this response was dose dependent.

Limb bud explants grown with 0.1 µg/ml TGF-β antibody exhibited an initial significant increase in height compared to untreated controls (Table 8; Fig. 39A). By day 8, limb bud explants exposed to 0.1µg/ml TGF-β antibody reached a mean height of 597 µm compared to 455 µm for untreated controls, a 31% difference. By day 20 however, growth had leveled off. No significant difference between untreated controls and explants exposed to low concentrations of antibody was seen at day 20 and only a very slight difference at day 24. Explants grown at low antibody concentrations actually showed a slight decrease in height at day 24 compared to controls (660µm versus 710µm respectively). At higher levels of antibody (1.0 µg/ml), there was no

significant difference between controls and treated explants at day 8. By day 15 however, treated explants were larger than controls, with an average height of 665 µm compared to 539 for controls. Between day 15 and day 20 of culture, explants grown at high TGF-β antibody concentrations exhibited a growth 'spurt'. By day 20, treated explants were approximately 55% higher than controls or low dose explants (1068 µm versus 687 µm and 676 µm respectively). No growth was noted between day 20 and 24.

Linear regression indicates that the growth profiles for limb bud explants grown at high antibody concentrations approximated a straight line ($R^2 = 0.94$). Growth profiles for explants grown at lower antibody concentrations were not as well accommodated by a linear curve ($R^2 = 0.73$). The growth profile for this concentration may be better approximated by the curve obtained by log transformation (Fig. 42B), providing an R^2 value of 0.977. This indicates an initial increase in growth rate between days 1 and 8 followed by a decrease in growth rate for the duration of the culture period.

5.3.2.3. BMP-2 antibody treatment

Mandibular explants grown with either 0.1 or 1.0 µg/ml of BMP-2 antibody exhibited similar growth profiles (Table 9; Fig.40A). Explants grown for up to 15 days did not exhibit significant differences in explant height when compared to untreated controls. At day 8, mandibles grown with 1.0 µg/ml antibody were slightly larger than explants grown in 0.1 µg/ml or than untreated controls. This slight difference disappeared by day 15 when all three groups reached mean heights of near 430 µm. By day 20, both treatment groups were significantly taller than controls, with mean heights of 668 and 748 µm for concentrations of 0.1 and 1.0 µg/ml antibody respectively. Controls at day 20 averaged 490 µm in height. No data were available for heights of explants treated with 1.0 µg/ml BMP-2 antibody

at day 24.

When linear regression was performed on the data, calculated slopes indicated that as concentration of antibody in the medium increased, so did the rate of growth (Fig.43B). At 0.1µg/ml, the average rate of height increase of the explants was 14.6 µm/day, not significantly different from the control value of 11.7 µg/day. At 1.0 µg/ml BMP-2 antibody, the growth rate increased dramatically to almost 22 µm/day. This value represented a doubling in growth rate as compared to controls.

The growth of limb bud explants exposed to BMP-2 antibody is shown in table 10 and figure 41A. Overall growth profiles for control and 0.1 µg/ml BMP-2 antibody treated explants were almost identical, at least until day 20 of culture when treated explants exhibited what appeared to be a slight drop in explant height. Up until day 20, there was no significant difference between control and the 0.1 µg/ml test group. At a concentration of 1.0 µg/ml, limb bud explants experienced a significant increase in rate of growth after day 8. Up until this time, both treated and control explants grew at the same rate. By day 15 however, there was a noticeable difference in the mean heights of explants treated at concentrations of 1.0 µg/ml BMP-2 antibody versus either 0.1 µg/ml or untreated controls. At day 15, explants treated with 1.0 µg/ml BMP-2 antibody averaged 818 µm in height while controls and 0.1 µg/ml antibody groups both averaged near 550 µm. This was almost 50% higher than either of the other groups. By day 24, no significant increase over that achieved by day 15 was noted. Growth of limb buds for all three groups appeared to level off between day 15 and 20. Regression analysis indicated that at low levels of antibody, a log transformation fits the data well, with an R^2 value of 0.91. Plotting the data for 1.0 µg/ml suggested a logarithmic profile yet the R^2 value is low at 0.87. Examining the data shows what appears to be an overall drop in explant height from 15 to 20 days of culture. It is

unlikely that the explants actually decreased in size and this result may actually reflect a leveling of growth. Shrinkage due to cell death, especially peripherally, may have been a contributing factor.

When linear regressions were plotted, a pattern similar to that of mandibular explants treated with anti-BMP-2 protein was seen (Fig. 40B and 41B). Untreated controls had the lowest rate of growth at 13.6 µm/day. This value was close to that calculated for limb bud explants treated with the lowest level of antibody at 18.9 µm/day. At concentrations of 1.0 µg/ml, the rate increased to nearly 31 µm/day or approximately double that of explants grown in medium containing 0.1 µg/ml of the same antibody and almost 2.5 times the rate for controls.

Growth rates for limb bud explants were dose dependent. Rates for control, 0.1 and 1.0 µg/ml BMP-2 antibody were 11.7, 14.6, and 21.9 respectively. For limb bud explants, rates of growth were 20.3, 18.7 and 31.1 µg/day for control, 0.1µg/ml and 1.0 µg/ml antibody respectively. Again, analysis still yielded similar results to those calculated for figure 40B.

In general, the data indicated that limb bud or mandibular explants cultured in a medium containing antibodies against BMP-2 grow at higher rates and reach greater heights than explants grown in untreated medium. This effect was more pronounced at higher (1.0 µg/ml) levels of antibody. Low levels (0.1 µg/ml) of antibody either had no effect when compared to controls, or a minor one. There also appeared to be a 'lag' period when no (or a minor) effect was seen, with most increases in rate and height achieved after day 15. The maximum average peak height achieved by mandibular explants was 748 +/- 12.7 µm at day 20 and a medium concentration of 1.0 µg/ml BMP-2 antibody. Limb buds grew to maximal height of 818 +/- 17.9 µm, also at a

concentration of 1.0 µg/ml antibody. In contrast to mandibular explants however, the peak height was reached by day 15. Further growth approached a plateau.

Growth profiles for mandibular and limb bud explant controls were very well explained by simple linear regression, indicating a constant rate of increase over the culture period. Limb bud explants had 60% greater rate of growth than do mandibular explants. In general, the addition of either TGF-β antibodies or BMP-2 antibodies resulted in an increase in growth rate over controls. Mandibles cultured in the presence of BMP-2 antibody exhibited a dose dependent growth rate increase (Fig. 40B). TGF-β antibody at a concentration of 0.1 µg/ml also induced a mandibular growth rate increase to levels similar to that seen for BMP-2 antibody at the same concentration (Fig. 38B).

Limb buds responded similarly to both antibodies but unlike mandibular explants, did not respond in a dose-dependent manner. Low concentrations of either antibody appeared to inhibit growth and yielded a rate that was slightly lower than that of untreated controls (Fig.39B and 41B). Higher concentrations on the other hand dramatically increased growth rate. At high antibody concentrations, growth profiles for both mandibles and limb buds were linear (although data for mandibular explants grown at 1.0 µg/ml TGF-β are incomplete). Mandibular and limb bud explants grown at the lower antibody concentration however appeared to be more closely modeled by regressions performed on log transformed data.

Mandibular explants cultured in medium containing TGF-β antibody generally grew to a greater degree both in area and height than did those exposed to BMP-2 antibody. Control explants were generally smaller than

those in either treatment group. After 15 days of incubation, the difference in height was easily observable, even without microscopic examination. Externally, mandibles grown in the presence of either antibody appeared smooth, resembling the controls in this respect. Mandibular explants rarely exhibited the large growth peaks seen in limb buds exposed to these antibodies. Treated limb buds grew in a much more erratic fashion and presented a much rougher external appearance. Structures resembling projections were produced by treated limb buds, a phenomenon not observed in mandibular explants.

5.3.3. Tissue differentiation in explants

HBQ staining is one method of visualizing the presence of bone and/or cartilage in tissue sections. Cartilage associated extracellular matrix proteins stain blue while matrix secreted by osteoblasts stains red. Cartilage can also be recognized by its typical histological appearance, often forming distinct nodules or rods.

By day 8 of culture, mandibular explants have formed recognizable tissues including cartilage. Bone may just be beginning to appear as some cartilage nodules exhibited red peripheral staining, indicative of endochondral bone formation (Fig. 42A). By 15 days of culture, both bone and cartilage were well established (Fig. 42B). A higher magnification of 15 day old mandibular explants clearly showed both cartilage and membrane bone (Fig. 42C).

Similarly, control limb bud explants produce cartilage by day 8 of culture. Bone was not present at this point (Fig. 43). Cartilage staining was slightly more intense than seen in mandibular explants of the same age.

Mandibles grown in medium containing TGF-β antibody examined after 8 days of culture exhibited large amounts of both bone and cartilage (Fig. 44A). This was especially noticeable at final antibody concentrations of 1.0 to 10 µg/ml. Immunostaining for the presence of TGF-β and BMP-2 indicated the presence of growth factors in mutually exclusive areas. TGF-β was not present in the bone or cartilage but rather was found to outline these regions (Fig 44B). This is not unlike the situation seen in immunostained sections from whole embryos (See chapter 3). BMP-2 on the other hand, appears to localize in the cartilage itself, but not in bone (Fig. 47C).

TGF-β antibody added to limb bud explant culture appeared to accelerate the formation of bone and cartilage in much the same manner as seen in mandibular culture. Both cartilage and bone were well formed by 8 days of culture, to a much greater extent than seen in controls (Fig. 45A and B). Unusually, what appeared to be membrane bone formed in limb buds exposed to TGF-β. This type of bone is not generally seen in limbs (Fig 45C). The amount of matrix seen associated with both bone and, especially, cartilage were much greater than seen in control explants of similar ages.

The inclusion of BMP-2 antibody in medium used for culture of limb bud explants accelerated the formation of bone and cartilage when compared to controls of the same age. Endochondral bone was very well established and replacement of chondrocytes by osteocytes was clearly visible (Fig. 46A). This antibody may promote the development of membrane bone in limb bud explants albeit at low frequency (Fig 46B).

The addition of BMP-2 antibody to mandibular explant culture did not appear to significantly increase either bone or cartilage formation. While there

was a definite increase in overall size of the explant compared to controls, cartilage and bone appeared similar to that seen in controls. Bone development may have been slightly enhanced (Fig. 47).

Closer examination of bone and cartilage forming regions in explants confirmed that the addition of TGF-β neutralizing antibody to cultured limb bud explants resulted in the formation of membrane bone, not characteristic of limbs (Fig. 48). Endochondral bone formation in mandibular explants was apparent but whether or not this was atypical is difficult to confirm. Bone formation in the mandible (and much of the craniofacial skeleton) proceeds primarily through the process of intramembraneous ossification but endochondral bone formation is responsible for forming some craniofacial skeletal components (Atchley and Hall, 1991).

5.4. Discussion

5.4.1. TGF-β antibody addition

TGF-β is a powerful inhibitor of cell proliferation and may delay many events related to skeletal cell differentiation. TGF inhibits differentiation of rat fetal osteoblasts *in vitro* (Breen et al., 1994). The addition of TGF-β at concentrations as low as 0.1 ng/ml during the cells' proliferative phase is sufficient to prevent maturation of preosteoblasts into mature osteoblasts. This is accompanied by changes in expression for a number of genes including *c-fos, jun-B*, fibronectin and collagen, among others. This, along with observed changes in cell morphology may result in part from a change in the extracellular matrix environment required by the cells to initiate terminal differentiation. Cultures of rat fibroblast derived from TGF-β null (-/-) mice proliferate at approximately twice the rate of normal (+/+) fibroblasts synthesizing TGF-β (Sudarshan et al., 1998). Extracellular matrix deposition is lowered in -/-

fibroblasts as are levels of collagen I and fibronectin. +/+ fibroblasts exposed to TGF-β neutralizing antibody exhibit similar phenotypes to those of -/- fibroblasts. TGF-β inhibits the differentiation and mineralization of nodules of fetal rat calvarial cells *in vitro* (Ghosh-Choudhury et al., 1994). TGF-β also down regulates expression of *BMP-2*. Primary cultures of rat calvarial cells contain high numbers of osteoblasts and, following a phase of rapid proliferation, form mineralized nodules (Bellows et al., 1986). The addition of TGF-β to calvarial culture at a concentration of 2 ng/ml not only inhibits the expression of markers specific to osteoblast differentiation, (including type I collagen, alkaline phosphatase, osteopontin and osteocalcin) but also down regulates BMP-2 expression to below basal levels (Ghosh-Choudhury et al., 1994). Addition of TGF-β after the proliferative stage, i. e., after osteoblast cells have differentiated, has no effect. TGF-β stimulates bone formation yet is known to be an inhibitor of mineralizaton. While this appears to be contradictory, TGF-β may activate the cascade of events leading to initiation of bone formation. Once this process has proceeded sufficiently however, TGF-β is no longer required. In fact, the presence of TGF-β after this time may inhibit mineralization and complete bone formation (Bonewald and Dallas, 1994).

Limb bud and mandibular cultures treated with TGF-β antibody produce cartilage and bone sooner than do controls. This is especially apparent at high concentrations of neutralizing antibody (1 – 10 μg/ml). As has been shown in the above and other studies, TGF-β inhibit differentiation and matrix deposition. The removal or reduction of available cytokine by antibody neutralization may in fact allow cells to proceed through differentiation sooner than controls.

Membrane bone, unlike endochondral bone, forms without the requirement for an intermediate cartilage model. Limb bud explants treated with higher levels of antibody against TGF-β appear to produce membrane

bone, a type not normally found in limb buds (Fig. 48). For TGF-β antibody concentrations of 1.0 µg/ml, this occurs less than 10 % (2 of 23 explants studied between 8 and 20 days of culture) of the time and for concentrations of 10 µg/ml, approximately 25 % of the time (3 of 9 explants).

The clavicle is one of the few areas outside of the craniofacial region that forms bone by intramembraneous ossification (Precious and Hall, 1994). It is possible that in the process of dissection, cells destined to form clavicle membrane bone were present in the explant. This is unlikely, however, as cells destined to form clavicle originate much deeper in the body of the embryo than where the dissection was made. Limb buds were removed at the point where they joined the body of the embryo whereas clavicle cells form in close proximity to the spine and vertebrae. In addition, there is no evidence of girdle cartilage in the explants, as would be expected if deeper mesenchyme was included. Membrane bones have the potential to form cartilage although the mechanisms are not clear (Fang and Hall, 1997). Whether the cells which form endochondral bone can, under the right conditions, avoid a cartilage model and form membrane bone directly, remains to be seen.

5.4.2. BMP-2 antibody addition

The addition of both TGF-β and BMP-2 antibodies to explant cultures has a positive effect on overall growth. The most obvious sign of differential growth between control and treated explants was the observation that explants exposed to TGF-β or BMP-2 neutralizing antibodies form higher peaks. Growth occurs in three dimensions but while growth along the filter support was similar between controls and treatment groups, antibody challenged explants tended to increase in height to a much greater degree. By sectioning and physically measuring peak heights under a microscope, it was possible to elucidate

some general growth trends. The fact that explants exposed to antibodies grow faster as well as produce different morphologies than controls suggests that the lack or reduction of TGF-β and BMP-2 protein has a profound effect upon patterning. In order to verify the postulated increase in proliferation, incorporation of [^{3}H] thymidine by explants should be measured. In addition, a measure of overall mass or size of the explants would be useful, perhaps via a measurement of explant total protein.

Control mandible and limb bud explants increase in height in an approximately linear fashion, with limb buds growing faster and reaching greater heights than mandibular arches. Treatment of mandibular or limb bud explants with 0.1 µg/ml TGF-β antibody results in a growth profile approximating a logarithmic curve, suggesting that after an initial spurt, growth levels off and approaches that of controls. Higher levels of TGF-β antibody (1.0 µg/ml) produced a linear profile in limb bud explants similar to, although at a higher rate of height increase than controls. The flattening of the growth curve seen in explants treated with the lowest TGF-β antibody concentration may derive from the different levels remaining after a given period of time compared to explants grown at higher antibody concentrations. In other words, at lower concentrations, antibodies may be more quickly metabolized or otherwise removed from circulation than would be the case for higher levels. It is possible that at low levels of antibody, fewer individual cytokine molecules may be inhibited from binding to cell surface receptors. While this may stimulate growth initially, the remaining unneutralized growth factor molecules are able to eventually compensate and reestablish near control conditions. There was insufficient data for a similar comparison on mandibular growth at 1.0 µg/ml TGF-β antibody.

At final concentrations of 0.1 and 1.0 µg/ml of BMP-2 neutralizing

antibody, mandibular explant growth was linear. There is an initial increase in height in explants treated with 1.0 µg/ml after 8 days but a much greater increase occurs between 15 and 21 days. Neither R^2 value is particularly strong, so in fact a linear profile may not be the best fit to the data. Growth almost appears to be biphasic in challenged explants. Initial antibody neutralization may trigger cellular responses that ultimately results in a large increase as the explants mature. It is unlikely that antibody remains in the medium for 15 to 21 days due to degradation in the cellular environment. The growth spurt seen is possibly not directly related to BMP-2 neutralization.

The response of limb bud explants grown in medium supplemented by BMP-2 antibody are alike in many respects to mandibular explants similarly challenged. Once again, major height increases do not occur until late in culture, between 8 and 15 days. Interestingly, at low levels of 0.1µg/ml antibody, explants appear to grow less than controls, although the opposite is true for higher antibody levels.

In general, low levels of TGF-β antibody appear to stimulate growth in both mandibular and limb bud explants taken from stage 21 chick embryos. This effect is characterized by an initial growth spurt which tails off between 15 and 20 days of culture. Higher levels produce a more linear response which persists in limb bud explants to at least 20 days of culture. BMP-2 antibody added to explant culture also produces an initial increase in height, although not to the same degree as TGF-β antibody treatment. The growth response produced by BMP-2 neutralizing antibody is most apparent after 15 days of culture, with early responses minimal.

Little is reported about the effects of BMP-2 neutralization on cell proliferation and growth. There is TGF-β data however it is usually with

reference to cell lines rather than more complex *in vitro* systems such as explant culture. TGF-β protein affects cell proliferation *in vitro*. The addition of TGF-β to CD34+ (human bone marrow progenitor) cells significantly reduces the activity and number of proliferating cells, an effect augmented by the addition of retinoic acid (Lardon et al., 1996). Proliferation of cultured endothelial cells from mink lung is also inhibited by TGF-β (Merwin et al., 1991; Cheifetz et al., 1991; Càrcamo et al., 1995). The TGF-β receptor system and, by implication, the growth factor, are involved in the inhibition of murine epidermal cell growth *in vivo* (Wang et al., 1997). TGF-β is not strictly inhibitory to cell proliferation. In human fibroblast culture for example, exogenous TGF-β inhibits proliferation, although results are contradictory (Kim et al., 1998). Interestingly, the TGF-β1 promoter is regulated by the transcription factor AP-1 while TGF-β 2 and 3 may be regulated by AP-2 (Roberts et al., 1992). Recently, AP-2 has been found to be expressed in the distal mesenchyme of chick limb buds and found associated with the facial primordia (Shen et al., 1997). This leads to the possibility that the expression of TGF-β in the early mesenchyme of the limb and in the mandible reflects its activation via AP-2. This further implicates TGF-β 2 and/or 3, but not –1, as the isoforms responsible for limb and mandibular development in the early embryo. Inhibition of TGF-β by neutalizing antibodies then may be expected to inhibit the cytokine's antiproliferative effects. The localization of TGF-β in the subepithelial cell layers of the mandible and limb bud at stage 21 suggests a possible role in patterning by suppressing cell growth (chapter 3). The inhibition of TGF-β in this region during development may account for the increase in explant growth.

BMP-2 and –4 have been localized in the developing face of both the chick and the mouse. In the mouse, BMP-2 mRNA appears at around day 13.5, and is found in the neural crest derived mesenchyme exclusively (Bennett et al., 1995). Similarly in the chick, BMP-4 transcripts are found in the epithelia of all

face primordia early in development (stage 20). Expression subsequently moves to localized regions of mesenchymal cells in the distal tips of facial primordia. BMP-2 expression initially appears in the frontonasal mass (stage 16), localized to epithelia and subsequently shows up throughout the underlying mesenchyme of the maxillary and mandibular processes (Francis-West et al. 1994).

Immunohistochemical analysis of chick facial development localizes BMP-2 to the epithelial basement membrane at stage 17 and a subsequent increase in signal in the mesenchymal cells associated with the epithelium (chapter 3). The initial appearance of BMPs in epithelia and subsequent expression in the mesenchyme may indicate an epithelial-mesenchymal interaction typical of inductive processes. BMP-2 saturated beads implanted into cultured stage 22 mandibular mesenchyme do not however induce bone formation but rather produce localized cell death, suggesting that the BMP-2 is not an inducer of osteogenesis in the chick mandible, or acts earlier in development as an inductive factor (Ekanayake and Hall, 1997). BMP-2 has also been localized to the developing chick limb bud (Francis et al., 1994; Kawakami et al., 1996; Macias et al., 1997). Immunohistochemical localization in the chick limb indicate that, as in the mandible, BMP-2 expression is initially localized to the epithelium and later progresses to the mesenchymal cells (Chapter 3).

Analysis of explant sections from BMP-2 antibody treated cultures indicates that bone and cartilage formation is slightly accelerated when compared to untreated controls. Matrix deposition also appears to be augmented but not to the same degree as explants treated with TGF-β neutralizing antibody. This effect is seen in both limb and mandible, but is more pronounced in the former. Both TGF-β and BMP-2/4 are coordinately

expressed in many vertebrate embryos and both, as well as other members of the TGF-β superfamily, appear to utilize intracellular Smad proteins as part of the signal cascade (Baker and Harland, 1997; Candia et al., 1997).

In contrast to TGF-β, BMP-2 applied to rat osteoblast cells *in vitro* promotes bone cell differentiation (Harris et al., 1995; Hughes et al., 1995). Inhibition of BMP-2 then, might be expected to reduce the levels of bone formation seen in explant cultures. BMP-2 however is unable to change the fate of already commited cells (Komaki et al., 1996). It remains unclear why BMP-2 antibody appears to accelerate bone and cartilage formation. Matrix formation is not increased to the same degree as seen with TGF-β antibody application. It may be that inhibition of BMP-2 activity may also down regulate TGF-β signaling or that the removal of BMP-2 may affect some other components of the developmental program.

5.4.3. Okadaic acid

The addition of okadaic acid to explant culture does not, at any dose level tested, parallel results obtained with either BMP-2 or TGF-β antibodies. This suggests that the intracellular signaling cascade employed by at least these members of the superfamily does not utilize either protein phosphatase 1 or 2 as part of its intracellular signaling cascade. It is possible, given its potency, that much lower concentrations of okadaic acid may have elicited a different response. Nevertheless, addition of okadaic acid to culture medium does appear to result in some curious growth responses.

Two unusual features are of note. First, both mandibular and limb bud cultures lose much of their morphology within 24 hours and flatten into low discs of cells. These cells continue to live for up to two weeks in culture, yet little growth

is obvious. Secondly, at doses of 1.0 µg/ml, some explants display a second cell type adjacent to, but not overlapping, the first. This was found to occur in approximately 43 % of explants grown at concentrations of 1.0 µg/ml (7 of 16) and in approximately 10 % of explants grown in concentrations of 0.1 µg/ml (2 of 16). No occurences were recorded at a concentration of 0.01 µg/ml. There are some dead cells with pycnotic nuclei but in general the explants are in good condition and remain attached to the filter. Analysis of staining in explant sections raises the possibility that one cell type is mesenchymal while the other is epithelial, both held in an undifferentiated state (not shown). While morphology is drastically changed when compared to controls, cell adhesion remains to some degree as the explants do not separate into individual cells, even when submerged in medium. The change in morphology and flattening of the explant suggests that cell adhesion molecules or ECM is degraded or inhibited, possibly through an upregulation of collagenase gene expression.

In summary, TGF-β and BMP-2 antibodies accelerate bone and cartilage formation in explant cultures of limb and mandible derived form stage 21 chick embryos. In addition, both antibodies appear to alter growth and patterning of explants as seen in the overall increase in explant heights treated with the antibodies. The addition of okadaic acid at the levels used does not parallel the effects of antibodies and suggests that protein phosphatase 1 and 2 are not involved in mediating the effects of either TGF-β or BMP-2.

Figure 33: Appearance of control explants after 1 and 14 days of culture.

A) Mandibular explant taken from stage 21 chick embryo and grown on filter (F) substrate for 1 day. Left (l) and right (r) arches can be discerned. The mandible was initially placed so that the exposed mesenchyme was in contact with the filter. Note the mandible retained symmetry; an opaque midline denoting fusion of the two arches can clearly be seen. Scale bar = 600 μm.

B) Limb bud explant from stage 21 embryo after 1 day of culture retained much of its original shape. As with mandibular explants, limb buds were oriented with the cut portion of the structure in contact with the filter, allowing mesenchymal cells access to nutrients. The limb bud was typically slightly asymmetrical, with a hooked or curved appearance at the distal end (d). Scale bar = 500μm.

C) Mandibular (left) and limb bud (right) explants after 14 days of culture. Explants had increased greatly in size, covering much of the filter. Growth in the vertical plane away from the surface of the filter has taken place. Much of the original shape of the explants was lost, resulting in irregular and asymmetric final morphology. White arrowheads indicates cartilage. L = left; r = right; d = distal. Scale bar = 500 μm.

Figure 33

Figure 34: Appearance of limb bud explants after 15 days of explant culture.

A) Limb bud explant grown in medium containing a final concentration of 1.0 μg/ml TGF-β antibody. Explant grew to cover the filter almost completely and had increased significantly in height. Filter is indicated by the white arrowhead. Scale bar = 500 μm.

B) Limb bud explant grown in medium containing a final concentration of 1.0 μg/ml BMP-2 antibody. As with explants grown in the presence of TGF-β, the filter was completely overgrown. There was a pronounced increase in height. Filter is indicated by a white arrowhead. Scale bar = 500 μm.

C) Control limb bud explant cultured in medium alone. The explant had increased in overall size and height but not to the extent seen in explants grown in medium supplemented with either BMP-2 or TGF-β antibody. Scale bar = 500 μm.

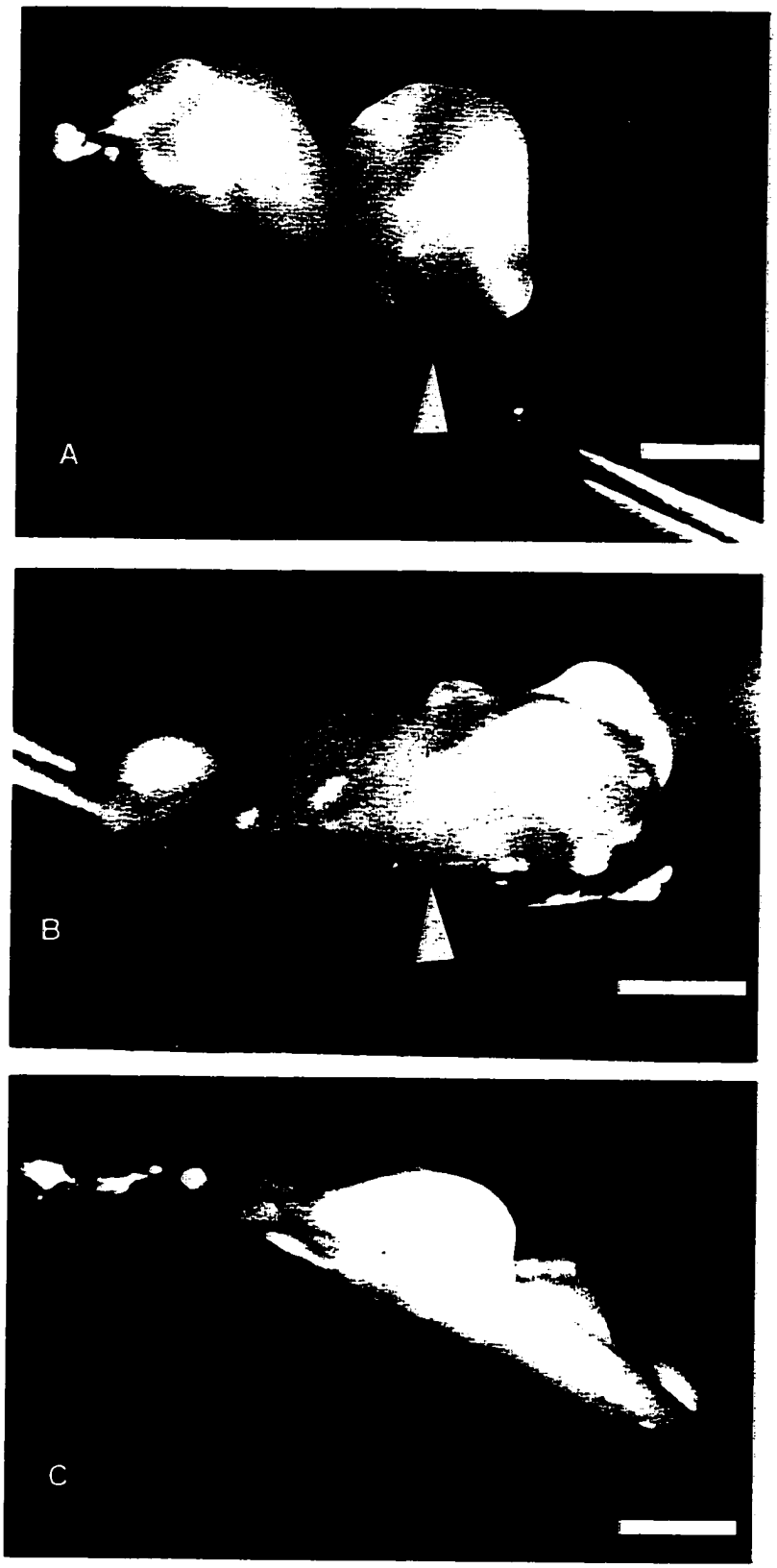


Figure 34

Figure 35: Appearance of mandibular explants after 15 days in culture.

A) Explant grown in medium with a final concentration of 1.0 μg/ml TGF-β antibody. Explant had grown over most of the filter and increased somewhat in height. From the angle shown, some symmetry appeared to be retained. Scale bar = 500 μm.
B) Explant grown in medium supplemented with BMP-2 antibody to a final concentration of 1.0 μg/ml. Explant size is much smaller in all dimensions than that seen with TGF-β antibody. Scale bar = 500 μm.
C) Mandibular explant control grown in medium alone. Overall growth was noticeably less than seen in explants grown in medium supplemented with BMP-2 and, especially, TGF-β antibodies. Scale bar = 500 μm.

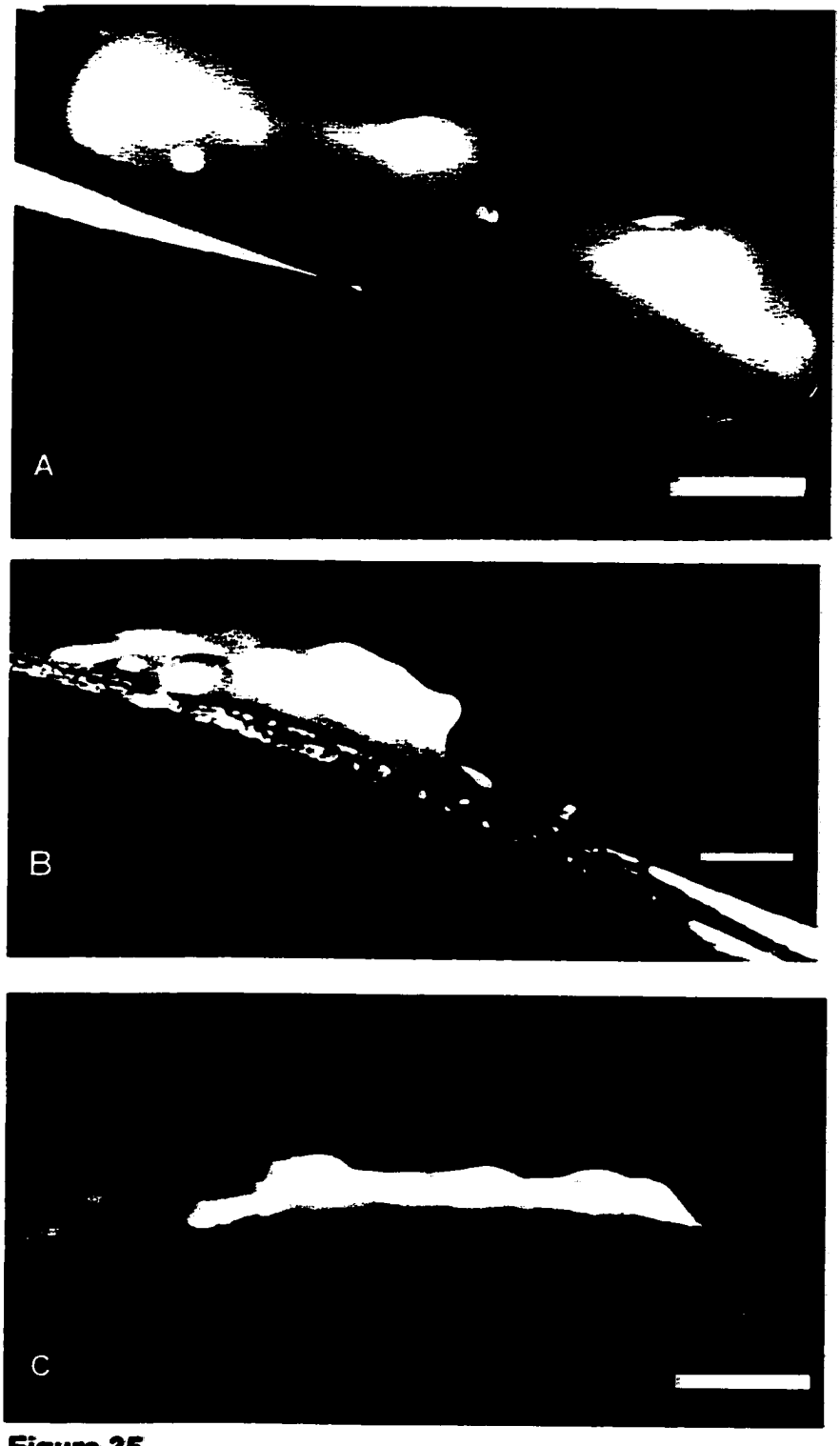


Figure 35

Figure 36: Limb bud explants cultured in medium supplemented with 0.1 μg/ml okadaic acid.

A) Appearance of limb bud explants after 1day of incubation. Explants lost much of their original morphology and flattened out on the membrane. Scale bar = 500 μm.
B) Appearance of limb bud explants after 8 days incubation. In many cases, the explant appeared to have separated into two distinct cell types, with a zone of minimal overlap in between (arrow). The opaque cells stain well with alcian blue, indicating extracellular matrix components (not shown). Scale bar = 500 μm.

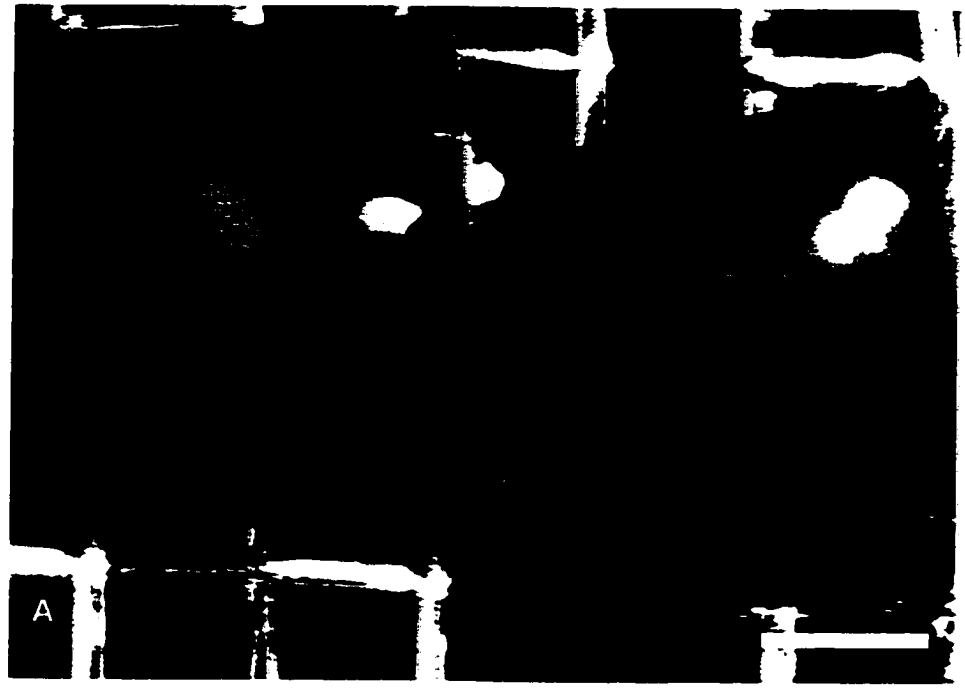


Figure 36

Table 6: Maximum heights reached by mandibular and forelimb bud explant controls grown in culture. S. E. = standard error.

days of culture	limb bud peak height +/- S.E. (μm)	n	mandible peak height +/- S. E. (μm)	n
1	280.0 +/- 20.0	8	294.0 +/- 22.3	8
8	455.0 +/- 31.0	10	366.5 +/- 7.7	10
15	539.0+/- 17.9	8	439.0 +/- 13.6	8
20	687.0 +/- 42.3	7	491.0 +/- 12.7	8
24	710.0 +/- 21.1	5	574.0 +/- 41.3	4

Figure 37: Growth of stage 21 chick limb bud and mandibular explant controls. lb = limb bud. Md = mandible.

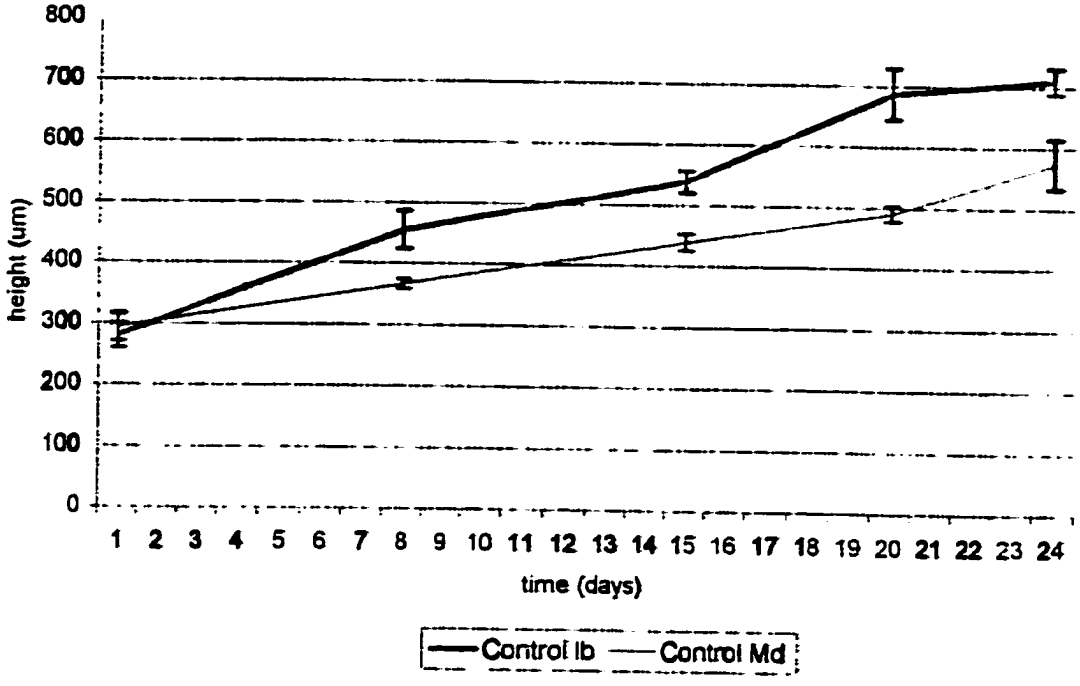


Table 7: Maximum heights reached by mandibular explants cultured in medium supplemented with TGF-β antibody to a final concentration of 0.1 μg/ml. S. E. = standard error.

days of culture	mandible control peak height +/- S. E. (μm)	n	+ 0.1 μg/ml TGF-β Ab +/- S. E. (μm)	n
1	294.0 +/- 22.3	8	267.0 +/- 27.5	5
8	366.5 +/- 7.7	10	487.3 +/- 23.4	9
15	439.0 +/- 13.6	8	528.7 +/- 44.0	7
20	491.0 +/- 12.7	8	653.0 +/- 19.9	8
24	574.0 +/- 41.3	4	619.8 +/- 28.4	4

Figure 38A: Growth of stage 21 chick mandibular explant exposed to 0.1 ug/ml TGF-beta antibody.

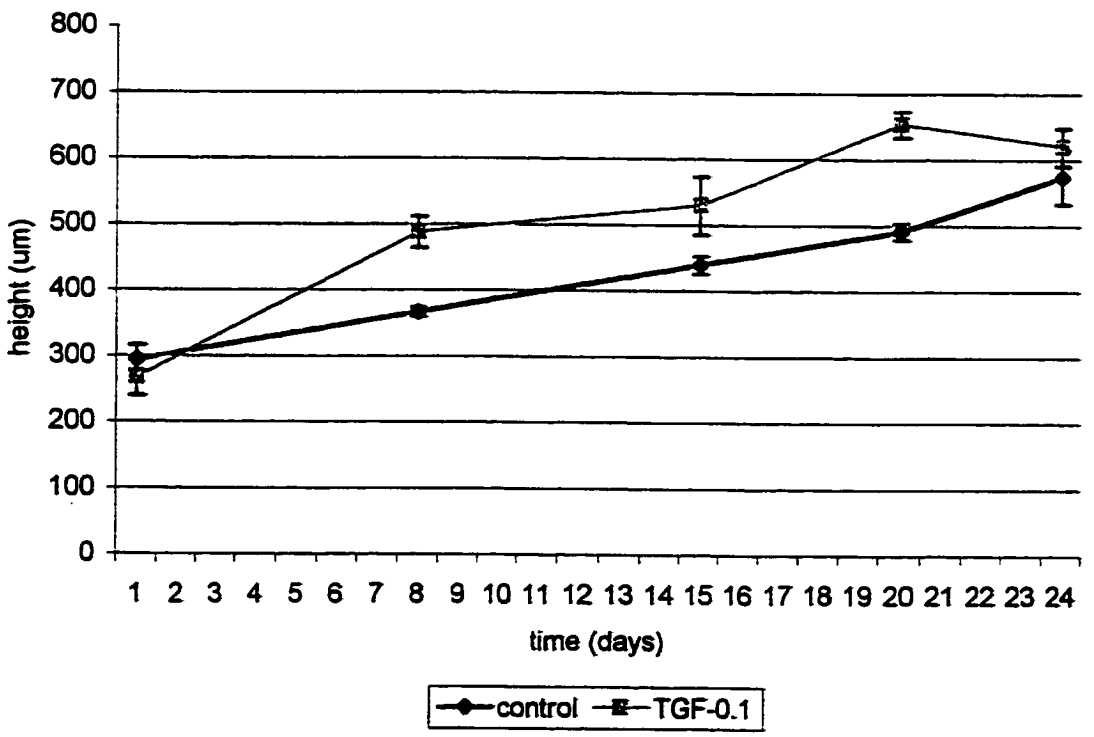


Figure 38B: Growth of mandibular explants treated with TGF-beta antibody (0.1 µg/ml) with linear and log regression analysis.

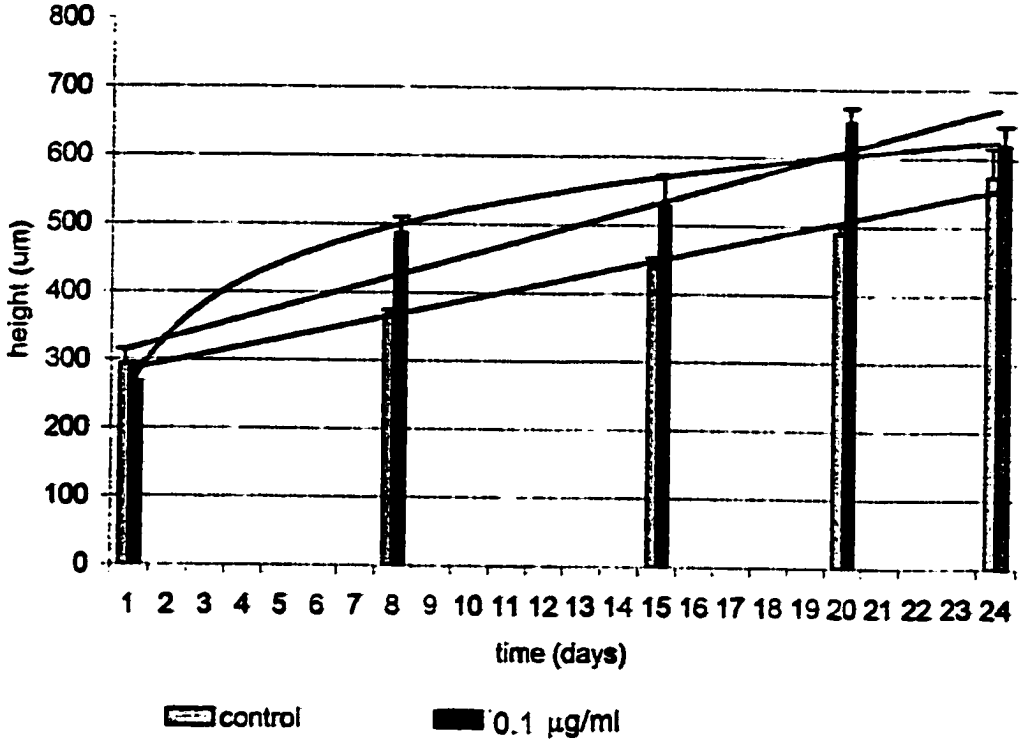


Table 8: Growth of limb bud explants cultured in medium supplemented with TGF-β antibody. Final medium concentrations are given in μg/ml. S. E. = standard error.

days of culture	limb control peak height +/- S. E. (μm)	n	+ 0.1 μg/ml TGF-β Ab +/- S. E. (μm)	n	+ 1.0 μg/ml TGF-β Ab +/- S. E. (μm)	n
1	280.0 +/- 20.0	8	287.0 +/- 36.7	6	249.0 +/- 27.0	6
8	455.0 +/- 31.0	10	597.0 +/- 42.6	8	442.0 +/- 20.0	8
15	539.0 +/- 17.9	8	643.0 +/- 81.0	7	665.0 +/- 19.6	8
20	687.0 +/- 42.3	7	676.0 +/- 29.0	7	1068.0 +/- 65.2	7
24	710.0 +/- 21.1	5	660.0 +/- 10.8	5	1040.0 +/- 41.0	6

Figure 39A: Growth of stage 21 chick embryo limb bud explants exposed to TGF-beta antibody.

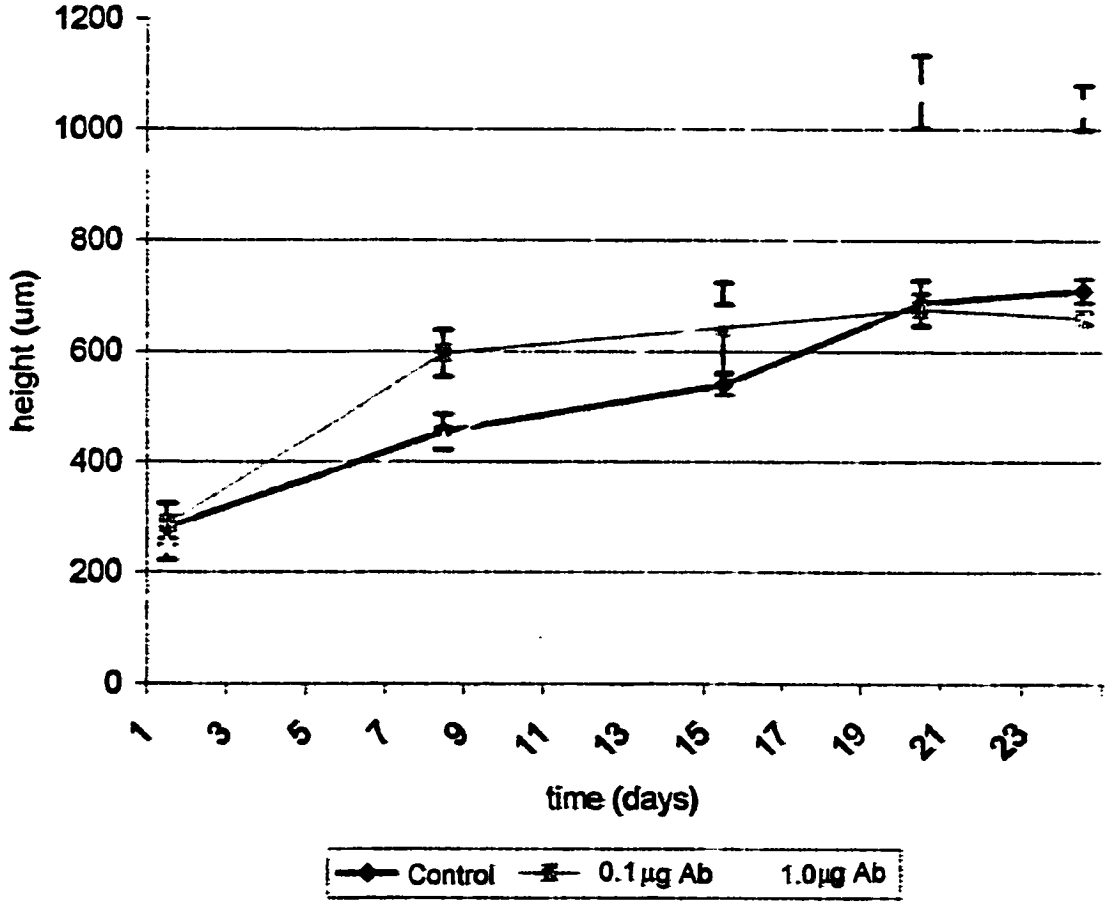


Figure 39B: Linear regression analysos of growth of mandibular explants exposed to BMP-2 antibody.

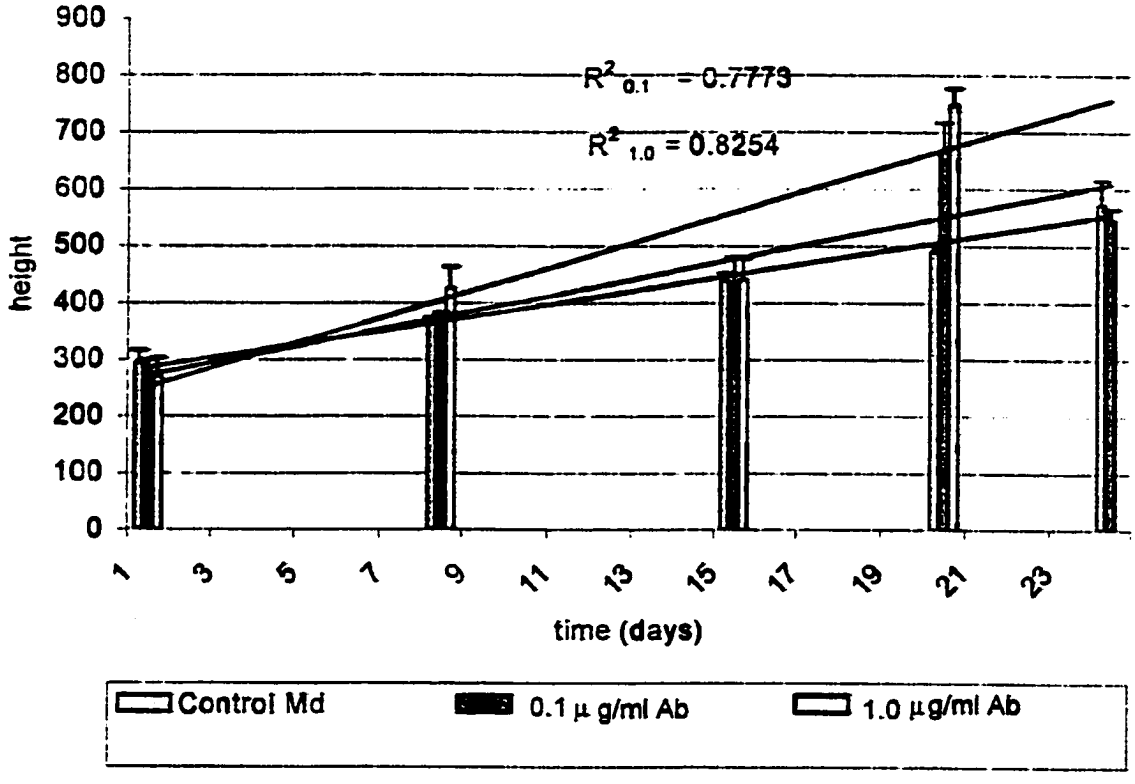


Table 9: Maximum heights reached by mandibular explants grown in medium supplemented with BMP-2 antibodies. Final antibody concentrations in growth medium are given in table. Heights are given in µm. S.E. = standard error.

days of culture	height of control +/- S. E. (µm)	n	+ 0.1 µg/ml BMP-2 Ab +/- S. E. (µm)	n	+1.0 µg/ml BMP-2 Ab +/- S. E. (µm)	n
1	294.0 +/- 22.3	8	277.0 +/- 23.0	6	270.0 +/- 33.4	6
8	366.5 +/- 7.7	10	368.0 +/- 13.6	8	426.0 +/- 37.2	8
15	439.0 +/- 13.6	8	424.5 +/- 56.5	6	441.0 +/- 36.9	8
20	491.0 +/- 12.7	8	668.6 +/- 47.3	7	748.0 +/- 29.7	8
24	574.0 +/- 41.3	5	545.0 +/- 20.9	6		

Figure 40A: Growth of stage 21 chick mandibular explants treated with BMP-2 antibody.

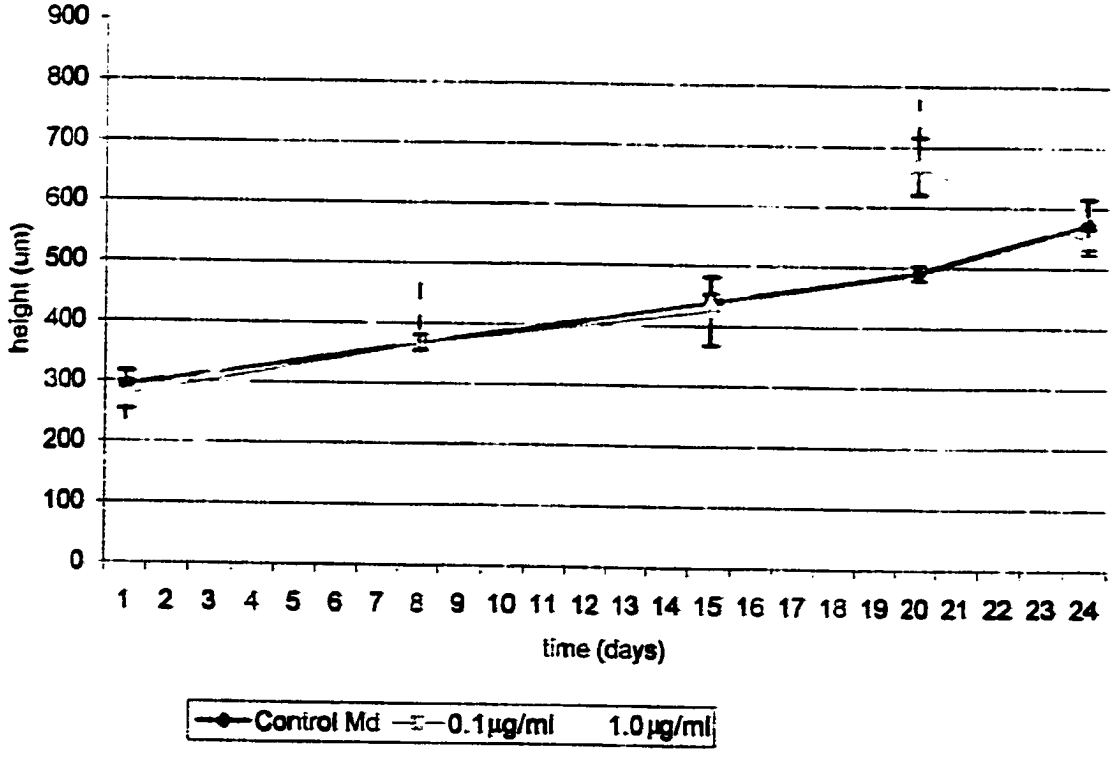


Figure 40B: Linear regression analysis of growth of mandibular explants exposed to BMP-2 antibody.

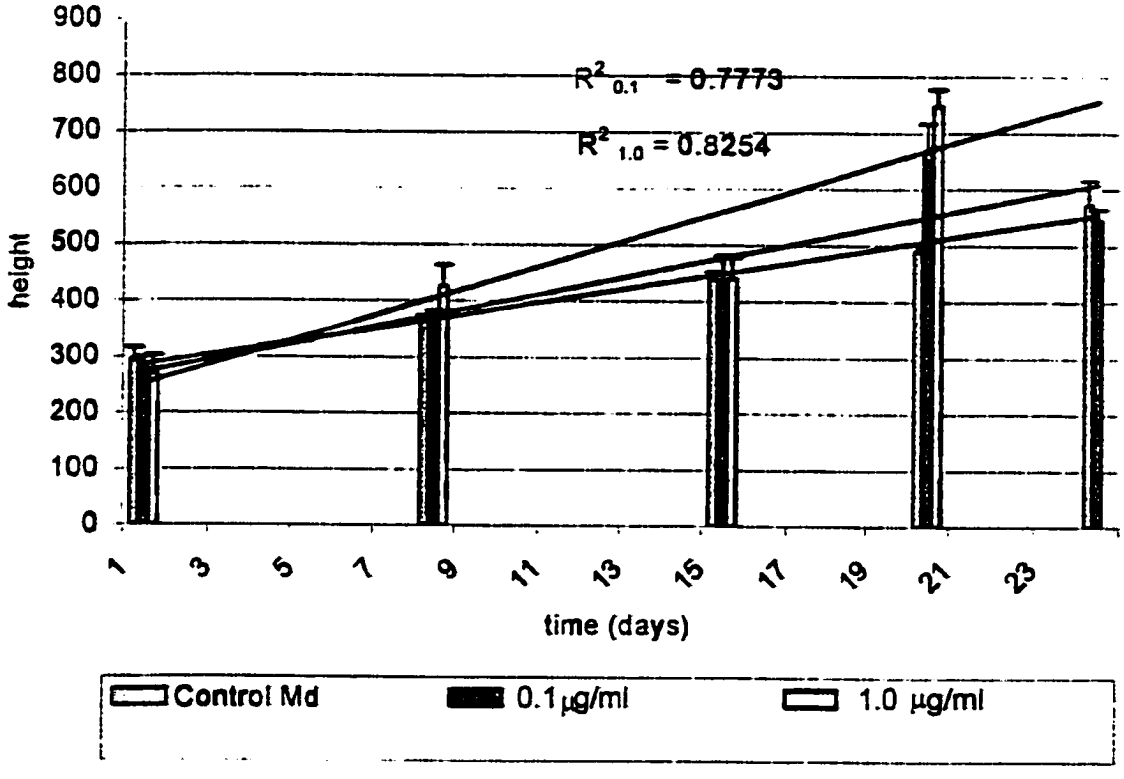


Table 10: Maximum heights of limb bud explants grown in medium supplemented with 0.1 or 1.0 µg/ml BMP-2 antibody (Ab). S. E. = standard error.

days of culture	height of control +/- S. E. (µm)	n	+ 0.1 µg/ml BMP-2 Ab +/- S. E. (µm)	n	+ 1.0 µg/ml BMP-2 Ab +/- S. E. (µm)	n
1	280.0 +/- 20.0	8	274.0 +/- 36.2	6	255.0 +/- 27.2	6
8	455.0 +/- 31.0	10	440.8 +/- 34.8	8	397.7+/- 23.5	6
15	539.0 +/-17.9	8	563.1 +/- 44.7	8	818.0 +/- 74.3	5
20	687.0 +/- 42.3	7	629.6 +/- 26.9	8	765.0 +/- 42.6	6
24	710.0 +/- 21.1	5	553.0 +/- 32.0	6		

Figure 41A: Growth of stage 21 limb bud explants treated with BMP-2 antibody.

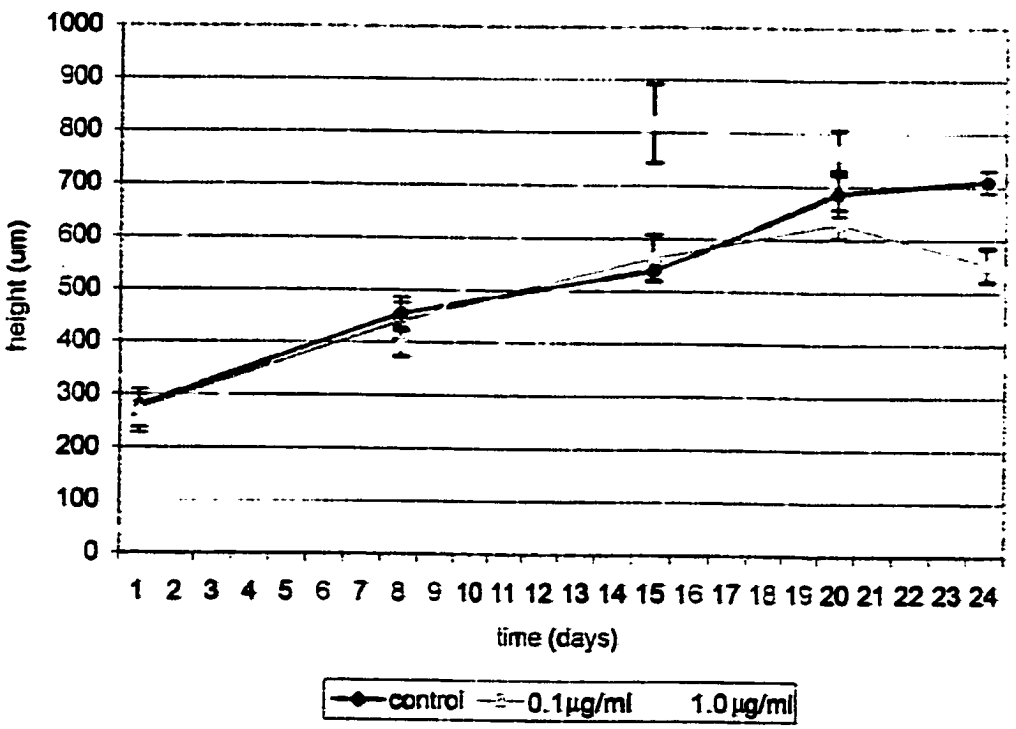


Figure 41B: Linear regression (1.0 μg/ml Ab) and log transformation (1.0 μg/ml Ab) of growthof limb bud explants treeated with BMP-2 antibody. BMP-2 antibody.

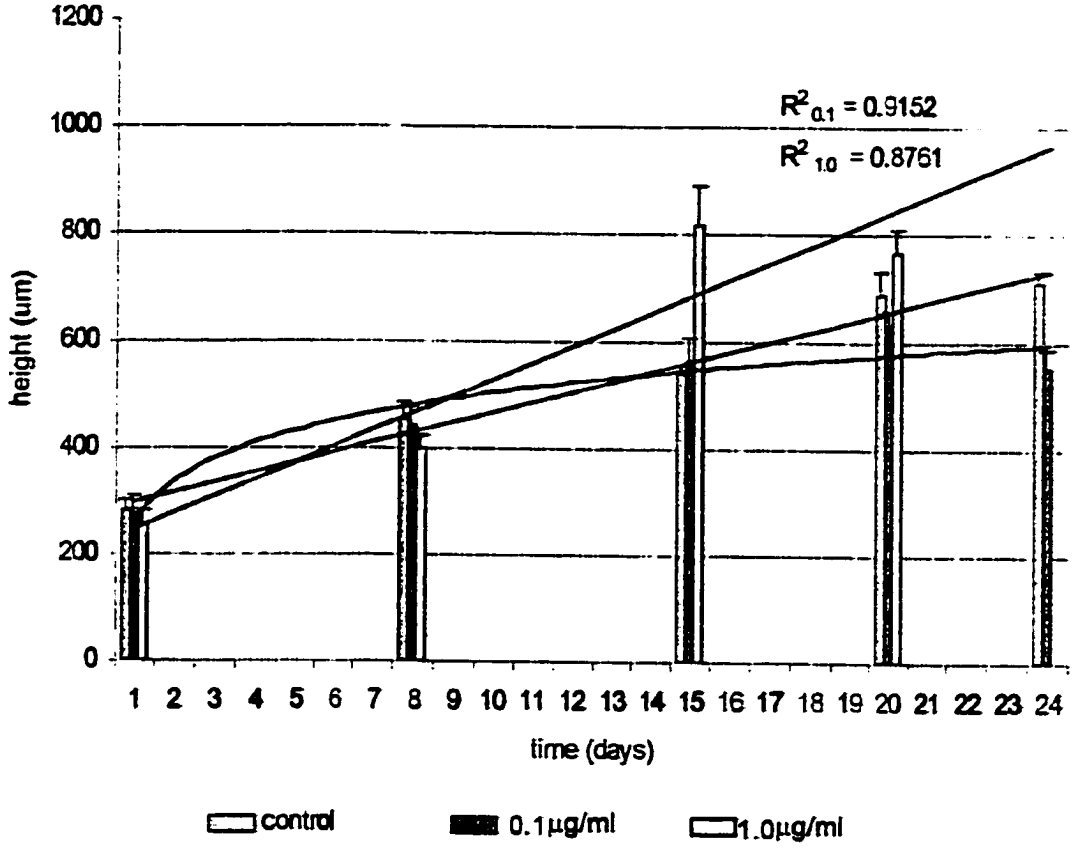


Figure 42: Mandibular explant controls stained with HBQ.

A) Mandibular explant cultured for 8 days covered much of the filter and exhibited the initial stages of cartilage (c) formation. Cartilage nodules stain lightly with alcian blue. Filter (F) in A) has became dislodged during sectioning and was bent away from the explant. Scale bar = 200 μm.
B) Mandibular explant grown in culture for 15 days exhibited increased blue staining, indicating dense cartilage matrix components. Bone (b) stains red. Scale bar = 200μm.
C) High magnification view of cartilage (c) and bone (b) formation in mandibular explants cultured for 15 days. Bone shown in this section was membraneous, as would be expected, and not endochondral in origin. Scale bar = 50 μm.

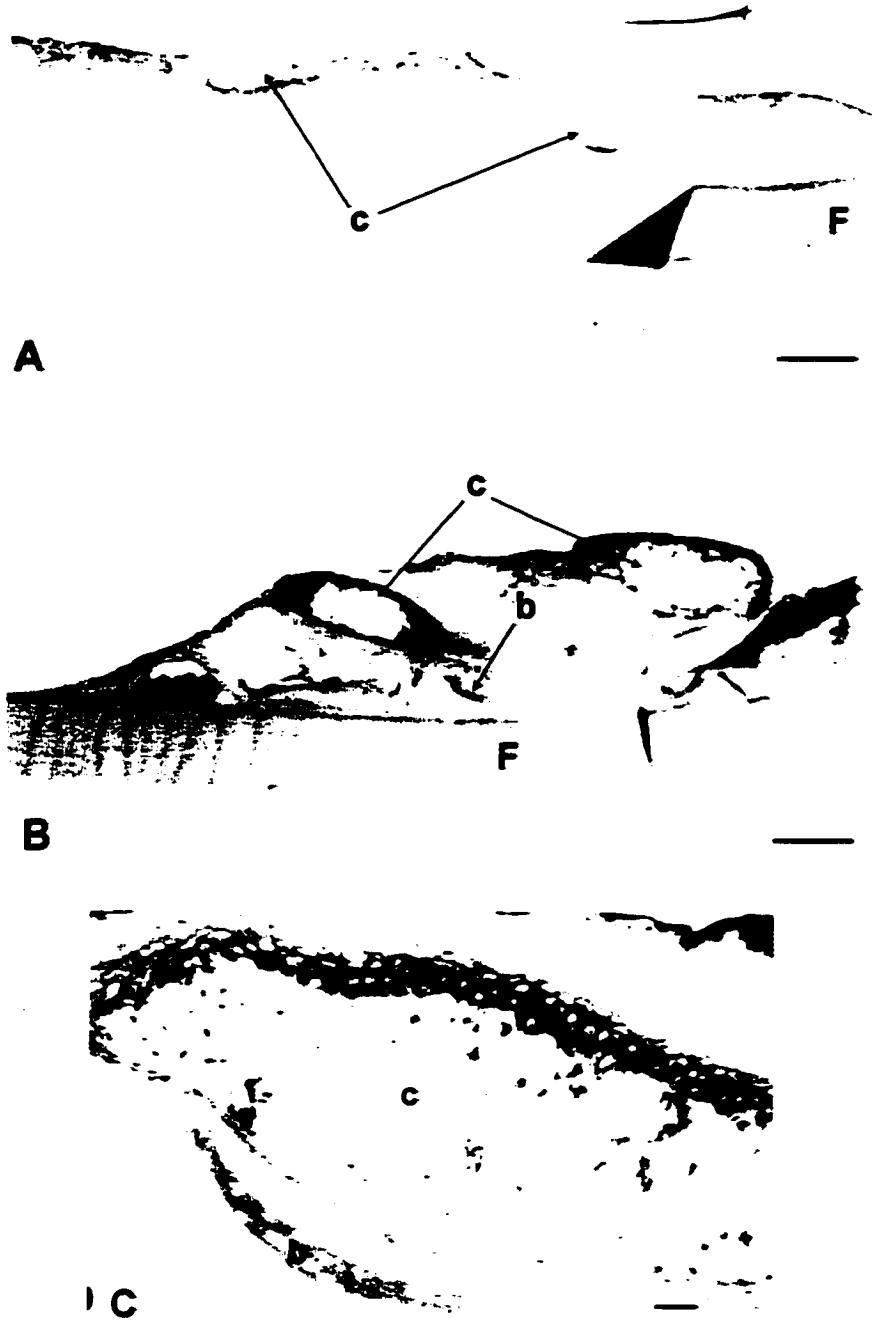


Figure 42

Figure 43: Limb bud explant control sections stained with HBQ.

A) Appearance of limb bud explant after 8 days of culture. Cartilage (c) was quite widespread but no bone was evident at this stage. The filter in this section has come completely away from the explant during sectioning. Scale bar = 175 μm.
B) Limb bud explant cultured for 8 days showing a cartilage nodule (c). F = filter. Scale bar = 200 μm.
C) High magnification view of nodule in figure (B) showing the cartilage (C) in blue. Scale bar = 100 μm.

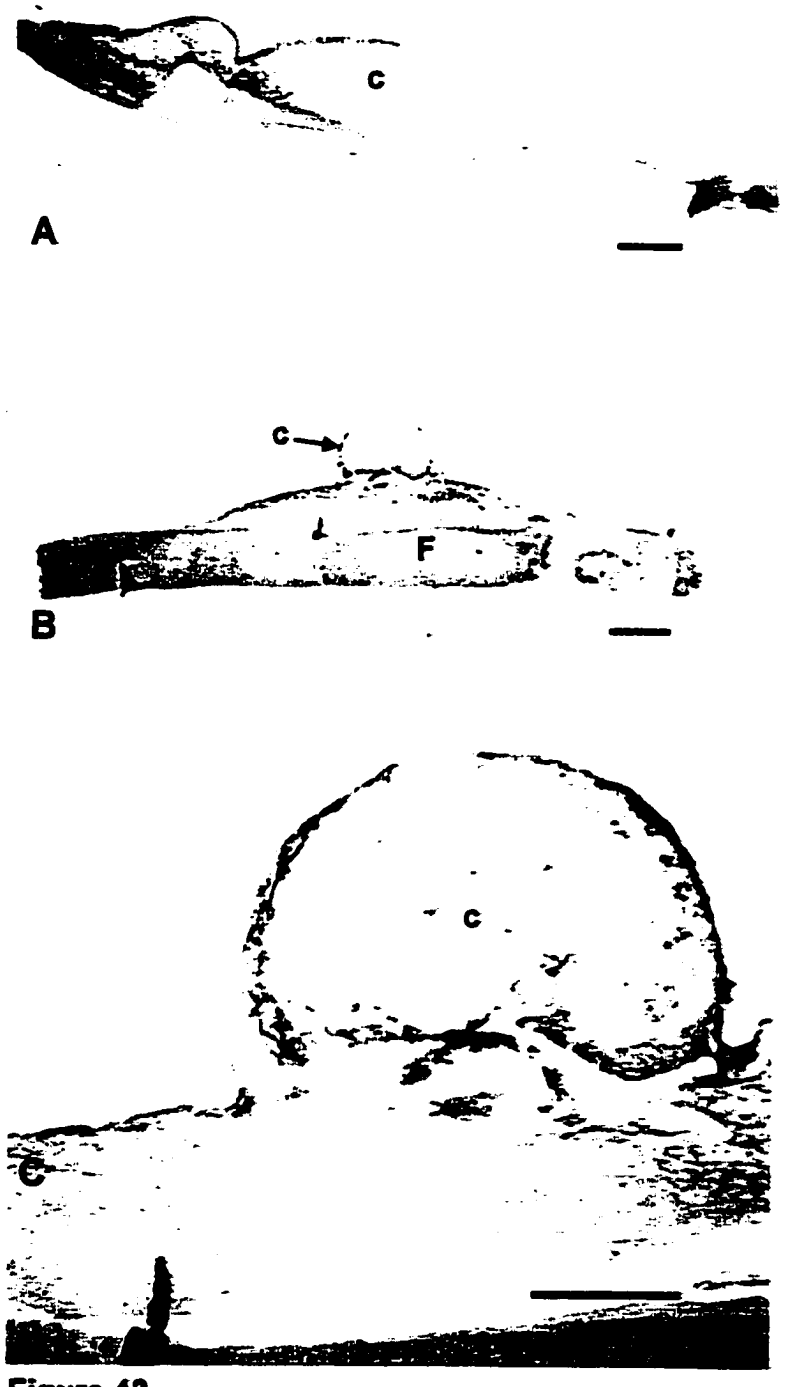


Figure 43

Figure 44: Sections through mandibular explants grown in medium supplemented with 10 μg/ml TGF-β antibody.

A) HBQ stained mandibular explant cultured for 8 days. Both cartilage (blue) and bone (red) appeared earlier than in control explants. Both endochondral bone (eb) and membrane bone (mb) were evident. Scale bar = 200 μm.
B) Adjacent section immunostained for TGF-β. Note staining around the periphery of the developing connective tissue elements but not within bone (b) or cartilage (c) forming regions. Scale bar = 500 μm.
C) Adjacent section immunostained for BMP-2. In this case, staining was localized to developing cartilaginous areas (c), correspondiong to the blue staining regions in (A). Bone remained unstained by BMP-2 antibody. Scale bar = 500 μm.

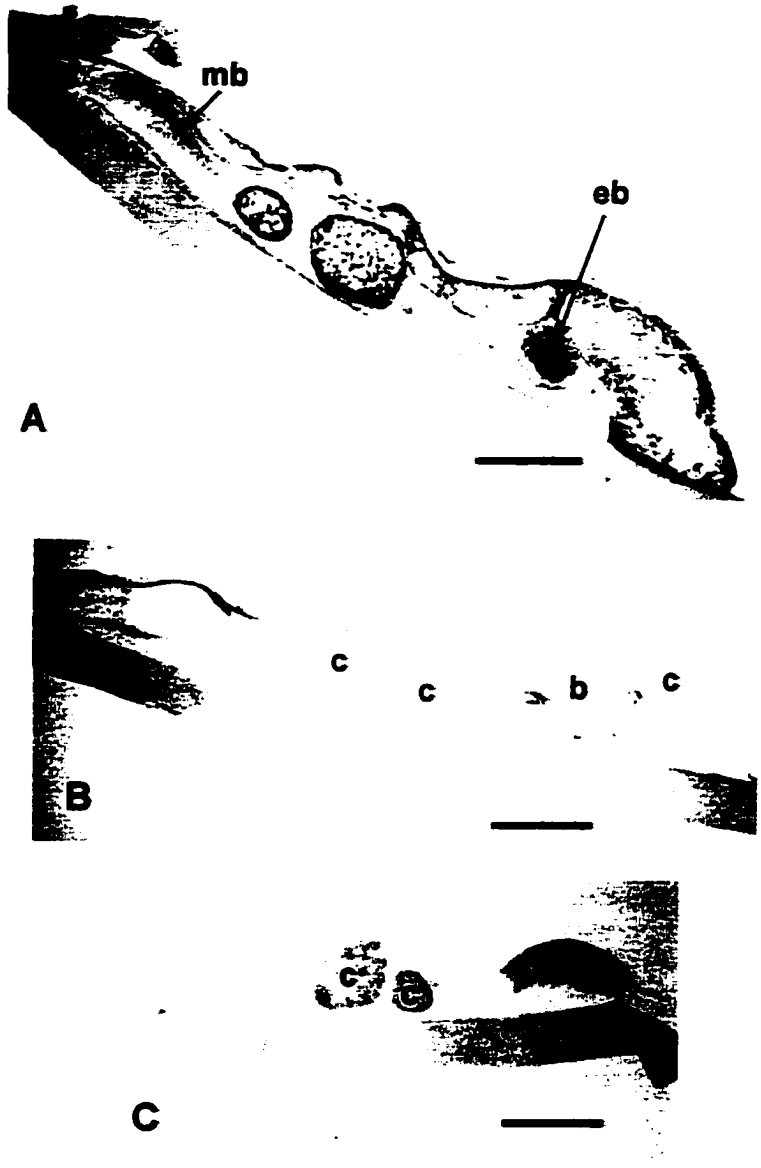
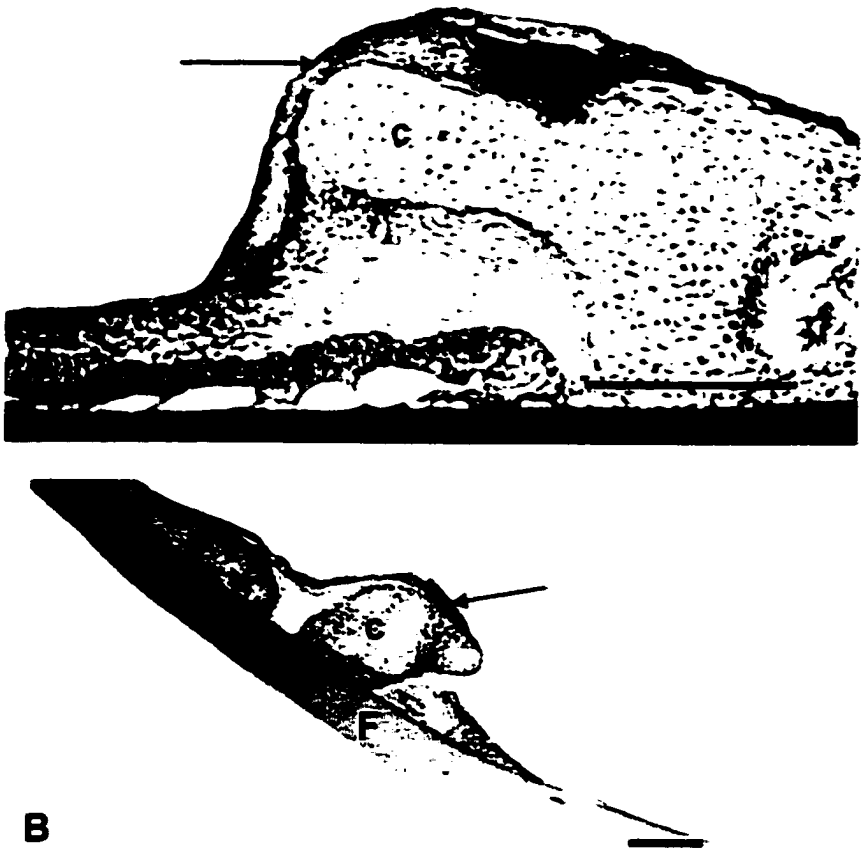


Figure 44

Figure 45: Sections through limb bud explants grown in medium supplemented with TGF-β antibody.

A) Limb bud explant cultured for 8 days in medium containing TGF-β antibody to a final concentration of 1μg/ml. Both bone (arrow) and cartilage (c) are present. The cartilage 'rod' in the picture was undergoing endochondral ossification and a red border, the periostium, is visible around the edge.. Scale bar = 200 μm.
B) Limb bud explant cultured for 8 days in medium containing TGF-β antibody. Arrow indicates an area of bone formation (possibly membrane bone), while (c) indicates cartilage. F = filter.Scale bar = 200 μm.
C) Explant grown under the same conditions as (B) indicating sites of cartilage (c) and possible membrane bone (mb), a type not usually found in the limb. F = filter. Scale bar = 250 μm.



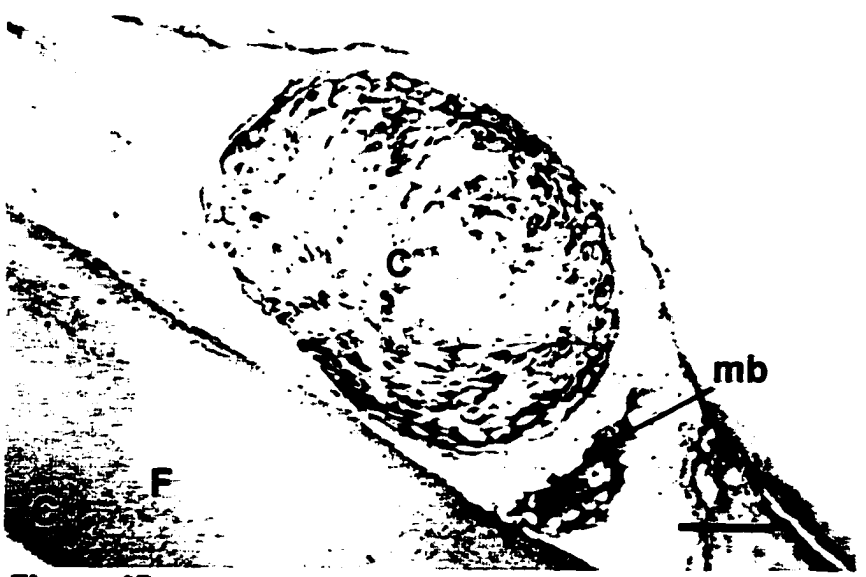


Figure 45

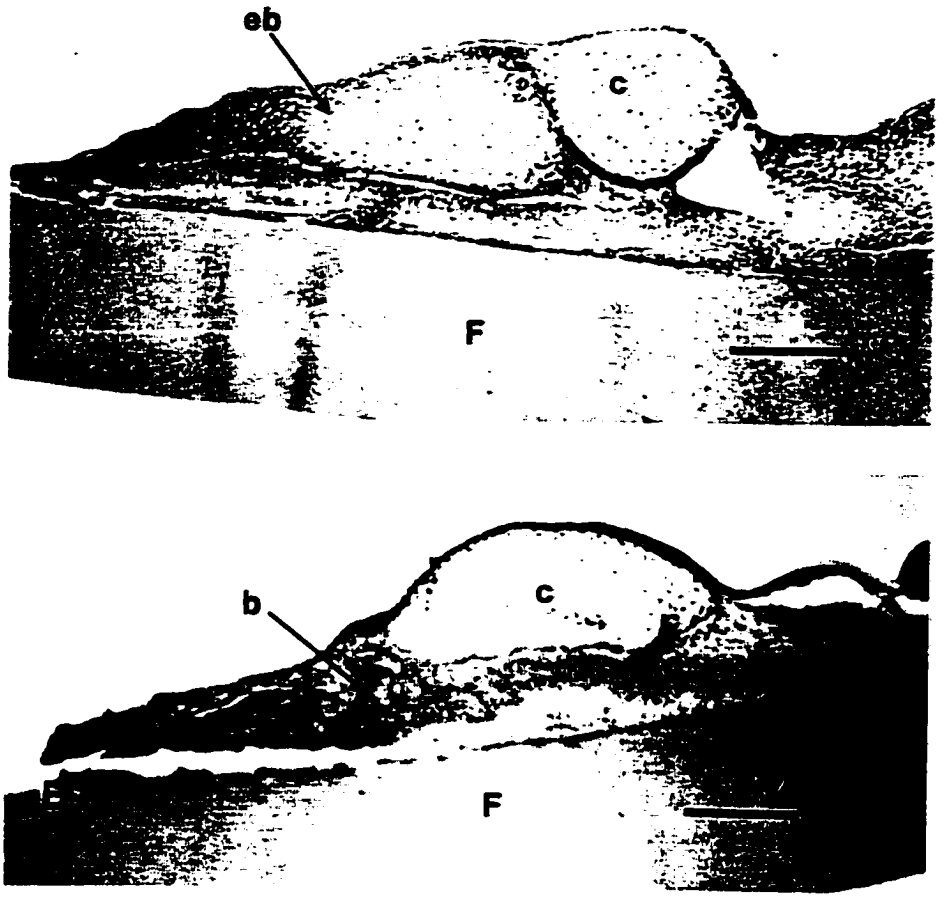


Figure 46: Limb buds grown in medium supplemented with BMP-2 antibody to a final concentration of 1 μg/ml.

A) Limb bud explant cultured for 8 days showed the formation of both cartilage (c) and endochondral bone (eb). Filter = F. Scale bar = 100 μm.
B) Limb bud explant grown under the same conditions as (A) showed the presence of cartilage and possible membrane bone (b). Scale bar = 100 μm.

Figure 47: Mandibular explants grown for 8 days in medium supplemented with either antibodies against BMP-2 or TGF-β.

Mandibular explant grown in medium with final concentration of 1 μg/ml BMP-2 antibody. Cartilage (c) and bone (b) were present. This explant had grown into a hole in the filter. In the photograph, the orientation has been reversed so that the top of the figure represents the side of the filter in contact with the medium. The size of the area projecting into the hole corresponds to the size of the stainless steel mesh. Of interest is the fact that the explant did not grow out around the mesh, nor has it invaded the medium, but remains compact. F = filter. Scale bar = 400 μm.

Mandibular explant grown under the same conditions as (A). Little bone was evident, although a large cartilage nodule (c) was clearly visible. Scale bar = 400 μm.

Mandibular explant grown in medium supplemented with TGF-β antibody to a final concentration of 0.1 μg/ml. Bone (b) was present next to cartilage nodule (c) and stained intensely. Scale bar = 400 μm.

Figure 47

Figure 48: High magnification views of bone and cartilage forming regions in explants treated with antibodies against BMP-2 or TGF-β and cultured for 8 days.

A) Mandibular explant grown in medium with a final concentration of 1 μg/ml TGF-β antibody. Cells forming the cartilage (c) and bone (eb) were visible. The morphology and location (within the cartilage nodule) of the bone producing region suggested endochondral formation. Scale bar = 100 μm.
B) Mandibular explant grown in medium with a final concentration of 1 μg/ml BMP-2 antibody. Cartilage (c) and possible endochondral bone (eb) are indicated. The periphery of the right hand cartilage nodule showed evidence of the early stages of endochondral bone formation in the form of a red staining in the developing periosteum. Scale bar = 100μm.
C) Limb bud explant grown in medium with a final concentration of 10 μg/ml TGF-β antibody.Intense staining was visible in both the cartilage (c) and bone (mb). The absence of evidence for a cartilage precursor suggests that membrane bone was being formed. Periosteum (p) was visible surrounding the cartilage nodule. Scale bar = 100 μm.
D) Limb bud grown in medium with a final concentration of 1 μg/ml BMP-2 antibody. Possible membrane bone (mb) is indicated. Cartilage cells showed evidence of matrix deposition although apparently less than seen in (C). Scale bar = 80 μm.
E) Mandibular explant grown in medium with a final concentration of 10 μg/ml TGF-β antibody with region of endochondral bone (eb) formation indicated. Scale bar = 100 μm.

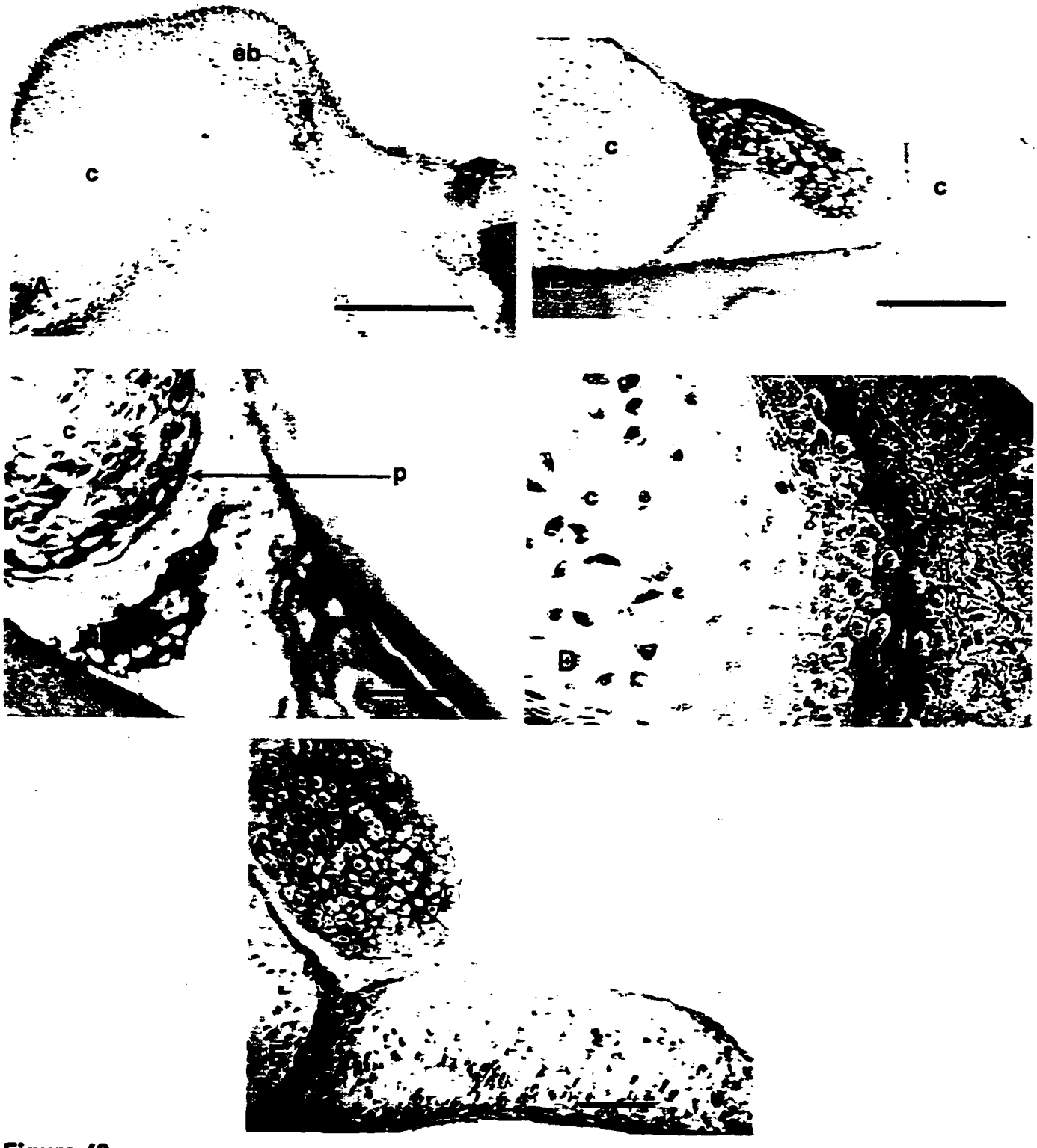


Figure 48

6.0 Summary

1. Northern blot analysis confirms that BMP-2 and TGF-β transcripts are present in the developing chick embryo well in advance of bone and cartilage formation. Since both factors are implicated in skeletogenesis, the appearance of transcripts at an earlier stage suggests they have a secondary role in the early embryo.

2. BMP-2 protein is present in the developing chick embryo prior to the formation of bone and cartilage, suggesting that its initial role in development is not primarily skeletogenic.

3. Staining of chick embryo sections indicates that both BMP-2 and TGF-β protein are immunohistochemically detectable in the early chick embryo. BMP-2 in the mandible and in the limb bud is initially localized to the epithelium and subsequently appears in the underlying mesenchymal cells. The domain of mesenchymal staining increases steadily from approximately stage 12 to 27, then decreases and becomes more diffuse during the latter stages. BMP-2 staining does not coincide with the regions of skeletal element formation during this time period. Exceptions include the staining seen within cartilage components of explants grown in culture for long periods. This suggests that *in vivo* BMP-2 may be found in developing or developed cartilage at later stages than tested here.

4. During the initial outgrowth of the limb bud and mandibular arch, TGF-β is localized to the edge of the primordia, appearing underneath, but not in, the epithelium. The domain of expression suggests that, in early stages of development, TGF-β is involved in initial pattern formation in these structures. This may result from the inhibition of outlying cells, thus setting up a border beyond which growth is limited. Later in development, coinciding with the onset of skeletal element formation, TGF-β is found in outlining regions destined to form skeletal components, especially around cartilage forming areas. During digit formation in the limb for example, TGF-β immunostaining patterns shift from being merely peripheral to appearing around forming cartilage models as well as in the interdigital spaces. That the protein is not produced by skeletogenic cells suggests that TGF-β is not directly responsible for their differentiation. The production of TGF-β by the interdigital cells, which will ultimately undergo apoptosis, suggests that factor this may limit their growth and differentiation rather than stimulate it and may set up initial conditions for their eventual senescence. The changing domains of expression over time indicate that TGF-β plays at least two roles on patterning, initially setting up the basic plan for limb and mandibular development and later directing the modeling of skeletal components.

5. Expression domains of TGF-β and BMP-2 rarely overlap during the developmental time frame surveyed. The two molecules do however often appear in adjacent regions. For example in the mandible, BMP-2 is initially confined to the epithelium and is absent in the mesenchymal tissue of the arch. TGF-β, on the other hand, is absent in the epithelium but is strongly expressed in the peripheral mesenchymal cells. There is, in some cases, a region of overlap a few cell layers thick. The overlap may indicate a transitional area where the two signals are co-expressed. The occurence of both factors in the same area may provide positional and patterning cues to neighboring cells. Removal of one or other of these signals through antibody neutralization may be responsible for the growth aberation seen in perivitelline injected embryos or explant cultures. In other words, by removing these signals, boundaries for expansion are no longer delineated and excessive or uncontrolled growth may occur.

6. Injection of antibodies against either TGF-β or BMP-2 into perivitelline spaces can cause perturbations of the developmental pathways, especially in craniofacial areas, limb and spine. Moreover, this effect is dose dependent. While LD_{50} for TGF-β was not approached at the doses administered, LD_{50} for BMP-2 was approximately 1.5 μg/ml.

7. Embryos injected early in development are more likely to have patterning disturbed by neutralizing antibodies than are older, more developed embryos. Embryos injected at around stage 14, when appearance and patterning of structures such as limb and mandible have just begun, are more sensitive to antibody inhibition than are stage 25 embryos, when limb and craniofacial development are well established. At earlier stages, embryos are still undergoing initial pattern formation and many cell populations have not yet been specified. Late stage embryos have received much of their positional information and cell populations destined to form skeletal elements, for example, have to some degree, differentiated. In early avian embryos whose cell fates are not yet completely determined, the addition of growth factor antibodies appear to interfere with future growth and differentiation.

8. Growth of limb bud or mandibular explants taken from stage 21 chicks is altered by the addition of neutralizing antibodies to the growth medium. Explants treated with either TGF-β and BMP-2 antibodies reach greater heights than do explants not challenged by antibody. The inhibition of either growth factor provides an environment where cell growth is less regulated. The microenvironment may thus no

longer provide sufficient inhibitory signals and growth is therefore not confined or restrained.

9. Explants treated with okadaic acid react differently than did antibody-challenged explants. Rather than growing excessively, explants flatten and lose much of their morphology. Microscopic examination reveals that cells remain undifferentiated. In addition, at high doses of okadaic acid, explants split into two non-overlapping cell types, resembling epithelium and mesenchyme. Okadaic acid is an inhibitor of protein phosphatases 1 and 2. Were these phosphatases downstream effectors in the either the BMP-2 or TGF-β signaling cascades, the addition of okadaic acid might have produced similar results to those seen with antibody neutralization. Since okadaic acid treated explants respond differently, these signaling intermediates are not likely to mediate TGF-β or BMP-2 actions.

10. Limb bud explants treated with TGF-β antibody and, to a lesser degree BMP-2 antibody, show strong evidence of membrane bone formation *in vitro*. Since this mode of osteogenesis is not normally seen in the limb, the removal of cytokine from the explant can radically affect the differentiation and development of bone.

11. In addition to the mandible and limb bud, both TGF-β and BMP-2 are expressed in other regions of the developing chick embryo. These regions include the heart and vascular system, thyroid primordium and dermatome (BMP-2) as well as the myotome (TGF-β). The latter two regions are of interest in that inhibition of the cytokines in vivo produces severely twisted embryos and may reflect a disruption of pattern formation along the spine. That BMP-2 localizes to the dorsal region of the neural tube may explain the occasional lack of neural tube fusion seen in avian embryos after *in vivo* injection of antibody.

12. In conclusion, the role of TGF-β and BMP-2 in the early development of the avian limb and mandible appears to be one of patterning and growth restriction. The factors provide boundary information to the mesenchymal cells and thus limit growth, possibly preventing out of control expansion of the limb bud and mandibular arch. Later in development, both factors appear to have important roles in skeletal element formation. That domains of expression change over time and both factors are additionally seen in other regions of the embryo not associated with skeletal element formation suggest that these factors play multiple roles during avian development which change with increasing developmental age.

			• tail bud a short cone
17	52-64 hrs.	29-32 somites	• both wing and leg buds lifted free of blastoderm; appear as swellings • lateral body folds extend around circumference of body • tail bud bent ventrally • nasal pits form • rotation to somites 17-18
18	3 days	30-36 somites	• limb buds enlarged with leg buds slightly larger than wing buds • rotation extends to far posterior of embryo, lifting legs out of horizontal plane • tail bud turned to right 90° to axis of trunk • somites extend beyond level of leg bud
20	3-3.5 days	40-43 somites	• maxillary process distinct, equaling or exceeding mandibular process in length • 4th visceral arch visible but indistinct • eye pigment a light gray • limb buds enlarged • tail bud beginning to point to lumbro-sacral region • rotation complete
21	3.5 days	43-44 somites	• limb buds enlarged and slightly asymmetrical, directed caudally • maxillary process longer than mandibular process • 4th arch and cleft distinct
24	4.5 days	Somites extend to tip of tail (stage 22)	• limb buds distinctly longer than wide • toes not demarcated but toe plate visible • tip of mandibular arch receding but three small protuberances faintly visible on arch • 3rd arch receding and overgrown by arch 2
25	4.5-5 days		• elbow and knee joints distinct; digital plate in wing distinct, but no digits demarcation; faint demarcation of third toe in leg bud • 3rd and 4th visceral clefts reduced to small pits; three protuberances of mandibular arch more distinct • Meckel's cartilage forms
29	6-6.5 days		• Mandibular arch and second visceral arch broadly fused; protuberances flattened; beak apparent • Wing bent at elbow; 1st, 2nd and 3rd digits apparent; 2nd digit longer than others

Appendix: Staging table for the first 7.5 days of chick embryogenesis based upon major morphological changes. Both Hamburger and Hamilton (H. H.) stages and corresponding developmental ages from time of laying are given as well as some of the major events occurring at a given time.
Note that not all intermediate stages are indicated. Normal incubation period to hatching is 20-21 days at 38° C.

stage	age	description	major developmental events
4	18-19 hrs.	definitive streak	• primitive streak at maximum length (1.9 mm). • Hensen's node, primitive pit, primitive groove present
6	23-25 hrs.	head fold	• head of embryo identifiable as folding in of blastoderm anterior to notochord • no somites visible
7	23-26 hrs.	1 somite	• neural fold visible in head • one somite (second of series) appears
8	26-29 hrs.	4 somites	• neural folds meet at midbrain • cephalic neural crest cells begin to migrate from region of posterior mesencephalon and anterior rhombencephalon (stage 8.5-11)
9	29-33 hrs.	7 somites	• optic vesicles begin to appear • paired heart primordia begin to fuse
11	40-45 hrs.	13 somites	• 5 hindbrain neuromeres distinct • slight cranial flexure • heart bent to right
14	50-53 hrs.	22 somites	• optic vesicles begin to invaginate; lens placode forms • axes of hind- and forebrain form right angle • body rotation extends to somites 7-9 • 1st and 2nd visceral arches distinct
15	50-55 hrs.	24-27 somites	• neural crest cells begin to populate 1st visceral arch • 3rd visceral arch visible but shorter than 2nd arch • regions on flank where future limbs will form are slightly flattened; limb mesoderm condensing
16	51-56 hrs.	26-28 somites	• wing primordium a thickened ridge formed by infolding of lateral body folds, lifting wings off blastoderm; leg primordium flat condensation of mesoderm • rotation of body extends to somites 14-15

			• 2nd and 4th toes appear as ridges with webbing between them
31	7-7.5 days		• membrane bones in mandible appear; beak enlarged; gap between mandible and beak reduced • webbing between digits of leg and wing • feather germs visible in rows extending from branchial to lumbro-sacral region
	20-21 days		• hatching

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