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CHONDROGENIC DIFFERENTIATION FROM MEMBRANE BONE PERIOSTEA AND ITS MOLECULAR REGULATION

by

Jianmin Fang

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

at

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Chondrogenic Differentiation from Membrane Bone Periostea and its Molecular Regulation"

by Jianmin Fang

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

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ABSTRACT

Membrane bones arise directly from mesenchyme through intramembranous ossification without intermediate cartilage development. However, under certain circumstances, cells of membrane bones express a chondrogenic phenotype, although regulation of such chondrogenesis is poorly understood. In this study, the quadratojugal (QJ), a membrane bone of the chick upper jaw, was used as model system. In response to embryonic movement, the periosteum of the embryonic QJ at the posterior hook switches from osteogenesis to chondrogenesis to form secondary cartilage. To look for the regulatory mechanism modulating movement to chondrogenesis, expression of five growth factors and one cell adhesion molecule (N-CAM) was studied *in vivo*. N-CAM exhibits a temporal and spatial expression pattern consistent with a role during secondary chondrogenesis. N-CAM expression persists in osteogenesis but is down-regulated when periosteal cells commit to chondrogenesis. Immobilization of embryos prevents down-regulation of N-CAM and secondary cartilage formation. To test cell differentiation potential, several cell culture approaches were developed. Differentiation pathways of QJ periosteal cells are greatly influenced by culture condition. High cell density favors osteogenesis. Low density monolayer, clonal, and agarose suspension cultures elicit chondrogenesis. Fully differentiated chondrocytes were obtained in monolayer culture of low density, which is the first demonstration that chondrogenesis can be achieved from membrane bone cells in primary monolayer culture. Neither osteogenesis nor chondrogenesis occurs in moderate cell density. Furthermore, the *in vitro* studies suggest that secondary chondrocytes differentiate from certain cell types of the osteogenic lineage. Intramembranous differentiation may include a transient stage in which cells are able to undergo both osteo- and chondrogenesis. Osteogenesis would be the normal pathway, but chondrogenesis can be evoked under certain circumstances. Unlike primary cartilage, in which mesenchymal condensation is a prerequisite and N-CAM is up-regulated, secondary chondrogenesis occurs without a condensation process or N-CAM expression. To assess the effect of N-CAM on periosteal cell differentiation, N-CAM was overexpressed by transfection of an N-CAM-encoding plasmid into cultured periosteal cells. Enhancement of N-CAM expression inhibited chondrogenesis in low density monolayer culture. N-CAM seems to play different roles in early and later stages of chondrogenesis: it enhances cartilage formation at the mesenchymal condensation stage by increasing numbers of potential cartilage cells, but inhibits chondrocyte phenotypic expression when cell differentiation starts. In membrane bones, N-CAM may inhibit the chondrogenic pathway of bipotential periosteal cells. Down-regulation of N-CAM is a prerequisite when periosteum transforms to perichondrium.

ABBREVIATIONS

ABC	Avidin-biotin-peroxidase complex
aFGF	Acidic fibroblast growth factor
APase	Alkaline phosphatase
BCIP	5-Bromo-4-chloroidolyl phosphate
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
bp	Base pair
CAM	Cell adhesion molecule
DAB	Diaminobenzidine
DMEM	Dulbecco's modified Eagle's medium
DMFA	N,N-dimethylformamide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediamine tetracetic acid
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
HH	Hamburger and Hamilton
IGF	Insulin-like growth factor
kb	Kilobase pair
kD	Kilodalton
mRNA	Messenger ribonucleic acid
NBT	Nitroblue tetrazolium
NC	Neural crest
N-CAM	Neural cell adhesion molecule

NCL	New cell layer
NGF	Nerve growth factor
PBS	Phosphate buffered saline
PNA	Peanut agglutinin lectin
QJ	Quadratojugal
RNA	Ribonucleic acid
rpm	Revolutions per minute
SV	Simian virus
TE	Tris-EDTA buffer
TGF	Transforming growth factor

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Chapter 1

General Introduction

Higher vertebrate skeletons are formed by either endochondral or intramembranous bone formation. Endochondral bones include most of the axial and appendicular skeleton as well as some boness at the base of the skull. They form through endochondral ossification in which mesenchymal cells develop into a cartilaginous template which is then replaced by bone. Intramembranous bones, also called membrane bones, are flat and mostly seen in the cranial vault and facial region. They develop directly from mesenchyme without intermediate cartilage formation.

1.1. Periosteum

Bone is surrounded by a periosteum. The outer layer of the periosteum is fibrous, containing fibroblasts and abundant collagen fibers. It provides attachment for bone to tendons, ligaments and muscles. The inner cambial layer contains cells at different stages of osteogenesis (Scott-Savage and Hall, 1980). Osteogenic differentiation is a multiple-step process during which cells progressively increase expression of osteogenic markers, but decrease proliferation rates (Stein et al., 1990, 1996). In periostea, osteogenesis starts from osteoprogenitor cells which differentiate sequentially into preosteoblasts, osteoblasts, and osteocytes.

Osteoprogenitor cells are fibroblast-like and undergo proliferation and renewal. However, they do not express osteogenic markers, which makes their identification difficult (Aubin et al., 1993). *Preosteoblasts* express alkaline phosphatase (APase) and some other osteogenic markers and undergo mitosis, but do not secrete bony matrix (Nijweide et al., 1988; Bruder and Caplan, 1990; Aubin et al., 1993). *Osteoblasts* are cuboidal cells lining the bone matrix front. They actively secrete bony matrix in which type I collagen is a major organic component. Osteoblasts exhibit strong APase activity and express other osteogenic markers such as osteocalcin, osteopontin, osteonectin and bone sialoprotein (Rodan and Noda, 1991; Aubin et al., 1993). Osteoblasts differentiate into *osteocytes* which are embedded in bony matrix. Hence, the periosteum is highly heterogeneous and contains cells in various transitional stages of osteogenic differentiation, as well as fibroblasts.

Osteogenic markers are expressed in osteogenic cells sequentially so that individual osteogenic cells may have different combinations of markers (Guenther et al., 1989; Liu et al., 1994). Moreover, so called osteogenic markers are not absolutely specific to osteogenic cells, but are present in other cell types. Almost all the osteogenic markers listed above are expressed in hypertrophic chondrocytes (Rodan and Noda, 1991; Roach, 1992). Thus, different stages of osteogenic cells are difficult to distinguish, especially early in differentiation.

1.2. Embryonic Origin and Condensation of Skeletal Cells

1.2.1. Embryonic origin of member bones

Most of the craniofacial skeleton is of neural crest (NC) origin (Le Lièvre and Le Douarin, 1975; Le Lièvre, 1978; Noden, 1975, 1978; Couly et al., 1993; Le Douarin et al., 1993). The neural crest is a group of cells located at the boundary between neural plate and epidermis in neurula stage embryos. They give rise to diverse cell types, including neurons and glia of ganglia, adrenal medulla cells, pigment cells, bone and cartilage cells, odontoblasts, smooth muscle and loose connective tissue cells (Bronner-Fraser, 1993; Le Douarin and Ziller, 1993).

All facial membrane bones, including those in the mandible, the quadratojugal (QJ) and their secondary cartilages, are derived from NC (Noden, 1975, 1978, 1988; Le Lièvre, 1978; Le Douarin et al., 1993; Couly et al., 1993). NC also contributes to the skull. The frontal and parietal bones of the skull, previously believed to be of both NC and mesoderm origins (Noden, 1975, 1978, 1988; Le Lièvre, 1978), were recently found to arise exclusively from NC (Couly et al., 1993; Le Douarin and Ziller, 1993).

The NC cells from different regions along the anterior-posterior axis of the neural tube migrate through characteristic pathways and reach specific sites where they differentiate into distinct cell types. For instance, to form mandibular skeleton in the embryonic chick, the presumptive mandibular NC cells detach from epithelium of the mesencephalon at Hamburger and Hamilton (H.H.) (Hamburger and Hamilton, 1951) stage 8.5 (28 hours of incubation) and reach the

mandibular arch at H.H. stage 15 (52 hours of incubation). They accumulate in the mandibular arch and differentiate into Meckel's cartilage at H.H. stage 25-26, or membrane bones at stage 31 (Tyler and Hall, 1977; Hall, 1978b). In order to generate a skeletal element, NC cells undergo two major events: tissue or cell interaction (Bee and Thorogood, 1980; Hall, 1991, 1992; Le Douarin and Ziller, 1993) and skeletal condensation (Hall and Miyake, 1992, 1995).

Interaction with epithelium is crucial for NC cells to differentiate along the the migration pathway. Premigratory NC cells from early chick embryos do not differentiate into bone or cartilage in organ culture, but form bone or cartilage when combined with epithelium (Bee and Thorogood, 1980; Hall, 1991, 1992). Although the mechanism of tissue interaction is not well understood, growth factors may instruct NC cells to generate diverse fates (Hall and Ekanayake, 1991; Hall, 1992). For instance, a TGF–β superfamily protein, bone morphogenetic protein-2 (BMP-2), induces neurogenesis from NC *in vitro*, while TGF–β1, another member of the same superfamily, promotes smooth muscle differentiation (Shah et al., 1996). In mandibular mesenchyme, signals provided by mandibular epithelium can be partially replaced by epidermal growth factor (EGF) (Hall, 1992).

1.2.2. Cellular condensation

After NC cells commit to a skeletal lineage and arrive at the presumptive site of a skeletal element, they generate a cellular condensation, either by undergoing increased mitotic activity (Fyfe

and Hall, 1983; Hall and Miyake, 1992) or by cellular aggregation (Thorogood and Hinchliffe, 1975; Ede, 1983).

Condensation is a basic cellular unit of skeleton morphogenesis through which the overall pattern of a particular skeletal element is established (Hall and Miyake, 1992, 1995). Timing, location and pattern of a condensation are intrinsically controlled by transcription factors encoded by *Hox* genes (Erlebacher et al., 1995). Inactivation or ectopic expression of those specific *Hox* genes results in loss of a particular skeletal element, ectopic addition, or change to another element (Erlebacher et al., 1995).

There are two kinds of skeletal condensations: precartilaginous condensations and membrane bone condensations. Precartilaginous condensations develop into primary cartilage in both higher and lower vertebrates (Hall and Miyake, 1992, 1995; McBurney and Wright, 1996). Membrane bone condensations develop into membrane bones. They occur in mesenchyme, followed by appearance of an ossification center (Hall and Miyake, 1992, 1995; Dunlop and Hall, 1995). In chick mandibles, the membrane bone condensation can be visualized with peanut agglutinin lectin (PNA) at HH stage 26 (5.75 days) (Dunlop and Hall, 1995). In the chick quadratojugal, condensation occurs by day 7.5 (Murray, 1963).

1.3. Chondrogenic Potential of Membrane Bone Periostea

Developmental data clearly demonstrate that membrane bone development does not involve cartilage formation, a major characteristic distinguishing intramembranous and endochondral

bones. However, one of the long existing problems in membrane bone research is how to interpret chondrogenic differentiation. Membrane bone periosteum is capable of expressing cartilage phenotypes under certain conditions. Appearance of this cartilage phenotype differs in various situations, from chondrogenic marker expression at the molecular level to fully differentiated cartilage at the cellular or tissue levels. The following section describes chondrogenic phenotypes appearing in different situations in membrane bones.

1.3.1. Secondary cartilage formation

Secondary cartilage is the cartilage arising from membrane bone periostea. Unlike other cartilages which develop from cellular condensation of mesenchyme, secondary cartilages are derived from preexisting periostea. Hence, the term secondary cartilage is used to distinguish it from most other cartilages, which are designated primary cartilage. Secondary cartilage was also called accessory, adventitious, or embryonic cartilage (Murray, 1963; Beresford, 1981) and is found in mammals, birds, and fish. In mammals, the dentary bone develops secondary cartilage in several processes, but the number of secondary cartilage sites varies among species. In mouse and rat mandibles, secondary cartilage is seen in the condylar, coronoid and angular processes (Beresford, 1981). In chick, secondary cartilage exists in the quadratojugal, surangular, pterygoid, squamosal, and palatine bones (Murray, 1963). Secondary cartilages in chick quadratojugal (Fig. 1) and mammalian mandibular condyle have been most extensively studied and characterized.

Fig. 1. Illustrations of a chick head and quadratojugal

(A). A lateral view of a chick head showing the position of the quadratojugal (black) in the upper jaw. The bone consists of a shaft and a posterior hook which articulates with the quadrate.

(B). A higher diagram of the quadratojugal hook in a day 13 embryo. Secondary cartilage has formed at both anterior and posterior sides of the hook, while osteogenesis continues at the tip and the shaft.

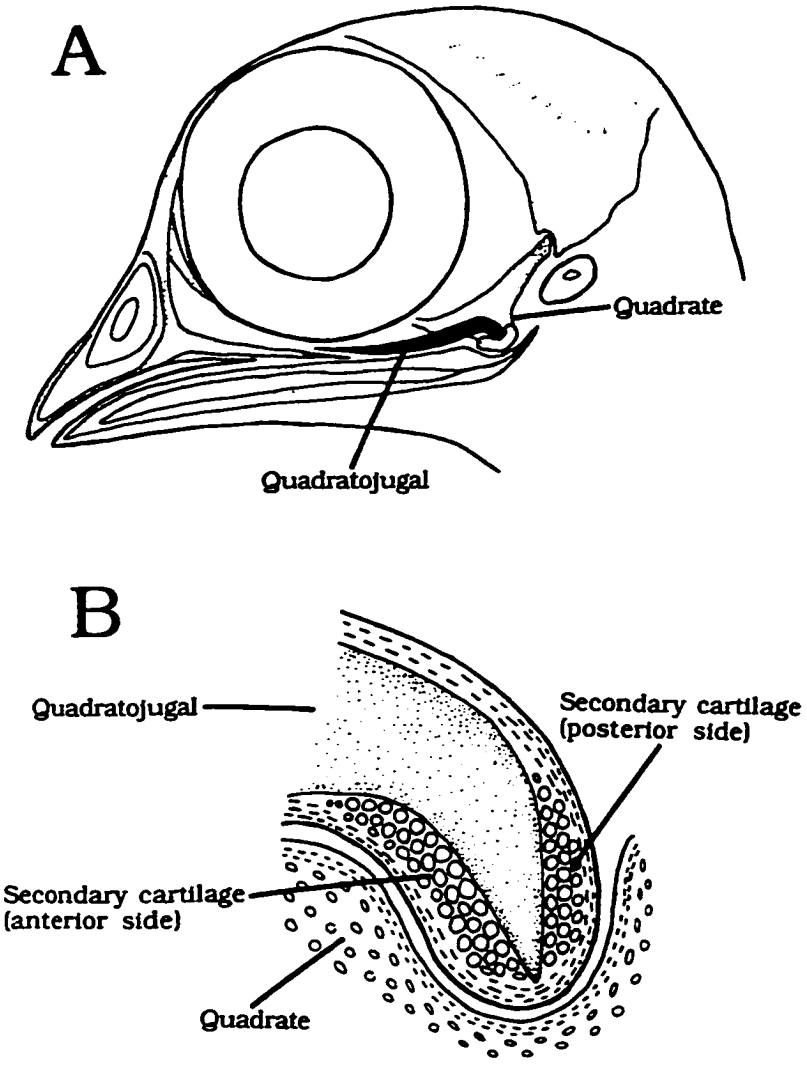

A
Quadrate
Quadratojugal
B
Quadratojugal
Secondary cartilage
(posterior side)
Secondary cartilage
(anterior side)
Quadrate

Figure 1

Since secondary cartilage develops from the periosteum of membrane bone, its cell differentiation varies from that of primary cartilage. Before committing to secondary chondrogenesis, the periosteum where secondary cartilage will develop is morphologically identical to other regions of the periosteum and undergoes intramembranous bone formation. However, when committed to chondrogenesis, the periosteum ceases bone formation and young chondroblasts arise from the periosteum, now a perichondrium (Hall, 1979; Fang and Hall, 1995). As chondrogenesis continues, a hyaline cartilage appears between the perichondrium and membrane bone. In chicks, the switch from osteo- to chondrogenesis in periosteum is dependent upon biomechanical stimulation generated by embryonic movement (see below for the precursor of chondrogenic cells). Secondary chondrogenesis fails to occur if embryos are paralyzed before cartilage formation is triggered (Murray and Smiles, 1965; Hall, 1972, 1979, 1986). Therefore, secondary cartilage formation is a response of membrane bone periosteum to the local mechanical environment. In general, secondary cartilage functions as a growth center of bone and an articular cartilage.

1.3.2. Cartilage formation during fracture healing

Under normal physiological conditions, secondary cartilage is the only genuine cartilage developing from membrane bone. However, in other circumstances, a chondrogenic phenotype can be evoked in

membrane bone where cartilage development normally does not occur. One such circumstance is during fracture healing.

Formation of callus cartilage during fracture healing is a well known phenomena in long bones (McKibbin, 1978). In intramembranous bone, several investigators reported an absence of cartilage during fracture repair (Richany et al., 1963; Radden and Fullmer, 1969; Alberius and Johnell, 1991), but cartilage was observed by other researchers in repair of mammal and bird membrane bones (Girgis and Pritchard, 1958; Craft et al., 1974; Hall and Jacobson, 1975; Granström and Nilsson, 1987; Precious and Hall, 1994). It seems that whether chondrogenesis occurs in membrane bone fracture healing is dependent on environmental conditions at the fracture sites; favorable conditions include poor vascular supply or low oxygen tension (Girgis and Pritchard, 1958; Henricson et al., 1987; Alberius and Johnell, 1991). Interestingly, in fracture sites where chondrogenesis is not found, some cartilaginous macromolecules are detected (Alberius and Johnell, 1991).

1.3.3. Cartilage formation in calcium-deficient embryos

Using long term culture of shell-less chick embryos to deplete the calcium supply, Tuan and colleagues demonstrated that calcium deficiency results in chondrogenic phenotypic expression in intramembranous bones (Tuan and Lynch, 1983; Jacenko and Tuan, 1986, 1995; Jacenko et al., 1995). Chick calvaria are typical intramembranous bones, but in calcium-deficient embryos they produce cartilaginous ECM characterized by type II collagen and

positive alcian blue staining (Jacenko and Tuan, 1986, 1995; Jacenko et al., 1995). Furthermore, genuine cartilage is found in calvaria of organ cultures in low calcium medium (Jacenko and Tuan, 1995) and in undermineralized regions of calvaria of both normal and calcium deficient embryos. These results suggest that calvarial cells have chondrogenic potential which is inhibited in normal embryos but can be induced.

1.3.4. Chondroid bone

Another cartilaginous phenotype is chondroid bone. Chondroid bone refers to various tissues which have characteristics of both bone and cartilage. The earliest description of such tissue is traced back to Schaffer in 1888, but its definition is sometimes confusing because of the variety of histological features and terminology used (see review by Beresford, 1981). Typically, chondroid bone is described as intermediate between bone and cartilage (Hall, 1978a).

Chondroid bone is observed in some developing membrane bones, such as cranial, lower facial and mandibular bones (Goret-Nicaise and Dhem, 1982; Goret-Nicaise, 1984; Goret-Nicaise et al., 1988; Lengele et al., 1990, 1996). This tissue has cartilaginous cells which are larger than osteogenic cells and which express some chondrogenic markers such as type II collagen (Goret-Nicaise, 1984), but its ECM is bony-like and is stained by methylene blue (Lengele et al., 1990, 1996) and contains type I collagen.

Chondroid bone often appears when membrane-bones are undergoing fast growth (Lengele et al., 1990). It does not become a

genuine cartilage and is absent from adults. For its further development, it remains unclear whether it transforms to a bony tissue or is replaced by bone.

1.3.5. Chondrogenic gene expression in developmental membrane bone

Absence of chondrogenic cell differentiation is a significant characteristic of intramembranous bone. However, gene products of the chondrogenic phenotype have been detected during the development of intramembranous bones *in vivo*. McDonald and Tuan (1989) reported that, in chick calvaria of normal embryos, type II collagen mRNA α1(II) was detected by *in situ* hybridization. Ting et al. (1993) investigated expression of several skeletal matrix genes during intramembranous bone formation in rat alveolar bone. They found that two cartilage genes, α1(II) of type II collagen and α1(IX) of type IX collagen, appeared during intramembranous bone development. At the protein level, Jacenko and Tuan (1986) found above-background levels of type II collagen immunostaining in the calvarium. Hence, membrane bones actually express some genes associated with the cartilage phenotype. The significance of expression of cartilage genes in intramembranous bones is not known, but expression of type II and IX collagen may represent an early phenotypic feature of osteoblast differentiation (Ting et al., 1993), i.e. an early osteogenic cell may have both osteogenic and chondrogenic features in terms of gene expression, chondrogenic genes being turned off in further osteogenic differentiation.

1.3.6. Chondrogenic phenotype expression of membrane bone periosteal cells *in vitro*

The above *in vivo* data provide evidence of chondrogenic potential in membrane bones, but chondrogenesis occurs only in certain circumstances. A question arises as to whether cells isolated from membrane bone undergo chondrogenic differentiation in culture.

Periosteal cells sequentially digested from calvaria represent an extensively studied model for skeletal cellular and molecular biology (Wong and Cohn, 1974; Aubin et al., 1993). The periosteal cells are a mixture of heterogeneous cell types which include fibroblasts, osteoprogenitor cells, preosteoblasts, osteoblasts, and young and mature osteocytes. When plated in monolayer culture, calvarial periosteal cells form mineralized bone nodules (Nefussi et al., 1985; Bellows et al., 1986; Bhargava et al., 1988). Each bone nodule is believed to arise from a single osteoprogenitor cell (Bellows and Aubin, 1989). In chick, calvarial periosteal cells express type I and V collagens at the beginning of culture but type II and X collagens are also expressed after 14 days (Berry and Shuttleworth, 1989). Since type II collagen is a marker for cartilage and type X is a collagen associated with hypertrophic chondrocytes (Gibson and Flint, 1985; Schmid and Linsenmayer, 1985a,b), calvarial cells have gene expression associated with chondrogenic phenotypes in monolayer culture. The chondrogenic phenotype genes seem to be expressed at a limited level and calvarial cells do not undergo further differentiation to give rise to chondrocytes.

To achieve chondrogenesis from membrane bone *in vitro*, various culture conditions and subpopulations of periosteal cells have been tested. The best culture condition for chondrogenesis is to suspend periosteal cells in agarose or other gels. In this condition, periosteal cells from membrane bones such as calvaria (Villanueva et al., 1989; Jacenko et al., 1995) differentiate into chondrocytes with high incidence. Agarose culture forces cells into a round shape that is a permissive condition for chondrogenic phenotype expression (Benya and Shaffer, 1982).

Since periosteal cells are highly heterogeneous, it was postulated that certain subpopulations may have higher chondrogenic potential. By separating subpopulations of calvarial cells, Wong and Tuan (1992, 1995) obtained chondrocytes in monolayer culture. In their studies, calvarial cells were fractionated by Percoll gradient isopycnic centrifugation into six fractions and plated in monolayer culture. One subpopulation, fraction F, is polygonal in shape at the beginning of culture and becomes rounded with a highly refractile ECM from day 12, a typical chondrocyte morphology. Type II collagen immunostaining and alcian blue staining confirm that they are chondrogenic (Wong and Tuan, 1992, 1995). Thus, this subpopulation of calvarial cells exhibits high chondrogenic potential and fully expresses the chondrogenic phenotype in monolayer culture. Other fractions are fibroblast-like in culture and do not show chondrogenic capacity. Furthermore, fraction F can enhance chondrogenesis of limb mesenchymal cells but other cell fractions inhibit it (Wong and Tuan, 1995). Thus, it is proposed that chondrogenesis is inhibited by other

cell subpopulations in calvaria *in vivo* or in unfractionated calvarial cells *in vitro* (Wong and Tuan, 1995). Their work demonstrates the chondrogenic capacity of calvarial periosteal cells and provides a possible regulatory mechanism for chondro- and osteogenic differentiation.

1.4. Progenitor Cells of Chondrogenesis in Periostea

To understand chondrogenesis in membrane bones, it is necessary to explore the origin of the chondrogenic cells. Although much effort have been made to clarify the origin of chondrocytes, where chondrocytes differentiate from remains obscure.

One may wonder whether the blood stream provides progenitor cells from marrow stroma since membrane bones are vascularized and bone marrow stroma contain chondroprogenitor cells (Berry and Grant, 1992). However, secondary cartilage in the QJ and mandible is derived from neural crest, not from mesoderm, which gives rises to bone marrow stroma. Furthermore, chondrogenesis in intact periosteum *in vitro* (Thorogood, 1979) also indicates a local origin of cartilage cells in periosteum. Hence, it is unlikely that bone marrow provides chondroprogenitor cells to QJ hooks via the blood stream.

Cells in both osteogenic and chondrogenic lineages are believed to arise from mesenchymal stem cells (Marks and Popoff, 1988), but it is unclear whether periostea contain such undifferentiated stem cells. Periosteal cells in intramembranous bones have undergone determination and commitment to osteogenesis but not to chondrogenesis, so what is the precursor for the cartilage cells in

membrane bone periostea? From previous studies, three cellular sources have been proposed: (1) a restricted chondroprogenitor cell population, (2) bipotential or multipotential stem cells, or (3) osteogenic cells (Fig. 2).

1.4.1. Restricted progenitor cells (Fig. 2A)

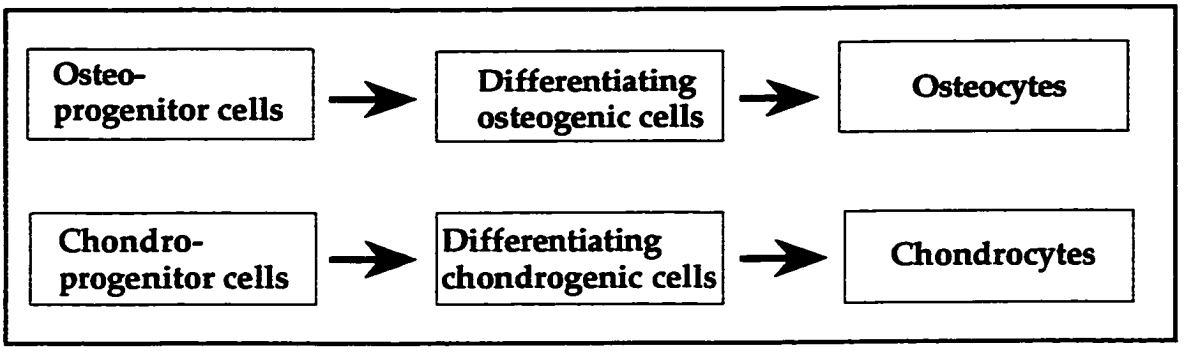
One possibility is that mesenchymal cells from neural crest have been committed to two restricted cell subpopulations in periostea: osteoprogenitor and chondroprogenitor cells. These two subpopulations coexist in the germinal layer of a periosteum but each has a restricted pathway of differentiation. During intramembranous bone formation, only osteoprogenitor cells proliferate and differentiate to form bone. Chondroprogenitor cells are inhibited. But in certain circumstances, as in secondary cartilage formation or bone fracture healing, differentiation of chondroprogenitor cells is evoked to lead to chondrogenesis. Thus, chondrogenesis from membrane bone can be explained as activation of chondroprogenitor cells and inhibition of osteoprogenitor cells. This hypothesis is favored by some investigators (Ben-Ami et al., 1993) and supported by some experiments. For instance, rat calvarial cells form only osteocyte or chondrocyte colonies, indicating a unipotential property of the progenitor cells (Bellows et al., 1989).

1.4.2. Bi- or multipotential progenitor cells (Fig. 2B)

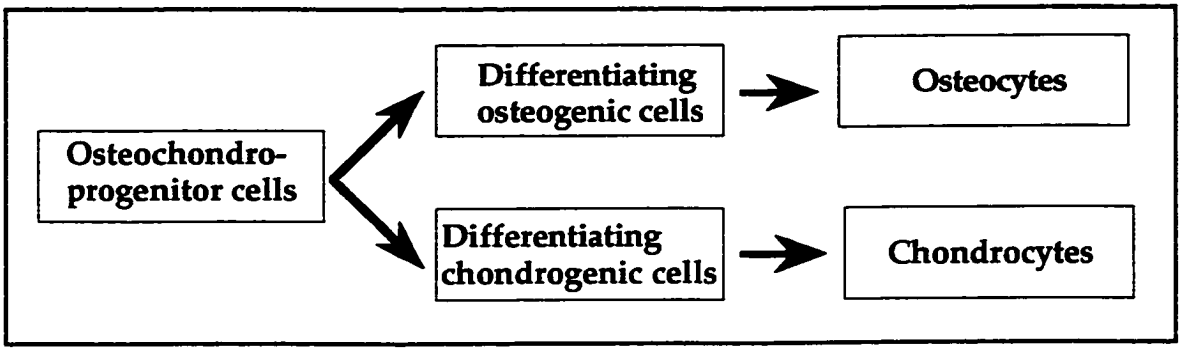
Another possibility is that progenitor cells are bipotential for both differentiation pathways, or multipotential for osteo- and

Fig. 2. Three possible origins of chondrogenic cells in membrane bone.

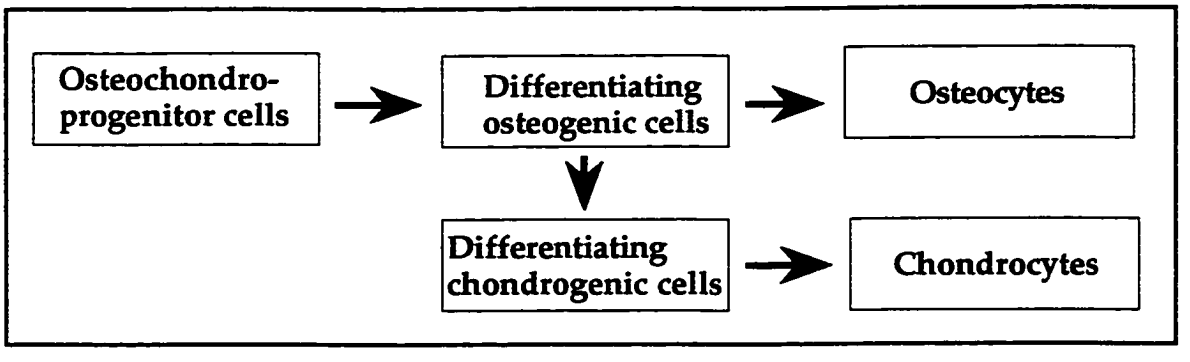
See text for details. The terms "differentiating osteogenic cells" or "differentiating chondrogenic cells" refer to all stages of differentiation between progenitor cells and osteo- or chondrogenic cells.



A. Chondrogenesis from restricted chondroprogenitor cells



B. Chondrogenesis from bi- or multipotential progenitor cells



C. Chondrogenesis from osteogenic cells

Figure 2

chondrogenesis plus other fates. In this case, initiation of chondrogenesis involves alteration of the differentiation pathway from osteogenic to chondrogenic. Multipotential capacity has been demonstrated in many systems, including differentiation of muscle, fat, cartilage, and bone cells in clonal culture of loose connective tissue cells (Young et al., 1993). Grigoriadis et al. (1988) demonstrated that a clonal cell line from calvarial cells can give rise to cartilage, bone, muscle, and fat cells in culture in the presence of dexamethasone. In membrane bone, bi- or multipotentiality of progenitor cells have been proposed based on observation at the tissue level that periosteum gives rise to both bone and secondary cartilage (Hall, 1979). While evidence is sufficient to say that the progenitor layers are bipotential, it is not adequate to ensure that a single progenitor cell is bipotential for both bone and cartilage tissues. In fact, the same data support the hypothesis that periosteum contains two subpopulations each restricted to one differentiation pathway.

1.4.3. Osteogenic cells (Fig. 2C)

The third possibility is that chondrogenic cells may arise from certain cell types of the osteogenic lineage, i.e. some osteogenic cells can switch to chondrogenesis through transdifferentiation. This hypothesis implies the existence of a transient stage between osteo- and chondrogenic cell differentiation, which is supported by some experiments in studies of morphology, biochemistry, and molecular biology (Fang and Hall, 1997).

1.5. Regulation of Chondrogenesis

As addressed above, local environmental factors can trigger chondrogenic differentiation in membrane bones. The triggering signals are quite diverse, for instance, biomechanical forces in secondary cartilage, fracture stimulation in callus cartilage, and low calcium concentration in calcium deficient embryos. How those factors act upon a periosteum and provide an initial signal which ultimately alters the cell differentiation pathway from osteogenic to chondrogenic is poorly understood. In order for chondrogenesis to occur, those environmental factors should activate intracellular biochemical signals. Then a cascade of gene expression changes may occur to lead to chondrogenic differentiation, in which only a few genes may play a key role.

Various factors play regulatory roles in the development, growth and remodeling of skeletal tissues. These include growth factors and their receptors, hormones and receptors, transcription factors, oncogene products, cell adhesion molecules, vitamins, inorganic components such as calcium and oxygen concentrations, etc. Among them, some transcription factors, such as homeobox gene products, contain information to pattern skeletal elements (Erlebacher et al., 1995) and play an important role in condensation and pattern formation. Hormones, such as growth hormone and glucocorticoids, have systematic effects on all the skeleton. Growth factors are important paracrine and autocrine factors. They regulate proliferation, differentiation, and metabolism of skeletal cells by binding to receptors on the plasma membrane (Hall and Ekanayake, 1991). Cell

adhesion molecules are surface molecules mediating cell-cell and cell-matrix interactions, which are required in many developmental processes.

It is not known how chondrogenesis is regulated in membrane bone. To date, there is no direct evidence showing that any particular molecule is a triggering molecule for chondrogenesis. Much investigation is needed to define the molecular mechanism. Since chondrogenesis is a local event responding to local microenvironment, it is likely that chondrogenic differentiation is mediated by locally produced factors in periostea. For this reason, growth factors and adhesion molecules are especially interesting.

1.5.1. Growth factors

Growth factors are potent regulators of cell proliferation and differentiation. The first growth factor, nerve growth factor (NGF), was discovered in the early 1950's (Levi-Montalcini and Hamburger, 1951). Since then, a large number of factors have been identified.

There are no specific glands secreting growth factors, rather they are produced by many cell types in various tissues of animal bodies. After synthesis, growth factors are secreted into extracellular matrices and reach other cells by simple diffusion, a phenomenon called paracrine regulation. On the other hand, growth factors can also regulate the same cells that secreted them, a phenomenon called autocrine regulation. Growth factors act upon target cells by binding specific receptors on their surfaces. Interaction between growth factors and their receptors varies based on the nature of the growth

factors, receptors, and cell types (Hall and Ekanayake, 1991). Once bound, second messengers are generated, which in turn influence cellular function and behaviors through various signal transduction pathways. For instance, FGF receptor-1 contains two tyrosine kinase domains in its cytoplasmic region (Miller and Rizzino, 1994). Upon binding to FGF, the intracellular tyrosine kinase of the FGF-receptor is activated, which stimulates phosphatidylinositol hydrolysis via the activation of phospholipase C-γl (Miller and Rizzino, 1994).

Growth factors play an important role in cell differentiation and proliferation during development. Skeletal tissues are rich sources of growth factors and numerous members of different growth factor families are expressed in bone and cartilage cells (Hall and Ekanayake,1991). They play various roles in regulating many cellular events of bone and cartilage formation during morphogenesis, growth, remodeling, and fracture healing (Hall, and Ekanayake, 1991; Bourque et al., 1993; Erlebacher et al., 1995). It is possible that growth factors play a role in chondrogenesis from membrane bone. In my study, particular attention was paid to five growth factors: transforming growth factor–β (TGF–β), acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF).

(A). TGF–β

The original described form of TGF–β is TGF–β1, a 25 kD protein. Four other proteins have 70-80% sequence identity to TGF–β1 and were assigned TGF–β2 to TGF–β5, which, together with TGF–

β1, are included in the TGF–β subfamily. A number of other molecules have 30-40% sequence identity to TGF–β1, including activins, inhibins, bone morphogenetic proteins (BMPs), and other molecules. They are categorized under the TGF–β superfamily (Kolodziejczyk and Hall, 1996).

Members of the TGF–β subfamily can stimulate or inhibit cell growth or differentiation of various cell types. They have a wide range of biological activities and their effects on differentiation and proliferation may be stimulatory or inhibitory, depending on cell types, differentiation stage, and physiological conditions (Akhurst, 1994).

In skeletal tissues, effects of TGF–β1 on bone and cartilage formation vary from system to system. Several *in vivo* studies indicated that TGF-β1 increases bone formation (Noda and Camilliere, 1989; Marcelli et al., 1990; Joyce et al., 1990, Beck et al., 1993). *In vitro*, TGF–β1 inhibits bone nodule formation and alkaline phosphatase activity in rat calvarial cells (Rosen et al., 1988; Antosz et al., 1989; Ber et al., 1991). An inhibitory effect of TGF–β1 on bone formation was also observed in high density culture of tibial periosteal cells (Iwasaki et al., 1993). Breen et al. (1994) found that the effects of TGF–β1 on osteoblasts are stage-dependent during cell growth *in vitro*; a negative effect during the proliferative stage, but a positive effect during the late matrix maturation and mineralization stage. In regards to chondrogenesis, both negative and positive effects have been reported. TGF–β1 inhibits type II collagen and cartilage proteoglycan expression in cultured articular and epiphyseal chondroblasts of fetal rat long bones (Rosen et al., 1988). In contrast, in tibial periosteal cells, TGF–

β1 stimulates chondrogenesis in high density culture (Iwasaki et al., 1993). In mesenchyme cells, it seems that TGF-β promotes chondrogenesis (Kulyk et al., 1989; Frenz et al., 1994).

(B). FGF

aFGF and bFGF belong to the FGF family which currently consists of nine structurally and functionally related polypeptides (Burgess and Maciag, 1989; Miyamoto et al., 1993; Miller and Rizzino, 1994). aFGF is an 18 kD protein. bFGF has four isoforms with molecular weights of 18, 22, 22.5, and 24 kD, all translated from a single mRNA transcript (Gualandris et al., 1994). Both aFGF and bFGF have strong affinity to heparin and most FGF molecules are bound to heparan sulfate on the cell surface and in the extracellular matrix after they are secreted. aFGF and bFGF bind to the FGF receptors and both have similar biological effects on many cell types. They are important regulators of cell differentiation, proliferation and function, and participate in induction of many development events (Dono and Zeller, 1994; Miller and Rizzino, 1994).

The response of osteoblasts to FGF varies depending on research models and cell differentiation status (Nicolas et al., 1990; Frenkel et al., 1992; Hurley et al., 1993, 1994; Tang et al., 1996). In cultured rat calvarial periosteal cells, aFGF increases proliferation during the initial stage of the culture, but blocks bone formation in late stage culture when bone nodules are forming (Tang et al., 1996). Chondrogenesis is stimulated by FGF in cultured mandibular condyle cartilage (Silbermann, 1990). During fracture healing, injection of aFGF into the

fracture sites increases the size of the cartilaginous soft callus (Jiangushi et al., 1990). In embryonic mesenchyme cells, Frenz et al. (1994) found that bFGF elicits chondrogenesis in cultured periotic mesenchyme. In general, it seems that FGF has a stimulatory effect on chondrogenesis, but this may vary in different stages of chondrogenesis. Furthermore, bFGF reduces alkaline phosphatase expression and mineralization of rabbit rib cartilage cells, indicating an inhibitory effect on terminal differentiation of chondrocytes (Kato and Iwamoto, 1990).

(C). EGF

EGF is a small protein with a molecular weight of 6,045 daltons (Carpenter and Cohn, 1990). EGF is initially synthesized as a 130 kD precursor molecule from which the small mature EGF is released (Rall et al., 1985). EGF binds to and activates the cell surface protein, EGF-receptor, which has an EGF–binding domain in its extracellular region and a tyrosine kinase domain in its intracellular region (Carpenter and Cohen, 1990). Binding of EGF to the EGF-receptor causes activation of the tyrosine kinase domain by which a signal transduction pathway is initiated (Liebl and Hoffmann, 1994).

EGF is a mitogen for many cell types. It can partially replace the molecular signal required for morphogenesis of mandible skeletons in epithelial-mesenchyme interactions (Coffin-Collins and Hall, 1989; Hall, 1991, 1992). EGF affects morphogenesis of Meckel's cartilage, limb skeletons, and teeth during development in chicks and mammals (Coffin-Collins and Hall, 1989; Shum et al., 1993; Canoun et al., 1993).

In rat articular chondrocytes, human recombinant EGF stimulates chondrocyte proliferation and DNA synthesis *in vitro* (Ribault et al., 1997). In calvarial cells, continuous exposure of osteoblasts to EGF reduces bone nodule formation, while pulsed exposure to EGF increases osteogenesis (Antosz et al., 1989).

(D). PDGF

PDGF is a growth factor first discovered in blood platelets with mitogenic effects on fibroblasts and smooth muscle cells (Ross et al., 1974, 1986). PDGF consists of two ligands: PDGF A-chain and PDGF B-chain. The two ligand proteins dimerize covalently to form three isoforms, PDGF-AA, AB, and BB. There are three types of PDGF receptors, aa, ab, and bb, which form by dimerization of a and b PDGF receptor chains (Bowen-Pope and Seifert, 1994). PDGF is expressed in many tissues derived from all three germinal layers and it plays significant roles in many developmental systems (Bowen-Pope and Seifert, 1994). In skeletal tissue, PDGF causes chondrocytes to increase DNA synthesis and proliferation rates in a dose-dependent manner *in vitro*, which is associated with an increase in cytoplasmic (intracellular) calcium concentration (Fukuo et al., 1989; Wroblewski and Edwall, 1992; Stojilkovic et al., 1994). It stimulates proliferation of mesenchymal progenitor cells from bone marrow *in vitro*, which differentiate into bone and cartilage cells in an appropriate environment (Cassiede et al., 1996). By contrast, all three isoforms of PDGF inhibit chondrogenesis in cell cultures of chick limb bud mesenchyme from early stage (HH stage 24) embryos, indicated by

synthesis of sulfated proteoglycans (Chen et al., 1992). During fracture healing, PDGF is expressed in both osteoblasts and chondrocytes (Andrew et al., 1995), and appears to have an influence on initiation of formation of cartilage and bone at the fracture sites (Bolander, 1992).

1.5.2. Cell adhesion molecules (CAMs)

Cell adhesion molecules are cell surface glycoproteins responsible for cell-cell and cell-matrix interaction and recognition. Although the phenomenon of cell adhesion has long been recognized to play a crucial role in embryonic development, only during the past two decades have the molecules responsible for cell adhesion been found (Cunningham et al., 1987). These molecules are classified on the basis of their sequence homology as belonging to several superfamilies, (Jones, 1996), including cadherin, integrin, selectin and immunoglobulin superfamilies.

An important CAM involved in skeletogenesis is neural cell adhesion molecules (N-CAM). N-CAM was the first CAM isolated by the strict criteria as a cell adhesion molecule (Edelman and Crossin, 1991). N-CAM was originally identified in neural tissue, but then was found in many other tissues. N-CAM belongs to the immunoglobulin superfamily of CAMs and its sequence contains 5 immunoglobulin-like domains in extracellular regions (Cunningham et al., 1987; Edelman and Crossin, 1991).

Three major isoforms of N-CAM have been isolated with apparent molecular masses of 170 kD (or 180 kD in mice), 140 kD, and 120 kD. The 170 kD N-CAM is primarily found in neural tissues.

The 140 kD and 120 kD N-CAMs exist in neural and other tissues. All three isoforms have identical sequences in their extracellular domains. Both 170 and 140 kD N-CAMs have membrane spanning segments and cytoplasmic sequences, which make these two isoforms the transmembrane proteins. However, the 120 kD N-CAM lacks membrane spanning and cytoplasmic sequences and it is linked to cell membranes by phosphatidylinositol-containing anchors (Hemperly et al., 1986; Cunningham et al., 1987; Edelman and Crossin, 1991). The 140 kD N-CAM differs from the 170 kD in lacking a 250 amino acid fragment in its cytoplasmic domain (Murray et al., 1986). It has been found that all three N-CAM isoforms are coded by a single gene with 19 exons. Different isoforms of N-CAM are translated from mRNAs alternatively spliced during transcription (Murray et al., 1986). In addition, polysialic acids attach to the fifth immunoglobulin-like domain (Edelman and Crossin, 1991), which may play a role in regulation of cell interaction (Rutishauser, 1996).

N-CAM expression is critical in many development systems. In the neural systems, it participates in several important events, which include neuron-neuron, neuron-glial, and neuron-muscle interactions, neurite fasciculation, and formation of retina (Edelman, 1988; Jessell, 1988; Edelman and Crossin, 1991). In other tissues, N-CAM is expressed during morphogenesis of many organs, especially during condensation of morphogenesis. In skeletal tissues, N-CAM may play an important role in limb precartilaginous condensation (Widelitz et al., 1993; Chuong et al., 1993). Based on adhesion assays of lipid vesicles containing N-CAM proteins, homophilic interaction between

N-CAM molecules of adjacent cells is suggested (Hoffman and Edelman, 1983). Furthermore, structurally, N-CAM contains several domains, such as those binding to type I collagen (Probstmeier et al., 1992) and heparin sulfate proteoglycan (Kallapur and Akeson, 1992), which provide a basis for interaction between cells and ECM. N-CAM can influence signal transduction pathways and thereby regulates cell behaviors (Schuch et al., 1989; Fagotto and Gumbiner, 1996; Sastry and Horwitz, 1996). An example of such regulation is that N-CAM stimulates neurite extension through the aFGF receptor (Williams et al., 1994; Fagotto and Gumbiner, 1996).

N-CAM expression can be regulated by growth factors and homeobox genes. *In vitro*, N-CAM expression is modulated by TGF–β in 3T3 fibroblasts (Roubin et al., 1993) and by nerve growth factor (NGF) and IGF-2 in chicken skeletal muscles (Lyles et al., 1993). Moreover, some other members in the TGF–β superfamily play significant roles in regulation of N-CAM expression. For example, BMP-2, -4, and -7 up-regulate N-CAM expression in neuroblastoma-glioma hybrid cell lines *in vitro* (Perides et al., 1992, 1994). Because BMPs play important roles in the regulation of cartilage and bone formation, it would be very interesting to determine if BMPs regulate osteogenesis and chondrogenesis through influencing N-CAM expression.

Recent evidence shows that the N-CAM gene is a downstream target of *Hox* genes. The upstream region of the N-CAM gene contains a promoter and a regulatory element (Hirsch et al., 1990, 1991). The regulatory element contains homeodomain binding sites of *Hox* genes. Activity of the *N-CAM* promoter can be greatly elevated by *Hox* 2.5,

and eliminated by *Hox* 2.4 (Jones et al., 1992). Moreover, two other *Hox* genes, *cux* and *phox* 2, bind with the regulatory element of the *N-CAM* upstream region. *Cux* strongly inhibits the *N-CAM* promoter but this inhibition is prevented by *phox* 2 (Valarche et al., 1993). *In situ* hybridization shows that *phox* 2 expression is restricted to the areas where N-CAM is expressed, while *cux* is expressed in many N-CAM-negative areas (Valarche et al., 1993).

1.6. Mechanical Stimulation and Signal Transduction

Mechanical loading has long been recognized to play an important role in bone and cartilage growth and regeneration since the emergence of Wolff 's law, which stated that mechanical stress is responsible for determining the architecture of bone (Wolff, 1892; Duncan and Turner, 1995). Skeletal cells are sensitive to local mechanical environments and they respond to various mechanical forces with alteration of cellular behavior and functions (Cowin et al., 1991; Burger and Veldhuijzen, 1993; Duncan and Turner, 1995). The molecular mechanism by which skeletal cells sense and transduce mechanical signals and finally change their differentiation, proliferation, or metabolism is undergoing extensive investigation. It is likely that several steps are involved in the process:

The first step is sensation and conversion of mechanical information into biochemical signals. Unfortunately, how external biomechanical signals cross cell membranes and transmit into internal biochemical signals are poorly understood. Several pathways have been proposed. One possible pathway is through a mechanism mediated by

integrins. Integrins are heterodimers of alpha and beta subunits. The alpha and beta chains of integrin form a transmembrane complex with their extracellular domains attaching to extracellular matrix components such as fibronectin, collagens, and laminin, and intracellular domains linking cytoskeletal actin through actin-associated proteins such as vinculin, talin, tensin, and alpha-actinin (Pavalko et al., 1991; Hynes, 1992). Integrins can transmit signals into cells which then further affect second messenger systems (Hynes, 1992).

Another pathway is through mechanosensitive ion channels. They include a large family of channels which can be further subdivided based on their activation properties, kinetic characteristics, and ion selectivity (Morris, 1990; Duncan and Turner, 1995). In osteoblasts, mechanosensitive channels may influence intracellular calcium levels and the cytoskeleton through the phospholipase C pathway (Duncan and Turner, 1995).

Besides these, a number of other mechanisms have been proposed as mechanotransductors, such as guanine nucleotide binding proteins (G proteins) and nitric oxide. In mandibular condylar chondrocytes, mechanical loading causes release of members of the *ras* superfamily of small GTP-binding proteins, indicating involvement of small G-proteins in mechanotransduction (Basdra et al., 1994). It is also possible that these pathways mutually interact during mechanotransduction.

The second step of mechanotransduction probably involves propagation of biochemical signals through second messenger

systems. Osteoblasts respond to mechanical stimulation with elevation of cAMP and inositol phosphates, including inositol-1,4,5-trisphosphate (IP3) (Sandy et al., 1993; Duncan and Turner, 1995). In mandibular condylar secondary cartilage, cAMP levels increase in response to mechanical stress in organ culture (Copray and Jansen, 1985).

The third step may include induction of immediate early genes, such as *c-fos*, *c-jum*, and *Egr-1* (Duncan and Turner, 1995; Dolce et al., 1996). Expression of these genes can be induced rapidly and transiently when osteoblasts are under mechanical deformations (Dolce et al., 1996). The immediate early genes encode transcription factors that bind to regulatory regions of late-response genes, which control phenotype expression, and regulate expression of the late-response genes (Sukhatme, 1992; Dolce et al., 1996). For instance, *EGR-1* gene products can interact with promoter regions of growth factors, such as PDGF and TGF-β genes (Dolce et al., 1996), and regulate the expression of PDGF and TGF-β. TGF-β is also released from calvarial cells when subjected to mechanical loading (Klein-Nulend et al., 1995b). These factors may further cause the cascade of changes in gene expression necessary for phenotypic alteration from one cell type to another. Consequently, mechanosensitive cells response to mechanical stimulation with a whole set of gene expression changes, by which cellular phenotype is modified.

It is worth pointing out that most information available for mechanotransduction in skeletal tissues is from osteoblasts or chondrocytes. Information related to how mechanical stimulation

influences differentiation in germinal cells is very limited. The regulatory pathways documented from osteoblast models are not necessarily the same as those in progenitor cells.

1.7. Experimental Plans

This study aims to use secondary cartilage in the chick quadratojugal as a model system to study chondrogenic differentiation and its regulatory mechanisms. The QJ periostea can undergo chondrogenic differentiation to form secondary cartilage in response to mechanical stimulation *in vivo* and differentiate into chondrocytes in explant culture *in vitro*. However, the differentiation pathway of secondary cartilage and its molecular regulation are poorly understood. This study will employ various *in vivo* and *in vitro* approaches to investigate the differentiation potentials of QJ periosteal cells and gene expression during onset of secondary chondrogenesis in normal and paralyzed embryos. Specifically, this research includes the following aspects:

1. Study the morphological development of secondary cartilage formation in the QJ of normal chick embryos. Carry out paralysis of chick embryos with decamethonium iodide and observe the effects of paralysis on secondary chondrogenesis in histological sections.

2. Investigate expression of potential regulatory molecules during secondary cartilage formation in the QJ hook *in vivo* with immunohistochemistry. A transient expression pattern of a particular molecule during initiation of secondary chondrogenesis would suggest a role for this molecule in regulation of differentiation. From previous

reports, it is likely that chondrogenesis is regulated by local factors. Those molecules may provide the initial signal that ultimately affects cell differentiation through a signal transduction cascade. For this reason, growth factors and cell adhesion molecules are chosen as candidates. Specifically, expression patterns of TGF–β, aFGF, bFGF, PDGF, EGF and N-CAM were studied by ABC immunohistochemistry during bone and secondary cartilage formation.

3. When an interesting expression pattern of a particular molecule is found, this molecule will be further investigated in detail *in vivo* and *in vitro*, particularly focusing on its correlation with chondrogenic or osteogenic differentiation pathways in normal and paralyzed chick embryos. Then, from these *in vivo* data, a hypothesis will be developed. In my immunolocalization study, it was found that down-regulation of N-CAM expression was coincident with the switch of QJ periosteum from osteogenesis to chondrogenesis. Therefore, further investigation was focused on N-CAM.

4. In order to study differentiation of periosteal cells and look for appropriate approaches for examining the role of N-CAM in periosteal cell differentiation, the differentiation potential of the QJ periosteal cells will be investigated in cell culture. An enzymatic digestion method which releases cells from the QJ periostea is adopted and periosteal cells are cultivated in various cell culture conditions to test their differentiation behavior. The *in vitro* study is expected to answer these important questions:

i) Do QJ periosteal cells retain chondrogenic potential in cell culture?

ii) What kind of *in vitro* environment favors chondrogenesis?

iii) What is the precursor cell for chondrogenesis?

Moreover, appropriate cell culture approaches will be explored to find the approaches by which the role of N-CAM can be tested *in vitro* with molecular manipulations.

5. Examine N-CAM expression during *in vitro* osteogenesis and chondrogenesis. This information will further define the expression pattern of N-CAM in skeletogenesis and provide insights into the role of this molecule on osteo- and chondrogenesis.

6. More direct evidence related to the effects of N-CAM on osteogenesis and chondrogenesis will be obtained by determining whether cell differentiation can be manipulated by overexpression or functional perturbation of N-CAM in culture. Overexpression will be achieved by transfection of plasmids encoding the N-CAM gene into the periosteal cells. Functional perturbation will be carried out with a monoclonal antibody against N-CAM.

Chapter 2

Secondary Cartilage Formation and Expression of Growth factors during Secondary Chondrogenesis

2.1. Introduction

Secondary cartilage is the cartilage that develops from membrane bone periostea. This form of cartilage often arises from the periostea of some craniofacial intramembranous bones at their articular surfaces. In chicks, secondary cartilage is observed in the quadratojugal, surangular, pterygoid, squamosal, and palatine bones (Murray, 1963). It is also seen in mammals and fish. The chick quadratojugal and mammalian mandibular condylar cartilage are the most extensively studied secondary cartilages (Fang and Hall, 1997).

There are three membrane bones in the chick upper jaw: the quadratojugal, jugal and maxilla. The quadratojugal is located at the most posterior end of the upper jaw and consists of a slender shaft and a posterior hook. The QJ hook articulates with a groove in the quadrate to form the QJ-quadrate joint (Fig. 1). This joint provides articulation for beak movement in birds. Embryonically, the membrane bone develops at day 7 as a condensation of ectomesenchymal cells, followed by appearance of ossification centers at 7.5 days (Murray, 1963; Thorogood, 1979). Both the shaft and hook of the QJ develop directly from a condensation of ectomesenchyme rather than from a cartilage template. The germinal layer of the QJ hook initially

undergoes osteogenic differentiation to form intramembranous bone. However, in embryos after 10 days, the progenitor cells in the periosteum of the QJ hook cease bone formation and switch to chondrogenic differentiation to form secondary cartilage (Murray, 1963; Murray and Smiles, 1965; Hall, 1972, 1979, 1981; Thorogood, 1979; Fang and Hall, 1995).

The mammalian mandibular condyle also arises from a periosteum. In mice, the condylar cartilage develops in day 16 embryos *in utero* from the tip of the condylar process of the dentary, which is a membrane bone. The secondary cartilage contributes to elongation of the condylar process and to articular cartilage in the squamosomandibular joint (Livne and Silbermann, 1990).

Since both bone and cartilage cells differentiate from the germinal layer of the periosteum, the periosteum is believed to have the capacity to undergo both osteogenesis and chondrogenesis (Murray and Smiles, 1965; Hall, 1979, Strauss et al., 1990). Osteogenesis and chondrogenesis in the QJ hook are precisely regulated during embryonic development. It has been long recognized that secondary cartilage formation in the QJ is a result of mechanical stimulation. This was noticed because secondary cartilage was formed only at the articulations, which may be either mobile or immobile, but receive contractile force from the adjacent musculature (Murray and Smiles, 1965). Requirement of mechanical stimulation during onset of secondary chondrogenesis in the QJ has been experimentally demonstrated by i) *in ovo* injection of paralyzing drugs such as tubocurarine or decamethonium iodide (Murray and Smiles, 1965;

Hall, 1972, 1979; Fang and Hall, 1995), ii) grafting QJs to the chorio-allantoic membrane (Murray and Smiles, 1965; Hall, 1972), and iii) organ culture of QJs at the medium-air interface (Thorogood, 1979). In all cases, movement or contractile pressure was abolished and the QJ hooks failed to form secondary cartilage. By contrast, bone formation in the QJ is not affected by immobilization. Hence, it has been concluded that secondary chondrogenesis in the QJ is evoked by biomechanical stimulation (Murray and Smiles, 1965; Hall, 1972; for review see Beresford, 1981).

In mammalian mandibular condyles, it seems that secondary cartilage can be initiated in the absence of biomechanical stimuli. The evidence supporting this claim is that secondary cartilage can form in mandiblar organ cultures prior to cellular commitment to chondrogenesis (Glasstone, 1971; Herring and Lakars, 1981; Vinkka-Puhakka and Thesleff, 1993). On the other hand, maintenance of secondary cartilage in mandibular condyles requires mechanical stimulation, for the germinal cell layer of the condylar cartilage switches to osteogenesis in organ cultures (Strauss et al., 1990; Ben-Ami et al., 1993; Takahashi, 1991; Takahashi et al., 1995). Therefore, secondary chondrogenic differentiation is highly influenced by biomechanical stimuli, although effects may vary in different species.

Mechanical loading plays important roles in bone and cartilage formation, repair, and remodeling. However, how biomechanical stimuli influence cell differentiation is not understood (Hogg and Hosseini, 1992). Generally, it is likely that biomechanical signals act upon progenitor cells and activate biochemical signals, which further

influence gene expression via signal transduction pathways. Consequently, a new differentiation pathways is triggered due to cascading gene expression changes, in which a few genes may play crucial roles during the switch. Although some molecules, such as growth factors and cell adhesion molecules, respond to mechanical loading in skeletal or non-skeletal cells (Ingber, 1991; Klein-Nulend et al., 1995a), our knowledge of molecular links between mechanical stimulation and secondary cartilage formation is very limited.

During the past two decades, tremendous progress has been made in identifying and characterizing regulatory factors of bone and cartilage formation and remodeling, as discussed in the General Introduction to Chapter 1. Among them, growth factors have a large influence in regulation of skeleton development, repair and remodeling, and some are involved in responses to biomechanical loading (Klein-Nulend et al., 1995b). It is interesting to determine if growth factors play a key role during the switch from osteogenesis to chondrogenesis in secondary cartilage formation. The QJ system provides an ideal system to examine the roles of growth factors in initiation of secondary chondrogenesis. Data about the expression of those molecules during secondary chondrogenesis would provide insights into their physiological roles in regulation of chondrogenic differentiation. In this chapter, expression of five growth factors, which play important roles in skeletal tissues, were assessed during secondary chondrogenesis in the QJ hook. These growth factors are TGF-β, aFGF, bFGF, EGF, and PDGF. Two experiments were designed and conducted:

(1). To establish the timing of secondary cartilage development in QJ hooks *in ovo*. The process of secondary cartilage formation was studied histologically in the QJs in normal embryos and in embryos paralyzed at different stages.

(2). Expression of TGF–β, aFGF, bFGF, EGF, and PDGF was examined by immunohistochemistry during secondary cartilage and bone formation in the QJ hook and shaft.

2.2. Materials and Methods

2.2.1. Antibodies and reagents

Rabbit anti-human pan-TGF–β IgG and goat anti-PDGF IgG were obtained from R & D Systems (Minneapolis, MN). Rabbit anti-EGF, anti-aFGF, and anti-bFGF IgGs were obtained from Sigma (St, Louis, MO). Biotinylated goat anti-mouse IgG was obtained from GIBCO BRL, (Burlington, ON). Biotinylated rabbit anti-goat IgG, avidin-biotin-peroxidase complex kit, normal goat serum, and normal rabbit serum were obtained from Vector Laboratories (Buringame, CA). Hydrogen peroxide (H_2O_2) and diaminobenzadine (DAB) were purchased from Sigma (St. Louis, MO). Decamethonium iodide was from Koch-Light Labs Ltd. (Colnbrook, UK.)

2.2.2. Chick embryos and incubation

Fertilized eggs of white leghorn chicken (*Gallus domesticus*) were obtained from Cook's Hatchery (Truro, NS) and incubated in a forced-draft Humidaire incubator at a temperature of 36 ± 0.5° C. These incubation conditions were used throughout experiments in this study, including those described in the following chapters.

2.2.3. Paralysis of chick embryos

Chick embryos were paralyzed by injecting a postsynaptic depolarising blocking agent decamethonium iodide dissolved in sterile saline (0.85% NaCl) into the air sac of embryonated eggs (Hall, 1972). The surface of the egg was swabbed with 70% ethanol and a pinhole

was made in the shell at the edge of the air sac. Decamethonium iodide was injected through the pinhole with a sterile syringe. The pinholes were sealed with Scotch tape and the eggs were returned to the incubator for further incubation. The effectiveness of paralysis was evaluated by observing absence of embryonic movement when opening the eggs. Embryos showing any movement were discarded prior to microdissection. Two experimental groups were established, varying in the time of paralysis and fixation.

(A). Paralysis of embryos before cartilage formation

Embryos were paralyzed by injecting 1.0 mg decamethonium iodide (0.5 ml of a 2 mg/ml solution) into each egg at day 9 of incubation. Day 9 is before initiation of secondary chondrogenesis (Murray, 1963; Hall, 1972). Embryos of the same stage were injected with sterile PBS as controls. The quadratojugals and associated parts of the quadrates from days 10 to 15 embryos were dissected at 24 hour intervals and fixed for histology and immunohistochemistry.

(B) Paralysis of embryos after cartilage formation

Decamethonium iodide (0.5 ml of a 3.5 mg/ml solution) was injected into the air sac of each egg at day 13 of incubation. By day 13 secondary cartilage is well developed in the QJ hook. Embryos of the same stage were injected with sterile PBS as controls. The QJs and parts of the quadrates from days 15, 17, and 19 embryos were removed and fixed for histology and immunohistochemistry. With this dose, about half of the embryos survived for 48 hours, and about twenty

five percent survived for six days. With lower doses, embryos were not paralyzed reliably, while higher doses were lethal.

2.2.4. Microdissection and tissue preparation

The QJs and parts of the quadrates were removed from the upper jaws of embryonic chicks with two fine #5 forceps under a dissecting microscope. Major portions of the quadrates were cut off carefully with forceps to leave the QJ-quadrate joint intact. Samples from at least five embryos per day for each group were fixed in periodate-lysine-paraformaldehyde (PLP) fixative (containing 4% paraformaldehyde) overnight (McLean and Nakane, 1974). The tissues were dehydrated in ethanol, infiltrated and embedded in low melting point paraffin at 52° C in a vacuum oven. Serial sections were cut with a microtome at 5 μm. To increase adhesion of sections to slides, sections were mounted on poly-L-lysine-coated slides.

2.2.5. HBQ staining

Histological sections were stained with the Hall and Brunt's quadruple (HBQ) stain (Hall, 1986). This method uses celestial blue B, alcian blue, Mayer's hematoxylin, and direct red, and it gave high contrast between cartilage (blue) and bone (red).

2.2.6. Immunohistochemistry

The avidin-biotin-peroxidase complex (ABC) immuno-histochemical technique was modified from Hsu et al. (1981). Sections were deparaffinized in histoclear and taken through graded ethanols

into distilled water. Then they were rinsed with Dulbecco's phosphate buffered saline (PBS) (Freshney, 1983) and incubated with normal serum (goat serum for TGF–β, aFGF, and bFGF; rabbit serum for PDGF) for 20 minutes at room temperature to block non-specific binding sites. After draining the serum, sections were incubated with primary antibodies, i.e. rabbit anti-TGF–β, aFGF, bFGF, or EGF, or goat anti-PDGF, for 2 hours at room temperature or overnight at 4 °C. The primary antibodies were diluted with PBS. Antibody against TGF–β was diluted to 1: 50, and the others to 1:500. Sections were rinsed and then incubated with 1:200 diluted biotinylated secondary antibodies, i.e. goat anti-rabbit IgG for TGF–β, aFGF, bFGF and EGF, and rabbit anti-goat IgG for PDGF, for 30 minutes at room temperature. After PBS rinsing, the sections were loaded with avidin-biotin-peroxidase complex (ABC) for 30 minutes at room temperature. The sections were then rinsed with PBS and a brown precipitate was produced by treating the slides with 3,3'-diaminobenzidine (DAB) [10 mg of DAB was dissolved in 20 ml of 0.05M, pH 7.6 Tris-HCl buffer containing 4 µl 30% H_2O_2 for 1.5-2 minutes at room temperature.

Two controls were used to check specificity of antibody binding. (1) A negative control, in which primary antibody was replaced with PBS. Non-specific reaction was not observed. (2) Control for endogenous peroxidase activity, in which sections were treated with the ABC and DAB steps. No endogenous peroxidase reaction was found in the QJs and quadrates, except for some reaction in associated blood cells.

2.3. Results

2.3.1. Secondary cartilage formation in the QJ hook during normal development

Secondary chondrogenesis in the QJ hook was studied histologically in normal (unparalyzed) embryos from day 9 to 21. Secondary cartilage was not observed in the QJ hooks of day 10 or younger embryos in any specimens. At day 10, the QJ consisted of a bone core surrounded by a periosteum. The periosteum in the QJ hook was morphologically identical to that in other regions of the bone and was undergoing intramembranous bone formation.

By day 11, secondary cartilage was still not seen in the QJ hook of most embryos. In approximately 80% of embryos, initiation of secondary chondrogenesis was not found and the QJ hook periosteum appeared similar in morphology to that seen in day 10 embryos (Fig. 3A). However, in some other specimens, a few weakly alcian blue-positive cells were present in the anterior side of the hook. These cells appeared more rounded with abundant ECM, indicating initiation of secondary chondrogenesis in these specimens. There was no sign of chondrogenesis in the posterior side of the hook in any specimen examined.

In the QJ hooks of embryos at day 12, cartilage tissue was present in all specimens. The posterior side always has a relatively larger volume of cartilage than the anterior side. Cartilage in both sides increased rapidly and, by day 13, became obvious on both sides of the hook, as seen in longitudinal sections after HBQ staining (Fig.

Fig. 3. Development of secondary cartilage in QJ hook between day 11-15.

(A). The QJ in day 11 embryo. Its hook consists of a bone core and the surrounding periosteum. No chondrogenesis is seen in the hook.

(B). QJ hook in day 13 embryo. Secondary cartilage formed in both sides of the hook. Osteogenesis is continuing at the tip (asterisk) of the hook as well as in the QJ shaft.

(C). QJ hook in day 15 embryo. A large piece of cartilage exists in both sides of the hook. Most chondrocytes are hypertrophied. An new cell layer is forming to separate the secondary cartilage from its perichondrium (arrowheads).

QJ: quadratojugal; QT: quadrate; b: bone; s: secondary cartilage.

Bar: (A) 100μm, (B, for B and C) 50μm.

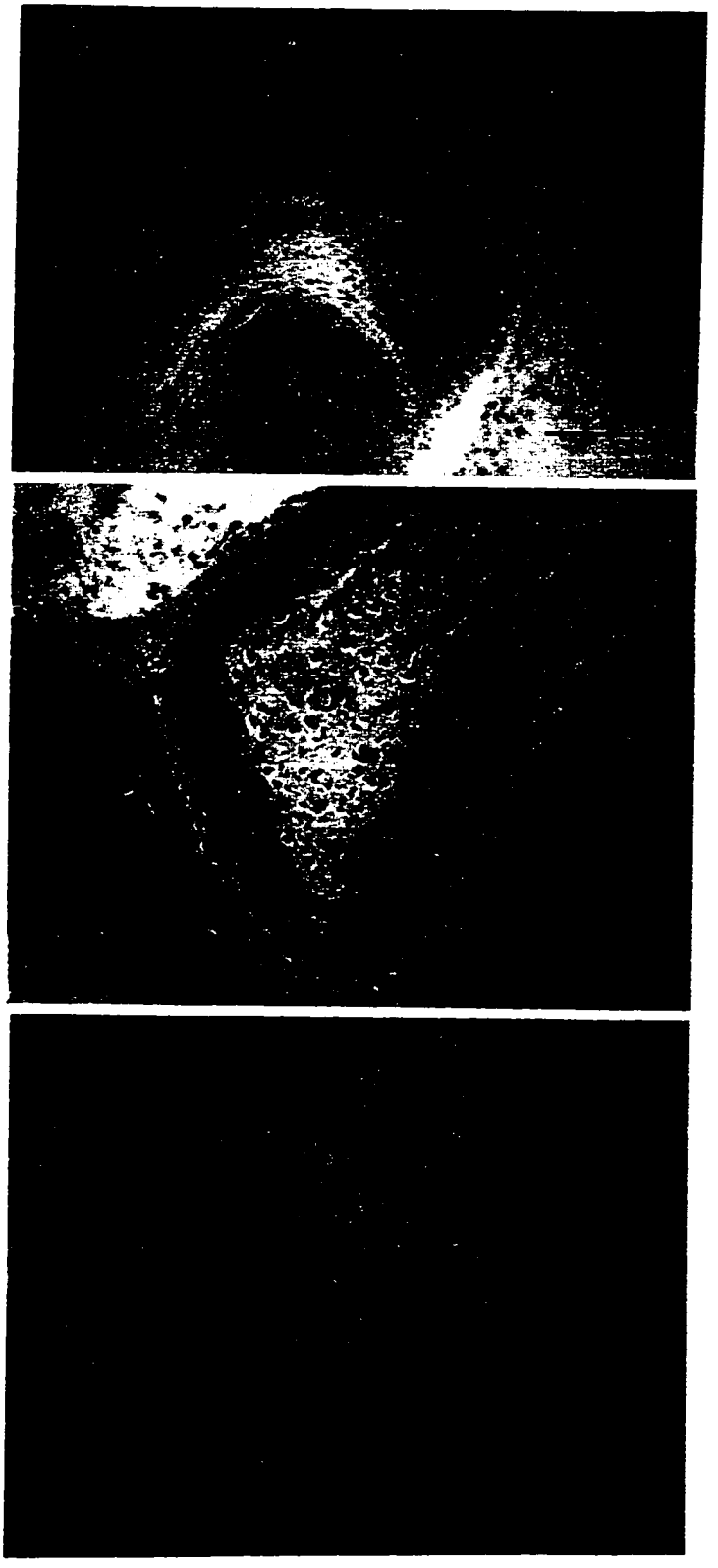


Figure 3

3B). Secondary cartilage was not found in the tip of hook where intramembranous bone formation continued (Fig. 3B). Because of this, the QJ hook grows in length from the tip at this stage. In embryos of day 14, secondary cartilage grew in both anterior and posterior sides of the hook.

In day 15 embryos, the hook had developed two pieces of cartilage with similar volume in the anterior and posterior sides (Fig. 3C). Overall, the hook was much enlarged due to secondary cartilage development. Most chondrocytes became hypertrophic morphologically at this stage. Hypertrophic chondrocytes are larger than chondroblasts or regular chondrocytes and have a round or oval shape. Meanwhile, around day 15, a new cell layer (NCL) formed between the existing secondary cartilage and the perichondrium. Cells in the NCL have some morphological features of chondroblasts, such as pericellular matrix staining with alcian blue, but are smaller than regular chondroblasts. Their alcian blue-positive ECM appears deteriorated. Such cells initially appeared at the dorsal edge of the cartilage, and then accumulated along the whole cartilage. In day 17 embryos, these cells covered the entire hook, including the tip, and separated both the preexisting cartilage from its perichondrium and bone from the periosteum at the tip (Fig. 4A, 4B).

As the NCL appeared, secondary chondrogenesis stopped at the hook. The already-formed secondary cartilage became hypertrophic and then most become an "intermediate tissue" [a tissue with features of both bone and cartilage, as described by Murray (1963) and Hall (1978)] (Fig. 4A). Meanwhile, blood vessels invaded the hook from the

Fig. 4. Development of secondary cartilage between day 17 and 19.

(A). QJ hook in day 17 embryo. Most secondary cartilage has become intermediate tissue (im) and the hook is undergoing endochondral ossification. The NCL is now more obvious and surrounds the entire hook (arrowheads).

(B). High magnification of (A). showing the new cell layer (ncl), intermediate tissue (im), and perichondrium (pc). Note that the NCL is located between intermediate tissue and perichondrium.

(C). QJ hook in day 19 embryo. The NCL resumed chondrogenesis as a second phase of secondary cartilage formation (arrowheads), which became articular cartilage.

Bar: (A, for A and C) 100 μm, (B) 10 μm.

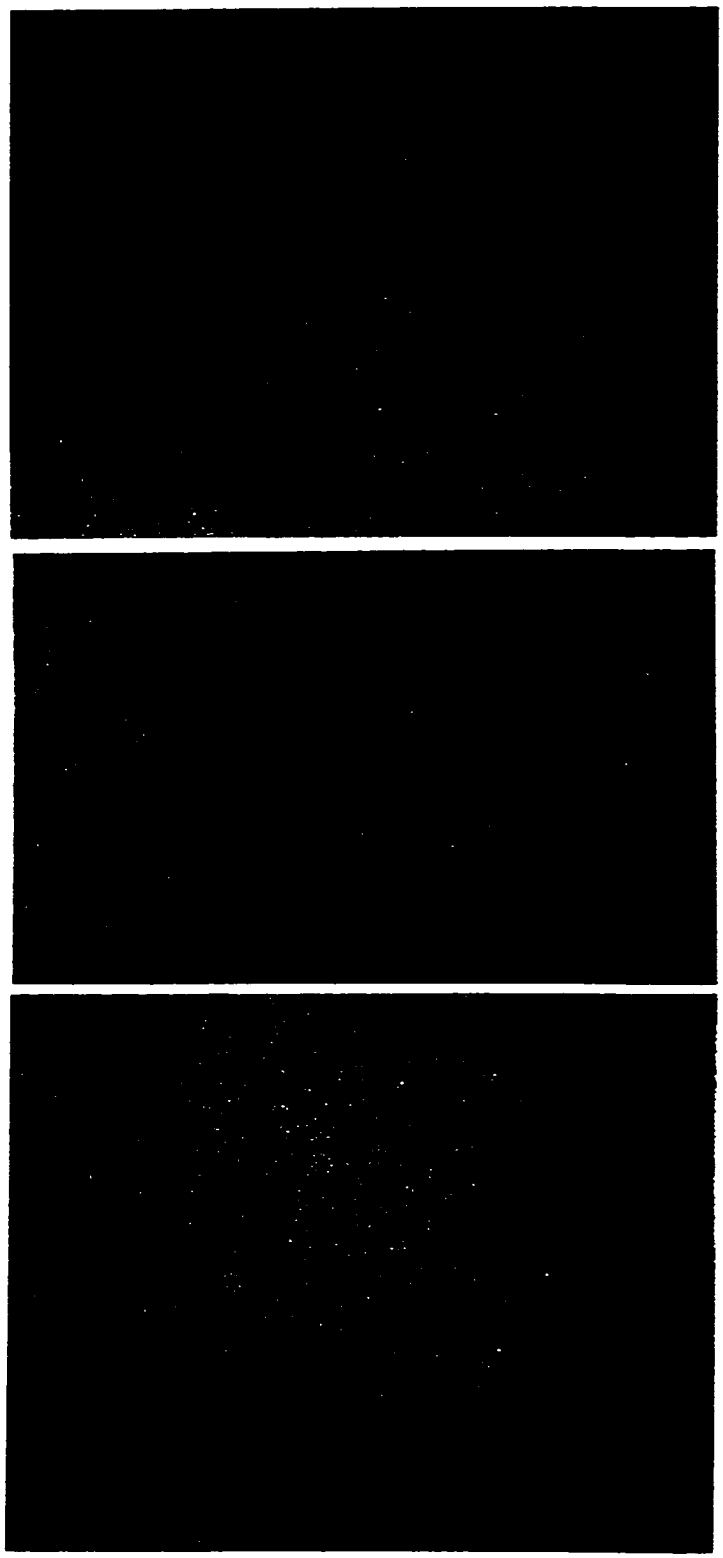


Figure 4

dorsal edge of the secondary cartilage, and multinucleated osteoclasts started to destroy the hypertrophic cartilage, intermediate tissue and bone in the center of the hook (Fig. 4A). The hook was at this stage undergoing endochondral ossification.

In day 19 embryos, most of the secondary cartilage in QJ hooks had been replaced by endochondral bone formation. In the middle of the hook, some intermediate tissue or cartilage remained and was being resorbed by osteoclasts with ensuing endochondral ossification. Meanwhile, some cells in the NCL exhibited the morphological features of chondroblasts and were forming cartilage again (Fig. 4C). This is a second phase of secondary chondrogenesis, which is not limited to the previous secondary cartilage region but from all the NCL covering the entire hook. In day 21 embryos, cartilage forming from the second phase of secondary chondrogenesis covered the entire QJ hook including the tip, which would serve as an articular cartilage for the quadratojugal-quadrate joint.

2.3.2. Inhibition of secondary chondrogenesis by paralysis

(A) Paralysis before secondary cartilage formation

In embryos paralyzed at day 9 and examined between days 10 to 14, no secondary cartilage was found in the QJ hooks. In the QJ hooks of day 13 embryos, when secondary cartilage can be found in normal mobile embryos, the bone was surrounded by periosteum and secondary cartilage was completely absent (Fig. 5A). Hence, secondary chondrogenesis was prevented by paralysis. Only in some specimens

Fig. 5. Absence of secondary cartilage in paralyzed embryos.

(A). The QJ hook of day 13 embryo paralyzed at day 9. This embryo was paralyzed before initiation of secondary cartilage formation. No secondary cartilage is seen in the hook.

(B). The QJ hook of day 19 embryo paralyzed in day 13. Secondary cartilage has completely transformed into bone. No second phase of secondary chondrogenesis occurs. The hook is smaller than in normal embryos.

Bar: (A) 30 μm, (B) 40 μm.

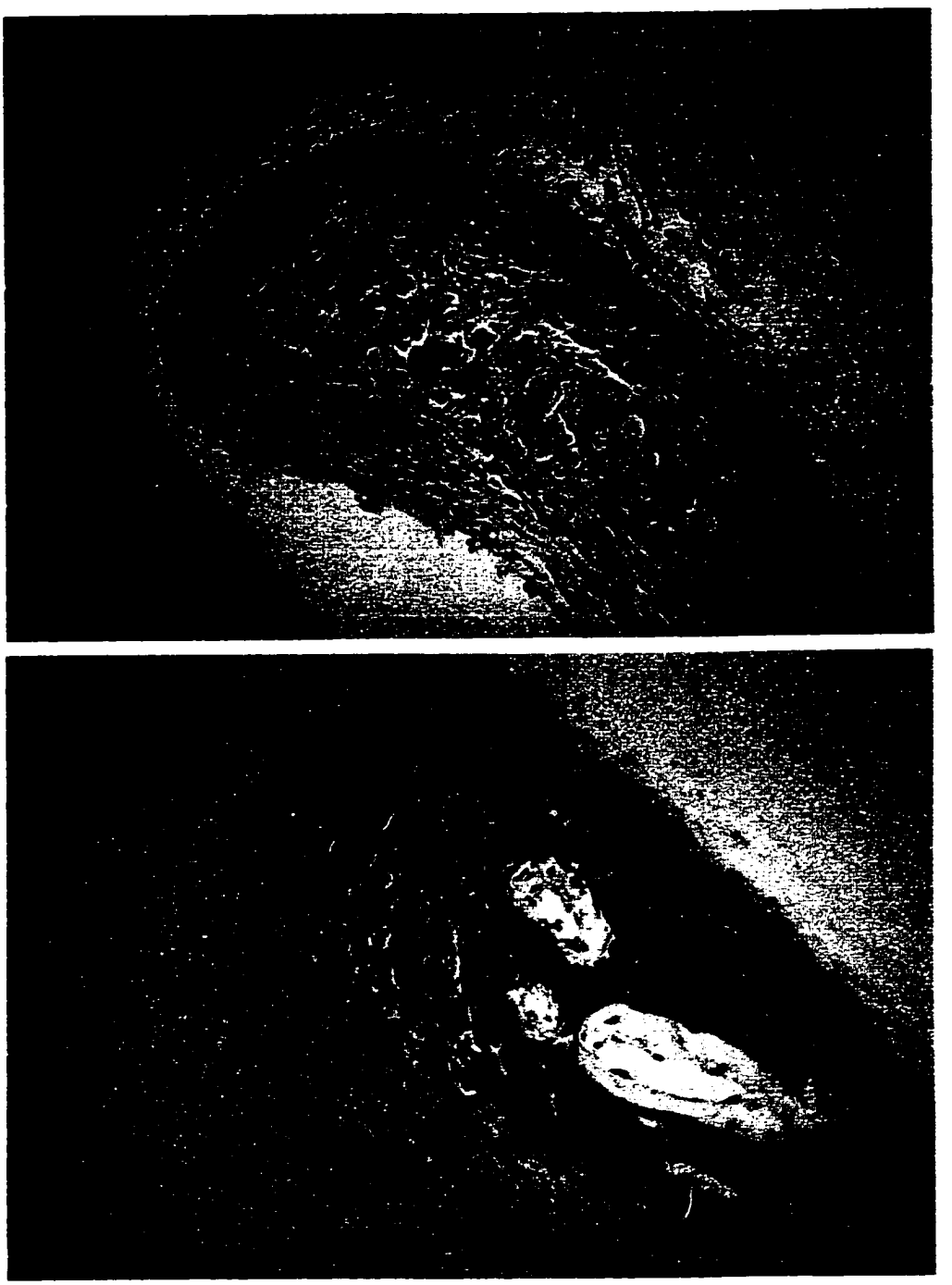


Figure 5

from embryos where paralysis was not complete (embryonic movement was still visible), was secondary cartilage seen in histology sections with HBQ staining.

(B). Paralysis after secondary cartilage formation

In embryos paralyzed at day 13 (i.e. after secondary chondrogenesis was initiated), cartilage developed in the QJ hook as in mobile embryos, though its volume could be smaller. The NCL layer also appeared at the hook between days 15 and 16. This cell layer has the same morphology as in normal embryos. The transformation from cartilage to bone also took place. In day 19 embryos, most secondary cartilage had transformed into bone in the paralyzed embryos. However, the NCL layer mentioned above was still distinguishable, and this cell layer did not resume chondrogenic differentiation to initiate the second phase of chondrogenesis seen in mobile embryos. As a consequence, the articular cartilage which covers the entire QJ hook was absent in embryos paralyzed in day 13 (Fig. 5B).

Development of secondary cartilage in QJ hook is summarized in Fig. 6.

2.3.3. Expression of growth factors in the cells of bone and secondary cartilage

Expression of five growth factors was studied both in bone and secondary cartilage in the QJs. To ensure that all cell types of secondary cartilage were present, the QJs were examined from day 13 embryos. With polyclonal antibodies against TGF-β, aFGF, bFGF, EGF,

Fig. 6. Illustration of morphological development of the QJ hook in control (normal embryos), paralysis 1 (paralyzed before cartilage formation), and paralysis 2 (paralyzed after cartilage formation).

Control. There is no morphological change between days 10 and 11. In days 12 to 14, secondary cartilage develops in the hook except in the tip where osteogenesis continues. At days 15 to 17, a new cell layer (NCL) appears between the cartilage and perichondrium. Chondrogenesis ceases and the already-formed cartilage becomes intermediate tissue (Im). By day 19, the NCL resumes chondrogenesis and new cartilage (C) forms at the surface of the hook. Intermediate tissue is being destroyed by endochondral ossification. Po, periosteum. Pc, perichondrium. B, bone. T, tip.

Paralysis 1. No cartilage is found in the hooks in the embryos of days 12 to 14. Paralysis completely prevents secondary cartilage formation in the hook.

Paralysis 2. From days 12 to 17, the hooks have the similar morphology as normal embryos. However, at day 19, the NCL still remains and there is no second phase of cartilage formation in the hook. Endochondral ossification in the hook is more rapid than in normal embryos.

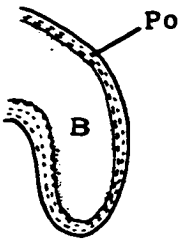
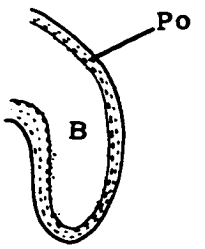
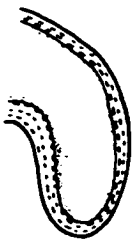
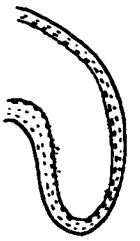
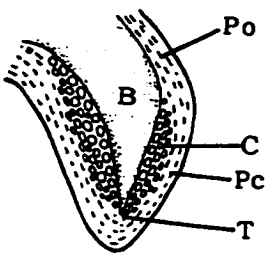
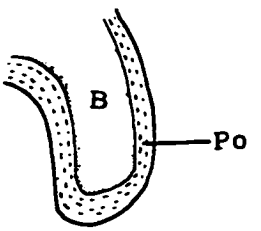
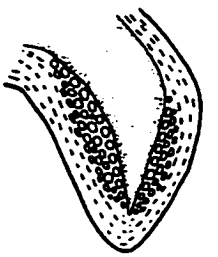
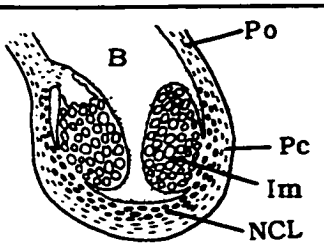
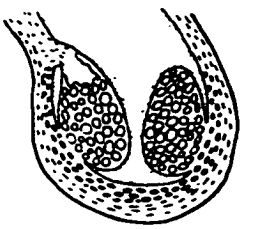
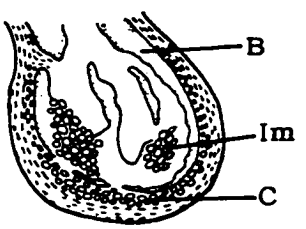
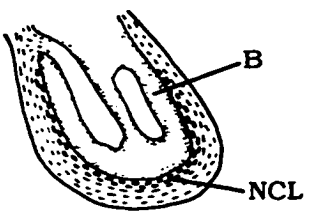
Days	Control	Paralysis 1	Paralysis 2
10			
11			
12-14			
15-17			
19			

Figure 6

and PDGF, the five growth factors were immunolocalized in the QJ hook and shaft with the ABC immunohistochemical technique. The results are summarized in Table 1. All five growth factors were expressed both in bone and in secondary cartilage. Distribution of individual growth factors varied with stage of differentiation in both bone and cartilage.

The polyclonal antibodies against pan-TGF-β recognizes all members of the TFG-β subfamily. The expression pattern of TGF-β was similar during osteogenesis and secondary chondrogenesis. It was most intensive in the outer fibrous layer of the periosteum and perichondrium (Fig. 7A, for general morphology of the QJ hook, see Fig. 3). It became negative in the progenitor cell layer of the periosteum and perichondrium. Then, TGF-β was weakly expressed in osteocytes and some chondrocytes (Fig. 7A).

aFGF was expressed during both osteogenesis and chondrogenesis (Table 1, Fig. 7B). In osteogenesis, aFGF was negative in the fibrous and progenitor cell layers. It was moderately expressed in preosteoblasts and became more intensive in osteoblasts. In the bone core, aFGF expression became weak in osteocytes; some osteocytes embedded in bony ECM were completely negative. In secondary chondrogenesis, aFGF was negative in the fibrous and progenitor cell layers. It was expressed in chondroblasts and had slightly more intensive staining in mature chondrocytes. bFGF was negative in ostoprogenitor and chondroprogenitor cells. It was expressed in chondroblasts and chondrocytes in secondary cartilage (Fig. 7C). In bone formation, bFGF was expressed in preosteoblasts,

Table 1. Summary of expression of five growth factors at various stages of cell differentiation in osteogenesis and secondary chondrogenesis in the QJ

Cell types	TGF-β	aFGF	bFGF	EGF	PDGF
Osteogenesis					
Fibrous layer	++	-	-	-	-
Progenitor cells	-	-	-	-	-
Preosteoblasts	+	++	+	-	-
Osteoblasts	+	+++	++	-	-
Osteocytes	+	+	+	+	+
Secondary chondrogenesis					
Fibrous layer	++	-	-	-	-
Progenitor cells	-	-	-	-	-
Young chondroblasts	-	+	+	-	-
Old chondroblasts	+	+	+	+	-
Chondrocytes	+	++	+	+	+

Notes: +: Positive, ++: stronger, +++: strongest, -: negative

Fig. 7. Expression of growth factors in QJs.

(A). Immunolocalization of TGF-β in the QJ hook of day 13 embryo.

(B). Immunolocalization of aFGF in the QJ hook of day 13 embryo.

(C). Immunolocalization of bFGF in the QJ hook of day 13 embryo.

(D). bFGF expression in periosteum of day 13 QJ shaft, showing both nuclear (arrows) and cytoplasmic localization of bFGF in osteoblasts.

b: bone; po: periosteum; s: secondary cartilage.

Bar: (A) 50 μm, (B, for B and C) 30 μm, (D) 10 μm.

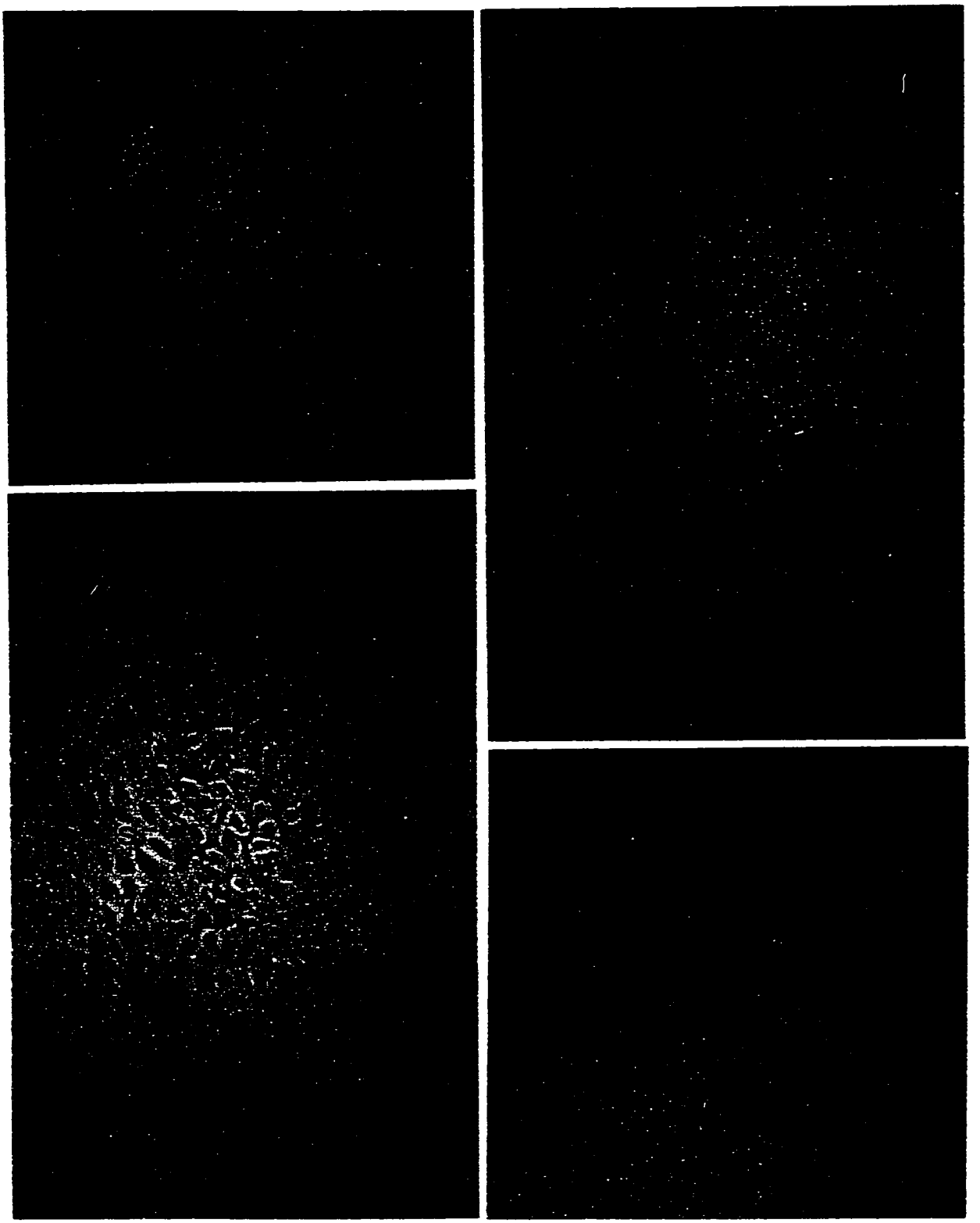


Figure 7

osteoblasts and osteocytes. In chondrogenic cells, both aFGF and bFGF were only localized in cytoplasm. However, aFGF and bFGF were localized in nuclei of osteogenic cells, including preosteoblasts, osteoblasts, and some osteocytes. Nuclear localization of bFGF was particularly obvious in some preosteoblasts and osteoblasts (Fig. 7D) and was not seen in chondroblasts and chondrocytes.

EGF expression was similar in both bone and secondary cartilage. It was negative in the periosteum and perichondrium, and become weakly-positive in osteocytes and chondrocytes (Table 1). PDGF was negative in most osteogenic and chondrogenic cell types, except for some weak expression in mature chondrocyte and osteocytes (Table 1).

2.4. Discussion

2.4.1. Initiation of secondary chondrogenesis

Secondary chondrogenesis was studied in histological sections. HBQ staining facilitates the discrimination of cartilage and bone in QJ hooks. Overall, the pattern of secondary chondrogenesis is consistent with previous descriptions (Murray, 1963; Hall, 1972). There is a slight difference in the timing of cartilage initiation compared with previous studies. In this study, secondary cartilage was visible in most embryos by day 12, while only a few embryos showed early cartilage features in the QJ hook by day 11. In contrast, previous reports showed that cartilage appeared by day 11 in most embryos. This delay is likely caused by slightly lower incubation temperature (36.5^{o} C) used in this study.

A major histological finding is that there are actually two phases of secondary cartilage formation in the QJ hook, separated by formation of a new cell layer between cartilage and perichondrium. That the NCL was not noticed by previous researchers is probably because it has some morphological features of chondroblasts. However, these cells are smaller than regular chondroblasts. More importantly, these cells express N-CAM (see next chapter for details), which excludes them from the chondrogenic cells. On the other hand, the morphology and location of NCL cells suggest their association with cells in the chondrogenic lineage. Accordingly, the NCL cells are probably dediffferentiated chondrogenic cells. It seems that the NCL stops further chondrogenesis and separates the already-formed

secondary cartilage from its perichondrium. The separation of secondary chondrogenesis by the NCL into two phases has physiological significance. The first phase forms two pieces of cartilage in the posterior and anterior sides of QJ hook. These cartilages hypertrophy and are subsequently replaced by bone through endochondral ossification, resulting in significant enlargement of the QJ hook. This process is very similar to endochondral bone formation in long bones. The second phase of secondary cartilage formation does not just simply resume chondrogenesis from the perichondrium, but it also includes cartilage formation at the tip of the hook where the first phase of second cartilage formation is not involved. As a result, the second phase gives rise to a layer of cartilage which covers the entire hook and serves as an articular cartilage. The articular cartilage exists in chicken until adults (Hall, 1968). Without the second phase, there will be no articular cartilage in the QJ hook, a situation seen in paralyzed embryos.

2.4.2. Effects of paralysis on secondary chondrogenesis

Immobilization of chick embryos was achieved by using *in ovo* injection of the paralysis drug decamethonium iodide. In embryos paralyzed at day 9 and examined at day 13, secondary cartilage was absent from the QJ hooks, confirming that secondary cartilage formation depends on biomechanical stimulation.

It is interesting that the secondary phase of secondary chondrogenesis is also dependent upon embryonic movement. In embryos paralyzed at day 13 and examined at day 17, the first phase of

secondary cartilage formation was not affected, but the second cartilage phase was absent. This result has two implications.

First, biomechanical signals are required only during initiation but not for the maintenance of chondrogenesis in QJ hooks. After initiation, secondary chondrogenesis can spontaneously occur without further stimulation. This is consistent with the conclusions drawn from QJ organ culture (Thorogood, 1979), but contrasts organ culture of secondary cartilage from mandibular condyles from newborn mice or human fetuses (Silbermann et al., 1983; Ben-Ami et al., 1993). In the latter case, progenitor cells switched from chondrogenesis to osteogenesis when the condyles were placed in culture. Since secondary cartilage studied in the condyle model was at a relatively late stage, its situation may differ from the QJ system.

Second, initiation of the second phase of cartilage formation also requires biomechanical stimulation. In the absence of embryonic movement, the NCL can not resume chondrogenesis resulting in absence of articular cartilage in the hook. It would be very interesting to see if the same molecular mechanism triggers the two phases of chondrogenesis. In addition, it is not clear whether there is a second phase of secondary chondrogenesis in mandibular condyles

2.4.3. Expression of growth factors on initiation of secondary chondrogenesis

In this study, five growth factors, TGF–β, aFGF, bFGF, EGF, and PDGF, were immunohistochemically studied during bone and secondary cartilage formation and during the switch from osteogenesis

to chondrogenesis in the QJ hook. All five growth factors were found in both bone and secondary cartilage. No transient expression pattern was found during the initial stage of chondrogenesis. There are some differences between bone and cartilage in expression patterns of the five growth factors; both aFGF and bFGF localize to nuclei in osteoblasts, but not in chondrogenic cells. However, overall, it seems that these growth factors are expressed and required by both osteogenic and chondrogenic cells. Hence, although growth factors may play important roles in chondrogenic differentiation in various systems (see Chapter 1), results from the present study fail to support involvement of those five factors in triggering secondary chondrogenesis in membrane bone. Those factors are probably important in maintaining normal proliferation and differentiation status for both bone and cartilage cells, but they are unlikely to be the factors evoking secondary chondrogenesis in the QJ hook.

Chapter 3

Differential Expression of N-CAM during Osteogenesis and Secondary Chondrogenesis in Quadratojugal Hook *in Vivo*

3.1. Introduction

Although osteogenesis and chondrogenesis in the QJ hook are precisely regulated by biomechanical factors that control the differentiation pathways of progenitor cells in the periosteum-perichondrium, how embryonic movement regulates the differentiation of skeletal progenitor cells is unknown (Hall, 1972, 1979; Hogg and Hosseini, 1992). Signal molecules are likely to be required to switch progenitor cells from osteogenesis to chondrogenesis. Embryonic movement may switch on (or off) the expression of signal molecules that regulate the pathway of progenitor cell differentiation. Such a switch would represent a commitment to secondary chondrogenesis.

As addressed in Chapter 1, several groups of molecules play regulatory roles in osteogenesis and chondrogenesis. In the previous chapter, expression of five growth factors was investigated in bone and cartilage formation and during the switch from osteogenesis to chondrogenesis in the QJ hook. However, data from immunolocalization failed to support the involvement of those factors in initiation of secondary chondrogenesis. Growth factors are important for differentiation and proliferation of both bone and

cartilage cells, but they seem to have no direct correlation with triggering secondary cartilage formation.

A second group of potent regulatory molecules are cell adhesion molecules (CAMs). Various CAMs have been identified in different tissues and shown to play important roles in cell differentiation, proliferation and morphogenesis (Takeichi, 1988; Linnemann and Bock, 1989; Edelman and Crossin, 1991; Edelman, 1992, 1993). More importantly, adhesion molecules may convert mechanical signals into a biochemical response in some non-skeletal cells (Ingber, 1991). As regarding skeletal tissues, neural cell adhesion molecule is particularly interesting. N-CAM was the first CAM identified (Jørgensen and Bock, 1974; Brackenbury et al., 1977; Thiery et al., 1977; Jørgensen et al., 1980; Hirn et al., 1981; Nobel et al., 1985), and is believed to play an important role in morphogenesis of the nervous system, skin, kidney, muscle and other systems (Linnemann and Bock, 1989; Crossin et al., 1985; Jiang and Chuong 1992; Knudsen et al., 1990; Soler and Knudsen, 1991). During chondrogenesis, N-CAM is expressed in precartilaginous condensations of mesenchyme and in the perichondrium, but is not expressed in chondroblasts and chondrocytes (Widelitz et al., 1993; Tavella et al., 1994). Moreover, *in vitro* assays showed that blocking N-CAM by antibodies partly inhibited chondrogenesis, while over-expression of N-CAM enhanced chondrogenesis in limb mesenchyme (Widelitz et al., 1993). Therefore, N-CAM is a possible candidate regulating periosteal cell differentiation and initiation of chondrogenesis.

N-CAM expression was examined during development of the QJ hook in normal and paralyzed embryos between 10 and 21 days of incubation, using a monoclonal antibody against all three isoforms of N-CAM. The results show that changing expression of N-CAM correlates with initiation of secondary chondrogenesis, with dedifferentiation of existing secondary cartilage, and with reinitiation of secondary chondrogenesis late in embryonic life. On this basis, it seems that N-CAM plays a role in regulating secondary chondrogenesis.

3.2. Materials and Methods

3.2.1. Chick embryos and tissue processing

Incubation and paralysis of chick embryos were as described in Chapter 2. The QJs were microdissected and processed for histology and immunohistochemistry following the protocols in Chapter 2.

3.2.2. Monoclonal antibody against chick N-CAM

A hybridoma cell line, 5e, was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University and Department of Biology, University of Iowa. This cell line produces mouse monoclonal antibody IgG1 which recognizes the extracellular domain of all three isoforms (120, 140 and 170 kD) of chick N-CAM polypeptides (Watanabe et al., 1986; Frelinger III and Rutishauser, 1986).

Frozen 5e cells were thawed in a 37° C water bath and suspended in 5 ml Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% L-glutamine (all from GIBCO, BRL, Burlington, ON), 1 mM sodium pyruvate (Sigma, St. Louis, MO). The cell suspension was centrifuged at 125g for 10 min. Then the cells were cultured in a 75 ml flask (Corning, Corning, NY) with 20 ml of DMEM medium. When cell density reached about 10^6 cells/ml, the hybridoma cells were transferred to 250 ml flasks (Corning, Corning, NY) and amplified through several subculture passages. The culture supernatant which contains antibodies was harvested at day 5 after

splitting without further medium change and centrifuged at 1000g at 4° C for 10 min to remove cell debris. Finally, the supernatant was filtered through a 0.25 μm filter and stored at -20° C or -70° C.

To purify monoclonal antibodies, immunoglobulin was first precipitated from the supernatant by addition of ammonium sulfate to 50% saturation (Harlow and Lane, 1988). Then IgG1 was further purified by protein-G affinity chromatography with a protein-G column based on the protocol provided by the manufacturer (Bio-Lab, Mississauga, ON). Purity of the IgG1 was determined by SDS-polyacrylamide gel and its concentration was measured with UV absorbance in a spectrophotometer at 280 nm (1 OD = approximately 0.75 mg/ml of purified antibodies). Purified IgG1 was stored at -20° C or -70° C without addition of sodium azide.

3.2.3. Immunohistochemistry

Immunostaining was carried out following the protocol in Chapter 2 in paraplast sections. Non-specific binding sites were blocked by normal goat serum. Supernatant or purified monoclonal antibody 5e was used as the primary antibody. The secondary antibody was biotin-conjugated goat anti-mouse IgG (GIBCO BRL, Burlington, ON). Adjacent sections to those processed for immunohistochemistry were stained with HBQ stain (Hall, 1986).

3.3. Results

3.3.1. N-CAM expression in QJ hooks of day 10 embryos

In normal day 10 embryos, no secondary cartilage was visible in sections of QJ hooks with HBQ staining (Fig. 8A). Immunohistochemistry revealed that N-CAM was expressed in the periosteum along the entire shaft and hook (Fig. 8B). N-CAM was localized on the cell surface of progenitor cells, preosteoblasts, and osteoblasts. Some young osteocytes were weakly N-CAM-positive in their cytoplasm, while old osteocytes were mostly negative. The pattern of N-CAM expression in embryos paralyzed in day 9 and examined in 10 day was similar to that seen in normal embryos. N-CAM was uniformly positive in the periostea in all specimens.

3.3.2. N-CAM expression in QJ hooks of day 11 embryos

In most specimens, initiation of secondary chondrogenesis was not found in the QJ hooks (Fig. 9A). However, N-CAM expression was down-regulated in the germinal layer of the hook. The timing of the switch from N-CAM-positive to negative differs slightly between the progenitor cells of anterior and posterior sides of the hook and between specimens. In the anterior side, most cells at the germinal layer of periostea had become N-CAM-negative in most specimens (Fig. 9B, arrowheads), while at the posterior side, only some progenitor cells appeared N-CAM-negative in most specimens (Fig. 9B, arrowheads). The tip of the hook remained N-CAM-positive (Fig. 9B) and osteogenesis was continuing from the tip.

Fig. 8. N-CAM expression in the quadratojugal of a normal day 10 embryo.

(A) A longitudinal section of the QJ, stained with HBQ to show the shaft (ST), hook (H), and tip (T). The periosteum surrounds the entire shaft and hook (arrowheads). No cartilage exists in the hook. Right is posterior, and left is anterior.

(B) N-CAM immunostaining of the section adjacent to that in (A). Note that N-CAM is localized on the cell surface of the osteoprogenitor cells of both the QJ shaft and hook (arrowheads) as well as of the osteoblasts in the inner side of the periosteum.

Bar: (A, B) 30 μm.

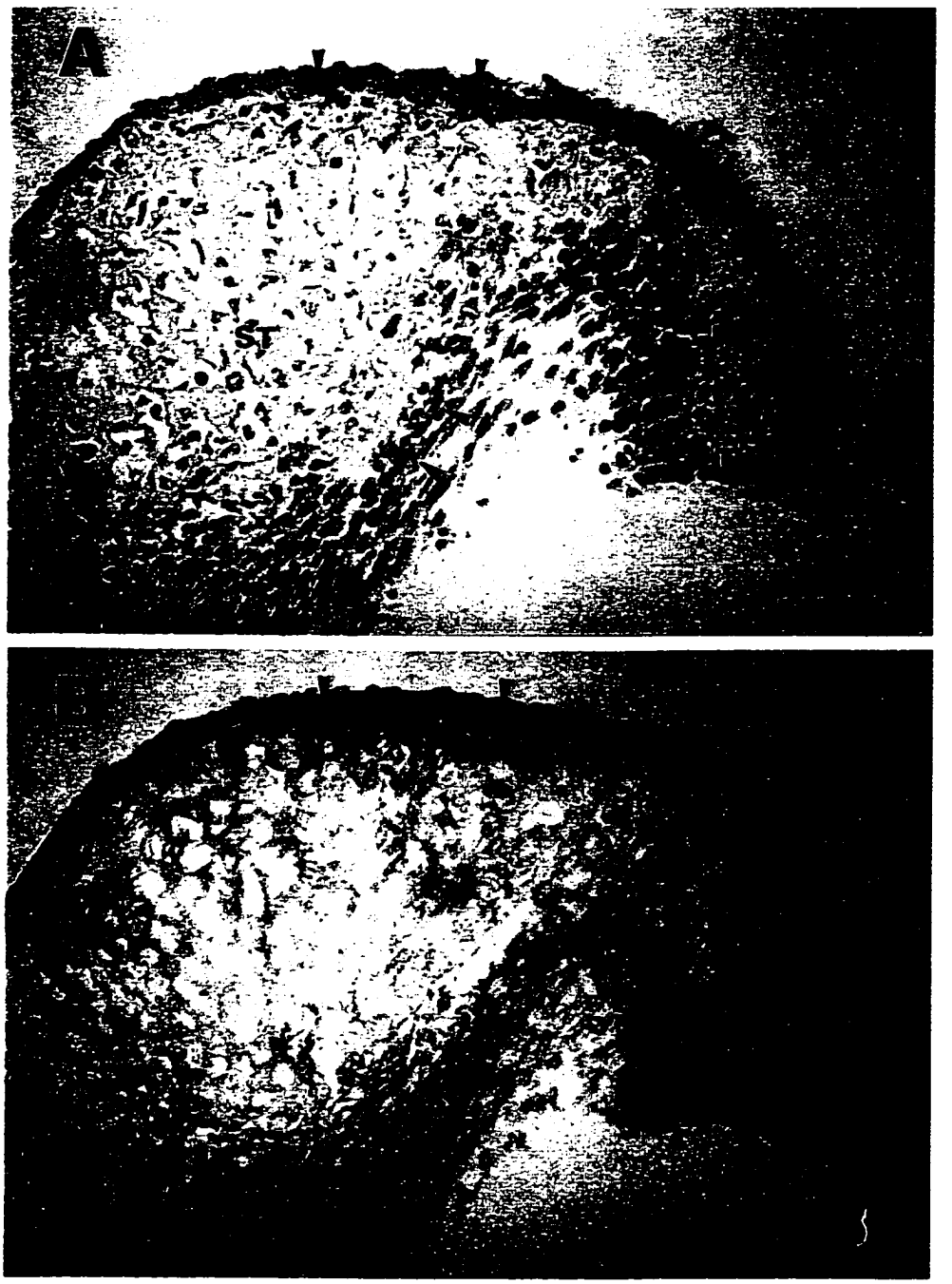


Figure 8

Fig. 9. N-CAM expression in QJ hook of a day 11 normal embryo and the embryo paralyzed in day 9.

(A, B). QJ hooks of a day 11 normal embryo. (A). HBQ staining. Bone core stained red. Secondary cartilage is not seen. Right is anterior. The cartilage (blue) surrounding the QJ hook is the quadrate cartilage (primary cartilage). **(B)** N-CAM immunostaining in the adjacent section to (A). Most of the progenitor cells at the anterior side of the hook and some at the posterior side have became N-CAM negative (arrowheads). The cells at the tip are still N-CAM positive.

(C, D) QJ hook of a day 11 embryo paralyzed at day 9. (C) HBQ staining. The bone core is surrounded by periosteum. **(D)** N-CAM immunostaining of the adjacent section to (C). Note that the progenitor cells in the hook are N-CAM-positive.

Bar: (A, for A, B, C, and D) 30 μm.

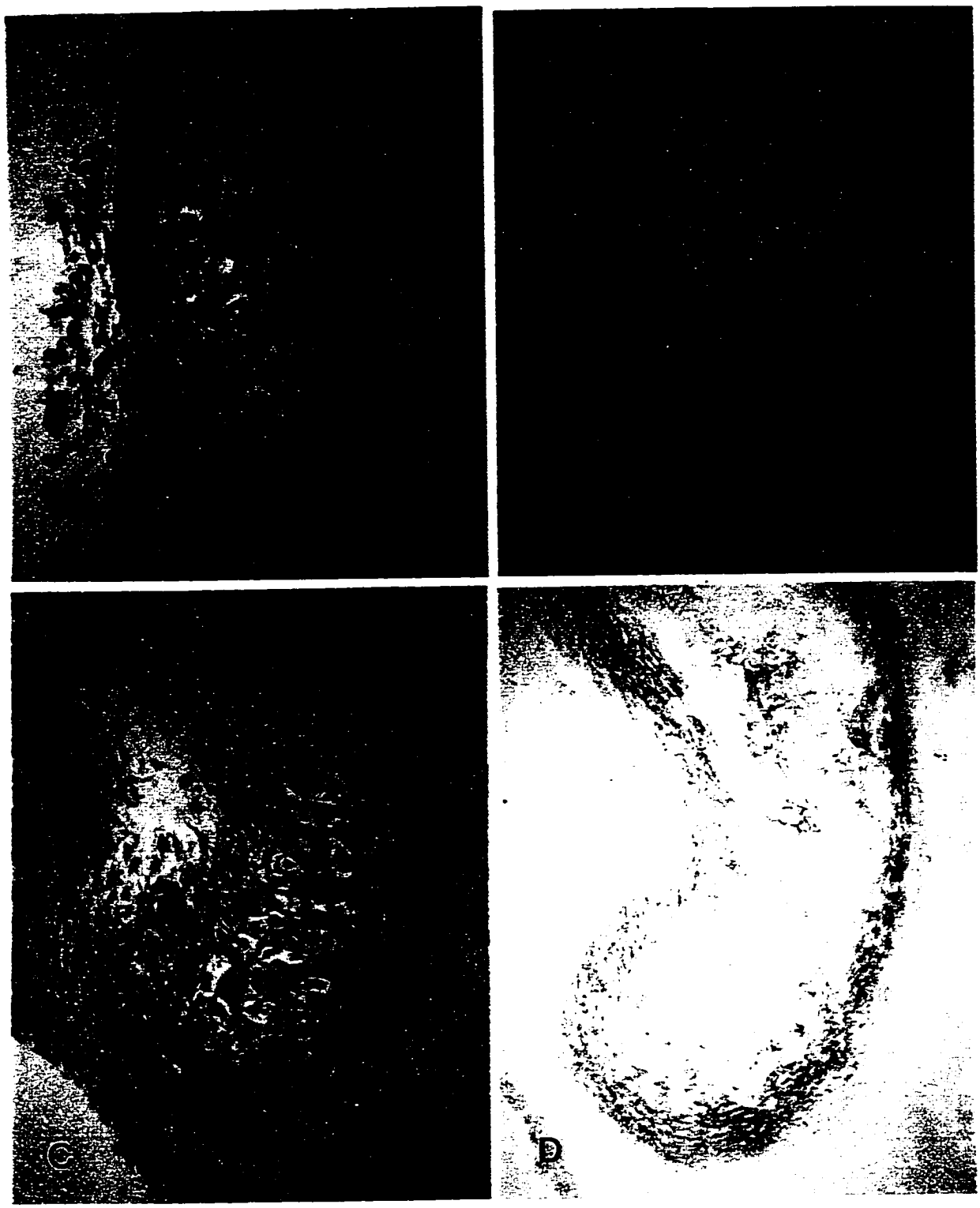


Figure 9

In embryos paralyzed at day 9 and examined at day 11, the hooks also consisted of a bony core and surrounding periostea (Fig. 9C). The overall morphology of QJ hooks from paralyzed embryos was very similar to those from normal embryos. However, N-CAM expression differed. N-CAM remained positive in the periostea surrounding the hooks of the QJs (Fig. 9D) and down-regulation of N-CAM (seen in normal embryos) did not occur in paralyzed embryos. Hence, paralysis prevented the switching off of N-CAM expression that would normally occur in the progenitor cells of mobile control embryos.

3.3.3. N-CAM expression in QJ hooks in embryos between days 12 and 14

In normal mobile embryos between days 12 and 14, N-CAM was negative in the progenitor cells of the hook except at the tip where osteogenesis was proceeding (Fig. 10A). The cartilage developed from the N-CAM-negative progenitor cells in what is now a perichondrium. All cell types in secondary cartilage — chondroprogenitor cells, chondroblasts, and chondrocytes — as well as their extracellular matrices, were N-CAM-negative. At the tip of the hook and along the shaft, osteogenesis still continued and N-CAM remained positive in the periosteum. Fig. 10B shows the pattern of N-CAM expression in the hook in a day 13 embryo.

As the QJ matured, two cell layers became distinguishable in the periosteum of the shaft — an inner, osteogenic cell layer, and an outer, fibrous cell layer. N-CAM was strongly positive only in the inner osteogenic cells and very weak or negative in the outer fibrous

Fig. 10. N-CAM expression in the QJ hook of a day 13 normal embryo and an embryo paralyzed in day 9.

(A, B) The QJ of day 13 normal embryo. (A) HBQ staining. Bone stained red. Secondary cartilage (s) has formed on anterior and posterior sides of the bone (b) at the hook. The progenitor cells of both sides are undergoing secondary chondrogenesis, while osteogenesis continues at the tip (asterisk) and the shaft. Right is anterior. **(B)** N-CAM immunostaining of the section adjacent to (A). Note that chondroprogenitor cells, chondroblasts and chondrocytes are N-CAM-negative (arrowheads), while osteoprogenitor cells in the QJ shaft (arrows) and the tip (asterisk) remain N-CAM-positive.

(C, D) The QJ of a day 13 embryo paralyzed at 9 days. (C) HBQ staining. The bony core is surrounded by periosteum and secondary cartilage is absent from the hook. **(D)** N-CAM Immunostaining of the adjacent section to (C). The hook is surrounded by N-CAM positive periosteum.

Bar: (A, for A, B, C, and D) 50 μm.

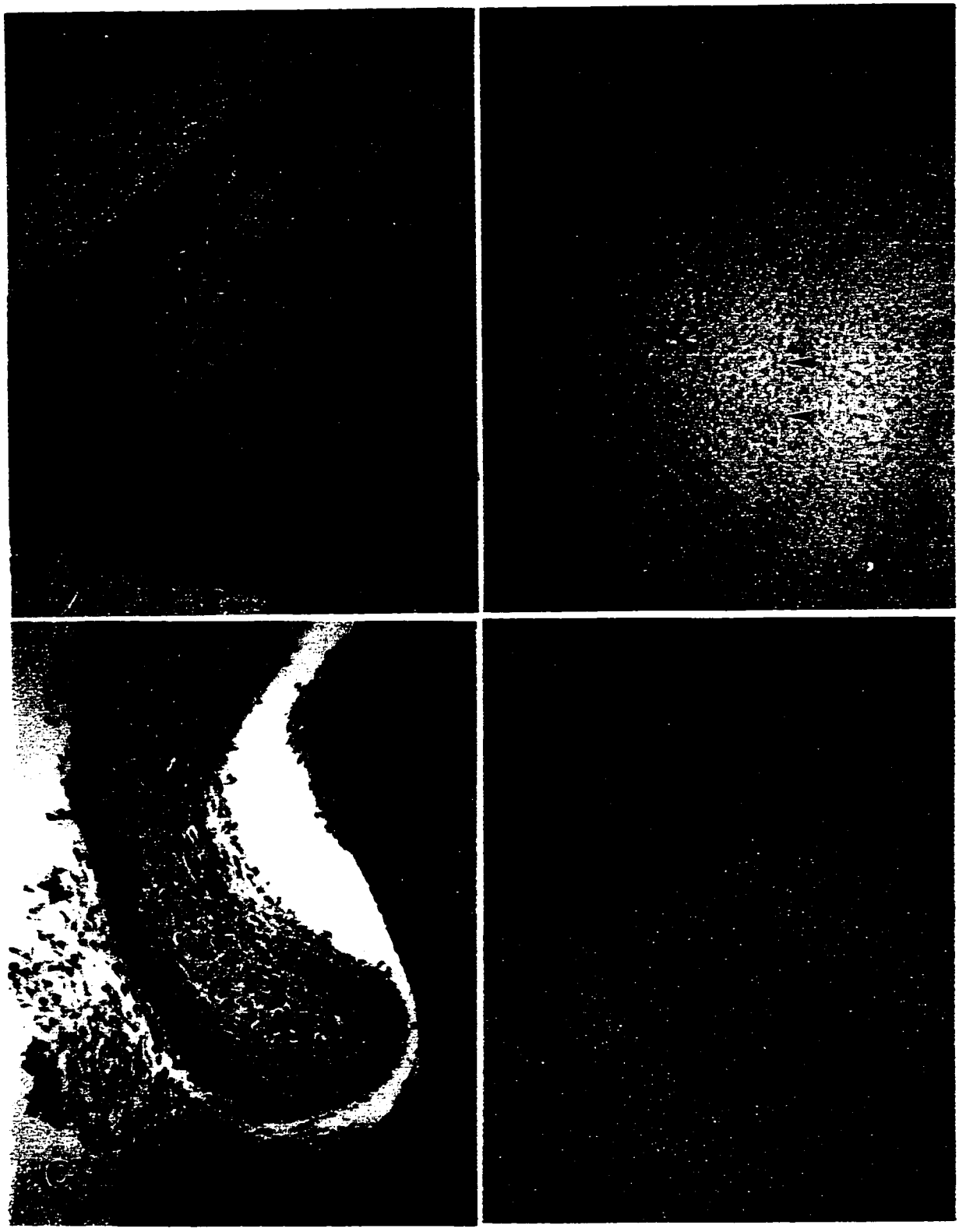


Figure 10

layer. As blood vessels extended into the bone in the shaft as well as into the bone core in the hook, endosteal ossification began. N-CAM was also expressed in osteoblasts at endosteal ossification sites.

In embryos paralyzed at days 9 and examined daily up to day 14, HBQ staining showed no secondary cartilage forming in the hooks (Fig. 10C). N-CAM was uniformly positive in the periosteum in the hook and along the shaft in paralyzed embryos examined between days 12 and 14 (Fig. 10D). Endosteal ossification can also be seen in paralyzed embryos as in normal embryos. Osteogenic cells at endosteal ossification sites were also N-CAM-positive (Fig. 10D), in both immobilized and normal control embryos.

3.3.4. N-CAM expression in QJ hooks in day 15 to 17 embryos

In this stage, the NCL formed between the existing cartilage and perichondrium. In the meantime, the secondary cartilage in the hook started to undergo endochondral bone formation (see Chapter 2). Immunostaining showed that the NCL cells were distinct from chondrogenic cells: they are N-CAM-positive (Fig. 11A, B). Thus, this cell layer became quite obvious at this stage since previously there were no N-CAM-positive cells between secondary cartilage and perichondrium. The N-CAM-positive NCL surrounded the hooks (Fig. 11B, arrowheads). Formed cartilage, perichondrium and "intermediate tissue" were all N-CAM-negative (Fig. 11B). Hypertrophic chondrocytes were also N-CAM-negative in the QJ hook. In the middle of hook, osteoclasts were destroying hypertrophic cartilage, intermediate tissue, and bone to remodel the hook through

Fig. 11. N-CAM expression in QJ hook of day 17-19 normal embryos and embryos paralyzed in day 13.

(A, B) The QJ hook of a day 17 normal embryo. (A) HBQ staining. This figure shows intermediate tissue (im), bone (b), NCL (arrowheads), and blood vessel invasion (arrows). **(B)** N-CAM immunostaining of the section adjacent to (A). Note that the new cell layer is N-CAM-positive (arrowheads).

(C, D) The QJ hook of a day 19 normal embryo. (C) HBQ staining. Most secondary cartilage has been destroyed by osteoclasts and endochondral osteogenesis is taking place in the hook (arrows). The NCL still exists (arrowheads), but in some area the cells have resumed chondrogenesis so that new cartilage (c) formation can be seen. **(D)** N-CAM immunostaining of the section adjacent to (C). The new cell layer has become N-CAM-negative (arrowheads). Cells in the areas of endochondral osteogenesis are N-CAM-positive (arrows).

(E, F) The QJ hook of a day 19 embryo paralyzed at 13 days. (E) HBQ staining. Show NCL (arrowheads), and bone core (b). No second phase of secondary chondrogenesis. **(F)** N-CAM immunostaining of the section adjacent to (E). Note that the new cell layer remains N-CAM-positive (arrowheads). N-CAM is also positive in endosteal sites (arrows).

Bar: (A, B, C, D, E, and F) 50 μm.

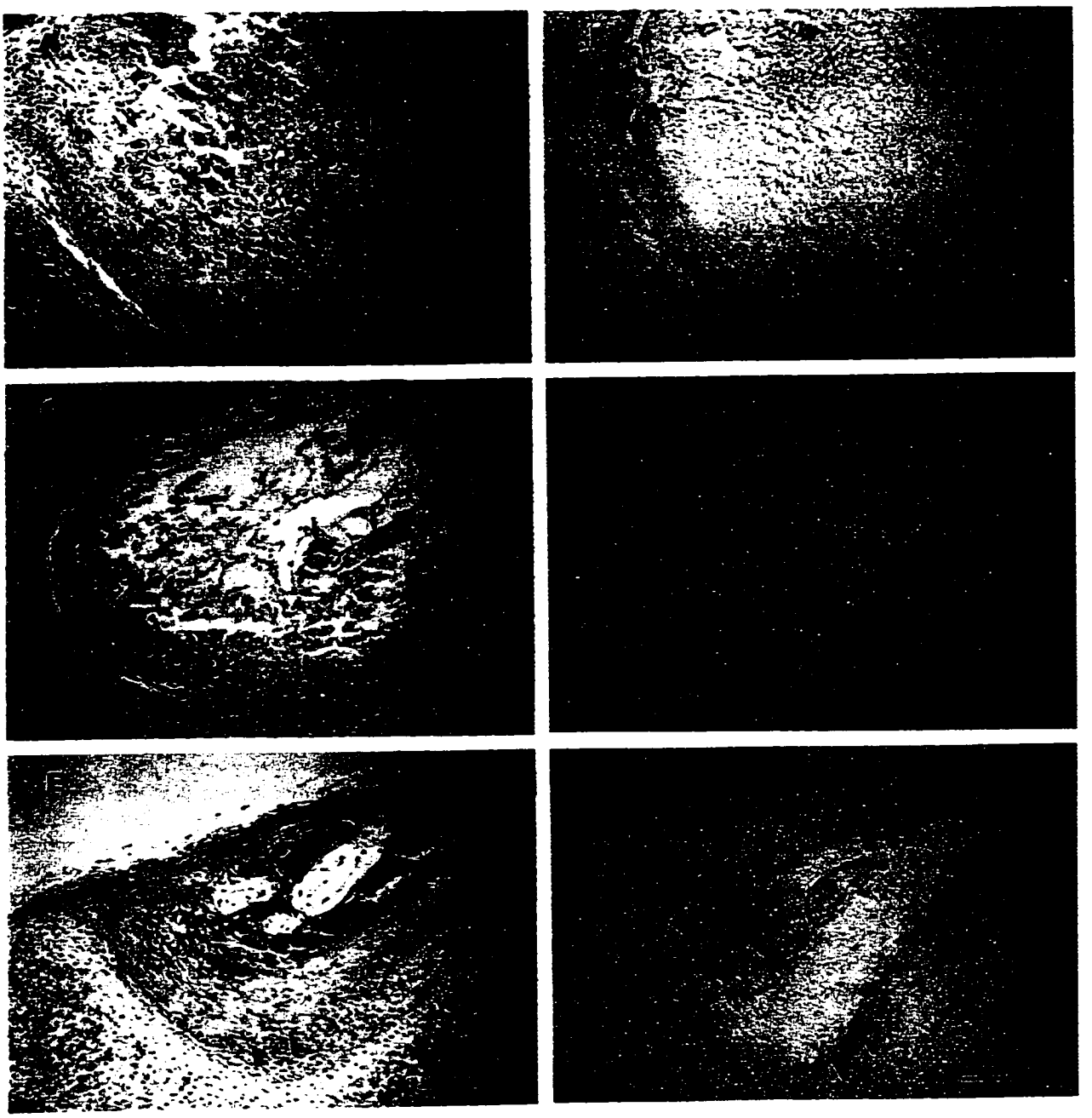


Figure 11

endochondral ossification. The osteoclasts were N-CAM negative, while the osteoblasts in ossification sites were N-CAM-positive.

In embryos paralyzed at day 13 (i.e., after secondary cartilage formation), the new cell layer also appeared at the hook and was N-CAM-positive (not shown), as in normal embryos.

3.3.5. N-CAM expression in the QJ hooks after day 19

In this stage, the NCL was still visible. However some NCL cells started to resume chondrogenesis to undergo the second phase of secondary chondrogenesis. This second phase cartilage become more obvious in day 21 embryos. Immunohistochemistry showed that the NCL had become N-CAM-negative in day 19 (Fig. 11C, D, arrowheads). Hence, N-CAM expression coincided with a temporary cessation of secondary cartilage formation in this cell layer. When this cell layer resumed cell differentiation, N-CAM expression was again lost. Moreover, down-regulation of N-CAM occurred earlier than the second phase of cartilage formation, as shown in Fig. 11 C, D, in which N-CAM had became negative in the entire NCL by day 19 but most cells had not yet resumed chondrogenesis. In areas undergoing endochondral osteogenesis, the N-CAM-positive osteoblasts (Fig. 11D, arrows) and N-CAM-negative osteoclasts still existed in the middle of the hook.

In embryos paralyzed at day 13 (i.e., after cartilage formation) and examined at day 19, the NCL was still distinguishable (Fig. 11E, arrowheads), but there was no sign of the second phase of chondrogenesis. Immunostaining showed that this cell layer remained

N-CAM-positive in embryos paralyzed at day 13 and fixed at day 19 (Fig. 11F, arrowheads).

3.3.6. N-CAM expression in the perichondrium of the quadrate

The perichondrium of the quadrate, a primary cartilage, is thicker than the perichondrium over secondary cartilage in the QJ. Both an inner, chondrogenic cell layer and an outer, fibrous cell layer were distinguished in the quadrate (Fig. 12A). The inner cell layer was N-CAM-negative (Fig. 12B), as was the perichondrium of secondary cartilage in the QJ. The outer fibroblast cell layer of the quadrate was weakly N-CAM-positive (Fig. 12B), although this N-CAM-positive layer was absent on the joint surface. Therefore, N-CAM expression patterns differ among the perichondrium of a primary cartilage which has negative chondrogenic and weakly positive fibroblastic layers, the perichondrium of secondary cartilage which has only a single negative layer, and the periosteum of bone, which has strongly positive osteogenic inner layer, and a negative fibroblastic outer layer (Table 2).

Fig. 12. N-CAM expression in the perichondrium of the quadrate, a primary cartilage.

(A) HBQ staining of the quadrate perichondrium. An outer fibroblast-like cell layer (OL) and an inner progenitor cell layer (IL) can be distinguished.

(B) N-CAM immunostaining of the adjacent section to (A). The outer fibroblastic cell layer (OL) of the perichondrium is N-CAM-positive, the inner progenitor cell layer (IL) is N-CAM-negative, as are chondroblasts and chondrocytes.

Bar: (A, for A and B) 10 μm.

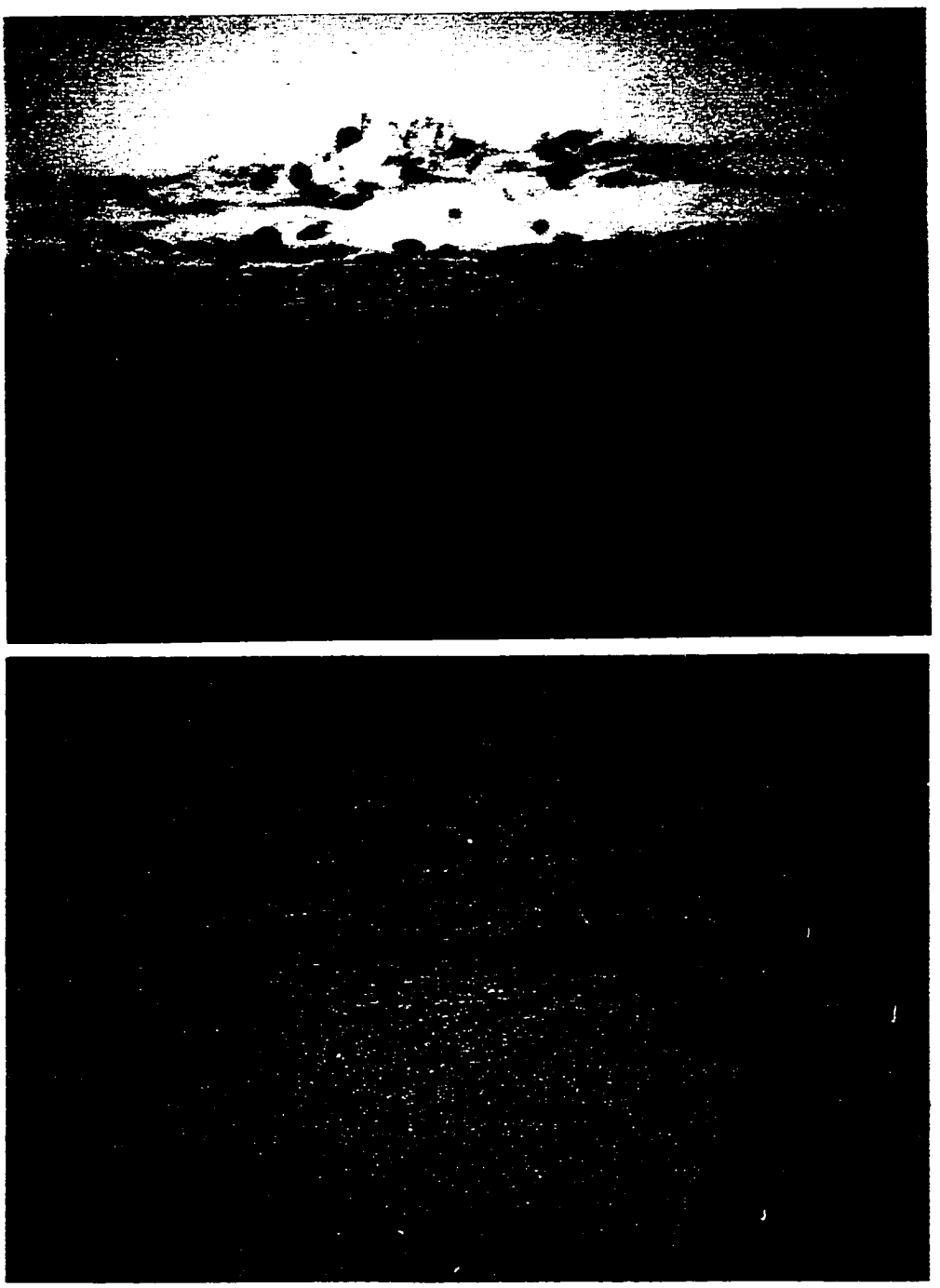


Figure 12

Table 2. N-CAM expression in quadrate (primary cartilage), quadratojugal hook (secondary cartilage), and quadratojugal shaft (membrane bone)

	Inner cambial layer	Outer fibrous layer
Quadrate	-	+
Quadratojugal hook	-	-
Quadratojugal shaft	+	-

Notes: +: N-CAM-positive, -: N-CAM-Negative.

3.4. Discussion

Using a monoclonal antibody against N-CAM, this study has demonstrated that N-CAM is transiently expressed during secondary cartilage development in QJ hooks of chick embryos. Before day 10, the progenitor cells in the periosteum express N-CAM and are undergoing osteogenesis. Around day 11, the progenitor cells become N-CAM-negative and switch from a bone- to a cartilage-formation pathway (although cartilage is not yet seen). N-CAM remains negative in all cell types during cartilage development in the hook between days 12 and 14. Then, an N-CAM-positive NCL accumulates beneath the perichondrium and chondrogenesis ceases in the hook. This cell layer ceases N-CAM expression in day 19 embryos and restarts differentiation toward chondrocytes as a second phase of cartilage formation, which is still ongoing when chicks are hatching.

N-CAM is expressed in osteoprogenitor cells, preosteoblasts, and osteoblasts during periosteal, endosteal and endochondral ossification. This result is consistent with a previous report of N-CAM expression in germinal cells and osteoblasts of tibia, vertebrae and calvaria (Lee and Chuong, 1992). The ABC method used in this study clearly revealed that N-CAM is localized on the cell surface of osteogenic cells. Young osteocytes have weakly-positive cytoplasm, while most osteocytes embedded in bony ECM are negative. On the other hand, N-CAM is not expressed in any cells associated with chondrogenic differentiation, including chondroprogenitor cells,

chondroblasts, and chondrocytes. Therefore, N-CAM expression is a feature of osteogenic but not chondrogenic cell lineages.

The physiological role of N-CAM in osteogenic cells is not clear. N-CAM is expressed in different situations of osteogenesis, including endochondral, intramembranous, and endosteal bone formation. This expression pattern suggests a requirement for this molecule in osteogenic cells. There are two possible roles for N-CAM in osteogenic cells. First, N-CAM is a factor required for osteogenic phenotypic expression. Since N-CAM is localized on cell surfaces and participates in gap junctional communication (Keane et al., 1988; Tavella et al., 1994), the homophilic interaction of N-CAM molecules between adjacent osteogenic cells may be important for osteogenesis. Second, N-CAM is most strongly expressed in activated osteoblasts and gradually disappears in osteocytes. This observation suggests that N-CAM probably participates in regulation of metabolism or secretion of bony matrix.

N-CAM expression switches from positive to negative in the progenitor cells of the QJ hook between 10 and 11 days. This change coincides with the timing of commitment for secondary chondrogenesis in the hook and is earlier than morphological initiation of chondrogenesis. Therefore, N-CAM expression seems to be an early marker of the switch from periosteum to perichondrium. Further, this transient expression of N-CAM in the progenitor cells is dependent on mechanical stimulation. In embryos paralyzed before the initiation of secondary chondrogenesis, the progenitor cells continue to express N-CAM and their differentiation pathway remains

osteogenic. Consequently chondrogenesis is completely prevented in paralyzed embryos. Therefore, N-CAM expression is influenced by embryonic movement, and may provide a molecular mechanism linking embryonic movement to the regulation of secondary chondrogenesis. It is not known whether N-CAM itself directly mediates the biomechanical signal: i.e. (1) embryonic movement —> N-CAM —> signal transduction —> secondary cartilage formation; or (2) embryonic movement —> other molecule(s) —> signal transduction —> N-CAM —> secondary cartilage formation. Interactions between cell and matrix have been suggested to be fundamental in transduction of biomechanical information into biochemical signals (Pavalko et al., 1991; Duncan and Turner, 1995). Given the fact that N-CAM contains extracellular domains capable of interacting with ECM components, such as type I collagen, and can influence intracellular signal transduction pathways, it is possible that N-CAM mediates the response to biomechanical stimulation. On the other hand, it is also possible that N-CAM cannot sense biochemical information but its expression is influenced by gene products in signal transduction pathways which respond to biomechanical forces.

Other interesting questions are whether N-CAM is a factor regulating chondrogenesis and what is the role of this molecule in determination of the switch from osteogenesis to chondrogenesis in the periosteum. As discussed before, a cascade of genes are likely to be turned on or off as progenitor cells change their pathway from osteogenesis to chondrogenesis in the QJ hook. Among them, one or a few genes may act as a switch regulating this change. Altering

expression of this switch gene should correlate with the timing of commitment for secondary chondrogenesis (between 10 and 11 days) and embryonic movement. Since the change of N-CAM expression in QJ hooks coincides with this commitment and requires embryonic movement, N-CAM satisfies some requirements for this switching molecule and, therefore, may play a role in controlling the alternative pathways of osteo- and chondrogenesis in the progenitor cells. Further investigation is needed to address this possibility.

It has been reported that over-expression of N-CAM enhanced chondrogenesis while exposure to N-CAM antibody partly inhibited chondrogenesis in limb bud mesenchymal cells *in vitro* (Widelitz et al., 1993; Chuong et al., 1993). N-CAM expression, therefore, is required for chondrogenesis in mesenchymal cells. However, my study demonstrates that N-CAM expression is present in osteogenesis but not in chondrogenesis in secondary cartilage formation, which seems to contradict the results gained from mesenchyme. It should be noted that specific roles of N-CAM in primary chondrogenesis from mesenchyme are not well understood. N-CAM is proposed i) as a molecule required for cellular condensation, and ii) as a mediating factor required for initiation of chondrogenic differentiation. In order to initiate chondrogenesis, mesenchymal cells have to undergo condensation (Hall and Miyake, 1992) and N-CAM is probably an important molecule which brings mesenchymal cells to condense (Widelitz et al., 1993). If N-CAM is needed merely for precartilaginous condensation, N-CAM would not be present in secondary chondrogenesis since cellular condensation is probably not a

prerequisite step in secondary chondrogenesis in membrane bone periosteum (see next chapter). From the present study, N-CAM seems not to be a signal required by chondrogenic differentiation due to the fact that N-CAM is down regulated during cartilage formation.

A surprising finding in this study is that the NCL cells, which were described in Chapter 2, express N-CAM. The NCL cells appear between the perichondrium and secondary cartilage. The position and morphology of this cell layer suggest an association with chondrogenic cells. However, since N-CAM is not expressed in any cells types in chondrogenesis, this cell layer apparently does not fit any cell types in cartilage. The only cell type in the chondrogenic lineage which express N-CAM are hypertrophic chondrocytes undergoing transdifferrentiation into osteogenic cell (Tavella et al., 1994). However, the NCL cells are not hypertrophic chondrocytes since they lack any morphological characteristics of hypertrophic chondrocytes. Moreover, the overall morphology of this cell layer suggests similarity to chondroblasts, except their proliferation, differentiation, and secretion seem to be arrested. Therefore, it is likely that this cell layer consists of dedifferentiated chondroblasts and it forms a barrier to chondrogenesis in the hook. This barrier has physiological significance: i) it prevents further chondrogenesis from perichondrium so that it separates two phases of secondary cartilage formation, ii) it serves as the border of already-formed secondary cartilage which will be transformed into bone through endochondral ossification, iii) later on it will reinitiate chondrogenesis to form the second phase of secondary cartilage formation. Hence, the NCL may

play an important role in the cessation and reinitiation of two phases of chondrogenesis and remodeling of the formed cartilage in the hook.

Expression of N-CAM in the NCL and its later disappearance would participate in this process. However, the exact role of N-CAM in NCL formation is unknown. N-CAM may prevent further differentiation and proliferation of chondroblasts and cause formation of the NCL. Furthermore, N-CAM expression ceases in the NCL when it restarts chondrogenesis in day 19 embryos. This switch from N-CAM-positive to negative is similar to the down-regulation of N-CAM during initiation of chondrogenesis in day 11 embryos and is also dependent on embryonic movement. This raised a further concern that N-CAM is probably an inhibitor to chondrogenic differentiation and the shut down of its gene expression is a prerequisite for chondrogenesis.

In summary, N-CAM is down-regulated during the switch from osteogenesis to chondrogenesis in the QJ periosteum. Whether N-CAM stimulates osteogenesis or inhibits chondrogenesis remains unclear. More investigation in cellular and molecular biology is necessary to further characterize the physiological role of N-CAM in skeletal cell differentiation. In addition, the *in vivo* model is not ideal for testing molecular mechanisms because cellular and molecular manipulations are difficult to conduct. An *in vitro* research model would provide major benefits for understanding N-CAM in osteo- and chondrogenesis.

Chapter 4

Differentiation Potential of the QJ Periosteal Cells *in Vitro*

4.1. Introduction

Chondrogenic potential is evidenced by secondary cartilage formation and other cartilage-associated phenomena in membrane bones (Fang and Hall, 1997). Secondary chondrogenesis represents a unique cell differentiation pathway in membrane bone, for cartilage arises from preexisting periosteum in response to biomechanical stimulation. Because chondroblasts of secondary cartilage arise from the germinal layer of the membrane bone periosteum, the periosteum is often stated to be bipotential for both osteogenesis and chondrogenesis (Hall, 1979; Ben-Ami et al., 1993). However, this statement sometimes causes confusion in interpreting the precursor for chondrogenesis. As addressed in Chapter 1, three possibilities exist in explaining the origin of chondrogenic cells in membrane bones (Fang and Hall, 1997). They include origins from unipotential progenitor cells, from bi- or multipotential progenitor cells, and from certain stages of osteogenic cells. Any of these origins could explain the bipotentiality of the periosteum.

It should be pointed out that all previous research on secondary cartilage was carried out either *in vivo* or in organ culture. In the past decades, organ culture studies on mandibular condyles and the QJs

have provided many insights into cell differentiation and gene expression during secondary cartilage formation (Hall, 1972; Thorogood, 1979; Hall, 1981; Strauss et al., 1990; Ben-Ami et al., 1993). However, further research has been limited because of the difficulty of cellular and molecular manipulation in organ culture. It is particularly true in attempts to manipulate cell differentiation with molecular approaches. For instance, in order to understand the physiological roles of a particular gene product in secondary chondrogenic differentiation, it is important to know how chondrogenesis is affected by enhancement or perturbation of gene expression *in vitro*. As addressed in the previous chapter, N-CAM is down-regulated during transformation of periosteum to perichondrium in the QJ hook, coincident with commitment of secondary chondrogenesis (Fang and Hall, 1995). This correlation implies a regulatory role for N-CAM in periosteal cell differentiation, but the exact mechanism still waits to be demonstrated.

In order to clarify the origin and regulation of chondrogenesis in membrane bone, a cell culture model is required. However, cell culture for secondary chondrogenesis has been difficult because of the small volume of the germinal cell layer and specific micro-environmental conditions required in membrane bone sites where secondary cartilage formation naturally occurs. An alternative means is to examine chondrogenesis in regular periosteal cells in membrane bones. As addressed in Chapter 1, chondrogenic potential widely exists in membrane bone and chondrogenesis can be evoked in response to certain microenvironmental condition. Thus, it should be

possible to achieve chondrogenesis *in vitro*. However, phenotypically fully-expressed chondrocytes are very difficult to achieve in monolayer culture of membrane bone cells. In fact, fully differentiated chondrocytes have never been achieved in primary monolayer culture of membrane bone periosteal cells. Calvarial cells are the most extensively studied cell culture model for skeletal cell biology. Although cartilage markers were expressed *in vitro*, overt chondrogenesis has never been obtained in monolayer culture. The only case where chondrocytes were observed in monolayer culture is a fractionated cell population of chick calvarial cells (Wong and Tuan, 1992, 1995), but unfractionated cells failed to produce chondrocytes in the same culture conditions. It seems that chondrogenesis is inhibited in cell culture by other differentiation pathways.

The QJ model has unique advantages for cell culture studies. The QJ is accessible and readily separated from other tissues in chick embryos. The QJ forms secondary cartilage *in vivo* in physiological conditions, while major portions of the calvaria do not. More importantly, chondrogenic capacity is not restricted to the QJ hook; chondrogenesis can occur along the entire shaft, which was demonstrated in organ cultures when intact QJs were submerged in culture medium (Thorogood, 1979). *In vivo*, chondrogenesis was also observed in QJ shafts during fracture healing (Hall and Jacobson, 1975).

In this chapter, I report several cell culture approaches that were employed to study differentiation of QJ periosteal cells enzymatically digested from their shafts. Results show that cell density

greatly affected the differentiation pathway of periosteal cells. In the high cell density of micromass cultures, periosteal cells undergo osteogenesis but not chondrogenesis. In monolayer culture at low density, periosteal cells develop into chondrocyte colonies with the characteristics of secondary cartilage cells *in vivo*. At moderate densities, the cells remain fibroblast-like, and neither osteogenic nor chondrogenic phenotypes occur. In order to analyze the lineage of chondrocytes, chondrogenic differentiation was further studied in clonal culture. Chondrogenesis was obtained in clonal culture. Data from both low density monolayer and clonal culture suggested that chondrogenic cells probably share a precursor with the osteogenic lineage.

4.2. Materials and Methods

4.2.1. Chick embryos and materials

Day 13 embryos were used for the experiments. Incubation of chick embryos is as described in Chapter 2. Monoclonal antibodies against chicken type II collagen (II-II6B3) and sheep type I procollagen (SP1.D8) were obtained from the Developmental Studies Hybridoma Bank. Biotinylated goat anti-mouse IgG, Ham's F-12 medium, BGJb medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin were obtained from GIBCO BRL (Burlington, ON). Collagenase type IA, bovine testicular hyaluronidase type VIII, low gelling temperature agarose (type VII), high gelling temperature agarose (type V), hydrogen peroxide (H_2O_2), BCIP (5-bromo-4-chloroidolyl phosphate), DMFA (N,N-dimethylformamide), NBT (nitroblue tetrazolium) were from Sigma (St. Louis, MO). Trypsin and ascorbic acid were purchased from BDH, Canada. Culture plates and dishes were from Fisher (Ottawa, Canada).

4.2.2. Dissections and enzymatic digestion of quadratojugal shafts

Quadratojugals were aseptically dissected from day 13 embryos with fine #5 forceps under a dissecting microscope. QJ hooks containing secondary cartilage were removed and the shafts pooled. After rinsing with phosphate buffered saline (PBS) three times to remove blood cells, QJ shafts underwent serial enzymatic digestion as in the sequential digestion of calvaria (Wong and Cohn, 1974). Shafts

from 2-4 dozen embryos were pooled and placed in 1.5 ml pH 7.2 PBS containing 200 units/ml collagenase type IA and 0.5% trypsin for consecutive periods of 30, 30 and 45 minutes with stirring in a vortex at room temperature. After each digestion, released cells were collected by centrifuging at 4000 rpm for 2 minutes in a microcentrifuge. Fresh enzyme solutions were added after each digestion. Cell populations 1, 2, and 3 were obtained from the 1st, 2nd, and 3rd digestions respectively. Each population was spun down and rinsed with Ham's F-12 + BGJb (3:1) containing 10% FBS to stop enzyme activity. Then the cell suspensions were spun down, resuspended, and passed through two layers of gauze to yield a single cell suspension. The suspensions were centrifuged again and suspended in 100 µl medium and cells were counted with a hemocytometer from 1 µl cell suspensions diluted to 20 µl with medium.

To monitor enzymatic digestion levels, the CJs were checked under an inverted microscope during each digestion. Generally, population 1 is mostly from the outer cell layer of the periosteum, population 2 from the intermediate layer, and population 3 from the inner layer. Because digestion rate varies among individual CJ and in different regions, each cell population may contain some fraction of cells from the other cell layers.

4.2.3. Micromass culture

After enzymatic digestion, each cell population was adjusted to a concentration of 2×10^7 cells per ml with culture medium. One drop

(10 µl, or 2 x 10^5 cells) of each cell suspension was placeed into a well of a 24-well culture plate. After 2 hours in a humidified CO_2 incubator to allow the cells to attach to the culture plate, the cells were flooded with 1 ml culture medium, which is Ham's F-12 + BGJb (3:1), containing 10% FBS, 1 mM L-glutamine, and 150 µg/ml L-ascorbic acid. Half the volume of the medium was replaced every 2-3 days. Morphology of the cultures was examined daily under an inverted microscope. After 14 days, the micromasses were peeled off the culture plates and processed for histology and immunohistochemistry.

4.2.4. Monolayer culture

Cell suspensions of populations 1, 2 and 3 were plated in 24 well culture plates at densities of 1 x 10^3, 2.5 x 10^3, 5 x 10^3, 1 x 10^4, 2.5 x 10^4, 5 x 10^4 and 1 x 10^5 per ml. Each well received 0.5 ml cell suspension. Culture medium is Ham's F-12 + BGJb (3:1), containing 10% FBS and 1 mM L-glutamine. In some cases, the medium was supplemented with 150 µg/ml ascorbic acid. The cultures were incubated at 37° C in a humidified CO_2 incubator with 5% CO_2 for up to 14 days; half the medium was replaced on days 6, 9 and 12. Cellular morphology in each culture was examined daily and photographs were taken with a Leitz phase contrast microscope.

To measure cell proliferation rates, each cell population was plated in a 24 well culture plate at 5 x 10^4 cells/ml in medium without ascorbic acid. Cultures were maintained for 12 days with medium changes at day 1, 3, 6, 9, and 11. Cells were trypsinized from 3 wells

and counted with a hemocytometer for each population at day 1, 3, 6, 9, and 12.

4.2.5. Clonal culture

To grow a single cell in a microwell in the 96-well plates, periosteal cells were collected from the second digestion and cells were counted following the procedure used for monolayer culture. the cell suspension was serially diluted to a final concentration of 20 cells/ml. Then, 100 µl of cells (which contains in average of 2 cells), was transferred into each well of 96-well culture plates. Then, another 100 µl of conditional culture medium was added to each well. The conditioned medium was collected from monolayer culture of the QJ periosteal cells and was filtered with a 0.25 µm Millipore filter. Culture medium was the same as in monolayer culture and did not contain ascorbic acid. After 2 hours, the culture was carefully observed under a Leitz inverted microscope and the wells containing a single cell were marked. The 96-well plates were incubated in 5% CO_2 and 37^{o} C for up to 14 days.

4.2.6. Suspension culture in agarose

To set up agarose cultures, three cell population were collected, rinsed, filtered, and counted as in monolayer culture. Cell suspensions were spun at 4000 rpm for 2 minutes with a microcentrifuge and resuspended in double strength (2 x) DMEM medium. Each population was adjusted to a concentration of 2×10^5 cells/ml with serum-free 2 x DMEM. Then, 0.5 ml of cell suspensions (2×10^5 cells/ml) from

each population was warmed at 37° C in a water bath and mixed with 0.5 ml of 1% low gelling temperature agarose prepared in sterile dH_2O, which was also warmed in a 37° C water bath, to yield a final concentration of 1 x 10^5 cells/ml. The cell-agarose mixtures were pipetted as 50 µl drops onto the surface of the 35 or 60 mm culture dishes, which were pre-coated with high gelling temperature agarose and stored at 4° C. The cultures were placed at 4° C immediately and left for 15 minutes to solidify in the agarose-cell drops. Then the agarose cultures were flooded with 2 ml complete medium to each 35 mm dish or 4 ml to each 60 mm dish. The complete medium contains 1 x DMEM with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cultures were incubated in a CO_2 incubator for 14 days and then fixed with 95% ethanol overnight. Chondrocyte colonies were determined by morphology and alcian blue staining at pH 1.

4.2.7. Histology and immunohistochemistry

Monolayer or clonal cultures were rinsed with cold PBS (pH 7.4) and fixed with 70% ethanol at 4° C for 30 minutes. Micromass cultures were peeled off the culture plates at day 14 and fixed with cold acetone at 4° C overnight. After dehydration and clearing, micromass cultures were embedded in low melting point paraplast at 52° C in a vacuum oven. Sections of 5 µm were prepared for histological and immunohistochemical analysis.

For assessing the expression of type I and II collagens, the avidin-biotin-peroxidase complex (ABC) immunohistochemical technique modified from Hsu et al. (1981) was used, as described in

Chapter 2. Both 70% ethanol-fixed monolayer cultures and cold acetone-fixed sections of micromass culture were pretreated with 5 mg/ml hyaluronidase in pH 7.2 PBS for 30 minutes at 37° C. Non-specific binding sites were blocked with 50% normal goat serum in PBS for 20 minutes at room temperature. After the serum was drained, specimens were incubated with anti-type I procollagen or type II collagen supernatant diluted 1:10 in PBS with 10% normal goat serum for 2 hours at room temperature or overnight at 4° C. The secondary antibody was biotin-conjugated goat anti-mouse IgG. Some sections from micromass cultures were stained with HBQ stain.

4.2.8. Histochemistry of alkaline phosphatase (APase)

To reveal alkaline phosphatase activity, ethanol-fixed monolayer cultures and acetone-fixed sections were rinsed with 0.1M Tris-HCl (pH 9.2) buffer and incubated for 25 minutes at room temperature in a matrix modified from Blake et al. (1984): 0.5 ml of 0.5% BCIP (5-bromo-4-chloroidolyl phosphate) dissolved in DMFA (N,N-dimethylformamide), 5 ml of 0.1% NBT (nitroblue tetrazolium) dissolved in pH 9.2, 0.1M Tris-HCl buffer, 44.5 ml of 0.1 M Tris-HCl (pH 9.2) buffer, and 50 µl of 2 M $MgCl_2$. The sections were dehydrated, mounted and examined in a Leitz microscope. Monolayer cultures were observed and photographed direct from culture plates in a Leitz inverted microscope with bright field illumination.

Double staining of type II collagen immunostaining and APase histochemistry were carried out on some monolayer cultures. Monolayer cultures were first treated for ABC immunostaining for type

II collagen. After rinsing with PBS, the samples were stained with the APase histochemical method. ABC and AP staining were easily distinguished by their brown and blue precipitates respectively.

4.2.9. Alcian blue staining

Monolayer cultures fixed with in 70% ethanol were rinsed in 3% glacial acetic acid (pH 1) and stained in 0.5% alcian blue in 3% glacial acetic acid (pH 1) for one hour. After rinsing in 3% glacial acetic acid and then PBS, the cultures were examined and photographed with an inverted microscope.

4.3. Results

4.3.1. Osteogenesis in micromass culture

To test the *in vitro* differentiation potential of periosteal cells enzymatically released from QJ periostea, the three cell populations were first tested in high density micromass culture. Ten microliters of cell suspension (2 x 10^5 cells) were plated per well. After 2 hours, cells attached and spread as a circular spot. After 24 hours, multiple-cell layers were visible in which cells gradually sorted out with polygonal cells at the top and fibroblast-like cells at the bottom. Cultures from population 2 and 3 had more polygonal cells, and cultures from population 1 more fibroblast-like cells. The general morphology of each population remained similar in the middle region of the micromasses throughout the 14 day culture period. Cells expanded at the edges and gradually covered the entire well.

To examine phenotypic expression, sections through the middle of the micromasses were prepared and stained with various methods. Generally, all three cell populations exhibited two cell types. One, located at the top, expressed APase (Fig. 13A). The upper portion of this APase-positive layer secretes bony extracellular matrix (ECM) that stained red by HBQ staining (Fig. 13B). The APase-positive layer is negative for both alcian blue staining and type II collagen immunostaining (Fig. 13C). These markers suggest that this cell layer is osteogenic.

The second cell layer is at the bottom of the cultures. It expresses neither APase (Fig. 13A) nor type II collagen (Fig. 13C), and

Fig. 13. Micromass culture of QJ periosteal cells. Paraplast sections were prepared through the middle region of day 14 cultures. Sections were stained with APase histochemistry (A), HBQ staining (B), or type II collagen immunostaining (C).

(A) APase expression in micromass culture. The top cell layer exhibits strong APase expression, and the bottom cell layer is negative. Broken line indicates the bottom edge of the micromass.

(B) HBQ staining. Upper region of the top cell layer is stained red; the bottom layer is fibroblast-like.

(C) Type II collagen immunostaining. No type II collagen is detectable in the micromass.

Bar: (A, for A, B, and C) 30 μm.

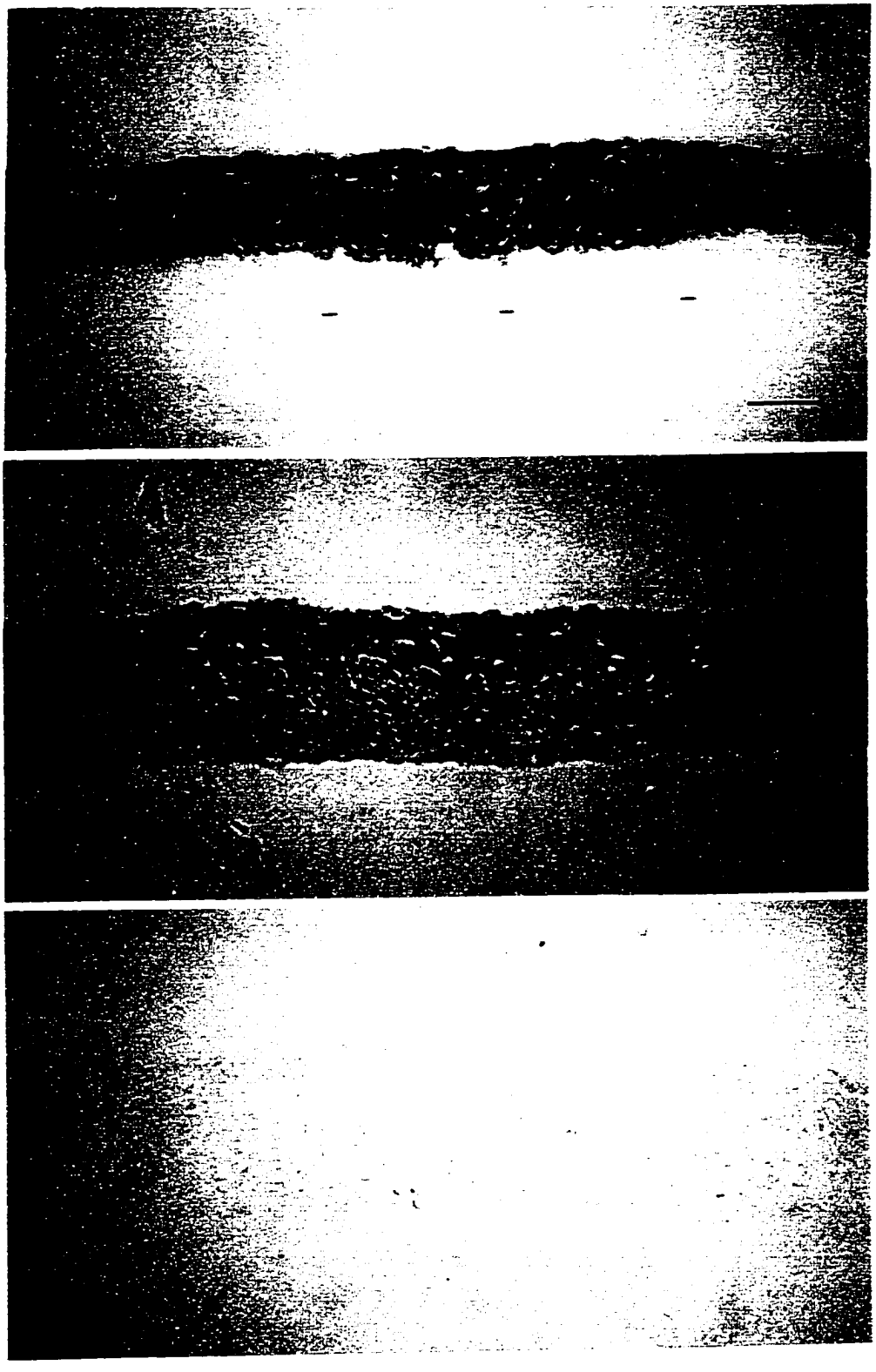


Figure 13

has an ECM that is not stained by alcian blue after HBQ staining (Fig.13B), indicating that these cells are neither differentiated osteogenic cells nor chondrogenic cells. The morphology of this cell layer is fibroblast-like.

Among the three populations, population 1 has a thick fibroblast-like cell layer and a thin bony cell layer, population 2 has both layers with similar thickness, and population 3 has a thick bony cell layer and a thin fibroblast-like cell layer. APase staining is intense in both population 1 and 2, and weak in population 3.

4.3.2. Chondrogenesis in monolayer culture

(A). Chondrogenesis in low cell density

To test chondrogenic potential in low cell densities, periosteal cells from the three populations were plated at densities between 1 x 10^3 and 1 x 10^5 cells/ml. Chondrogenesis occurred at low density (Fig. 14) for all three populations, but each had a different range of densities over which chondrocytes were elicited (Table 3). The density that allows for chondrogenesis is 1 x 10^3 to 2.5 x 10^3 for population 1, 1 x 10^3 to 5 x 10^3 for population 2, and 1 x 10^3 to 2.5 x 10^4 for population 3. When plated at these densities, periosteal cells divided very slowly and remained separated from each other during the first few days, when most cells were fibroblast-like and some were polygonal. Both gradually increased in number and either became small colonies or remained separated from each other. After 7-8 days, some round cells with pericellular refractile ECM appeared, often near

Fig. 14. Phase contrast photographs of periosteal cells in monolayer culture. Cells of population 2 were plated at low (5 x 10^3 cells/ml, A, B) and moderate (1 x 10^5 cells/ml, C) densities. The cultures were photographed at day 10 or 14.

(A) A small chondrocyte colony in a day 10 culture in low density, shows chondrocytes which have refractile ECM and a round shape.

(B) Chondrocytes in a large colony in a day 14 culture.

(c) No chondrocytes appear in a day 14 culture in moderate density. Cells have reached confluence and are fibroblast-like.

Bar: (A, for A and B) 40 μm, (C) 100 μm.

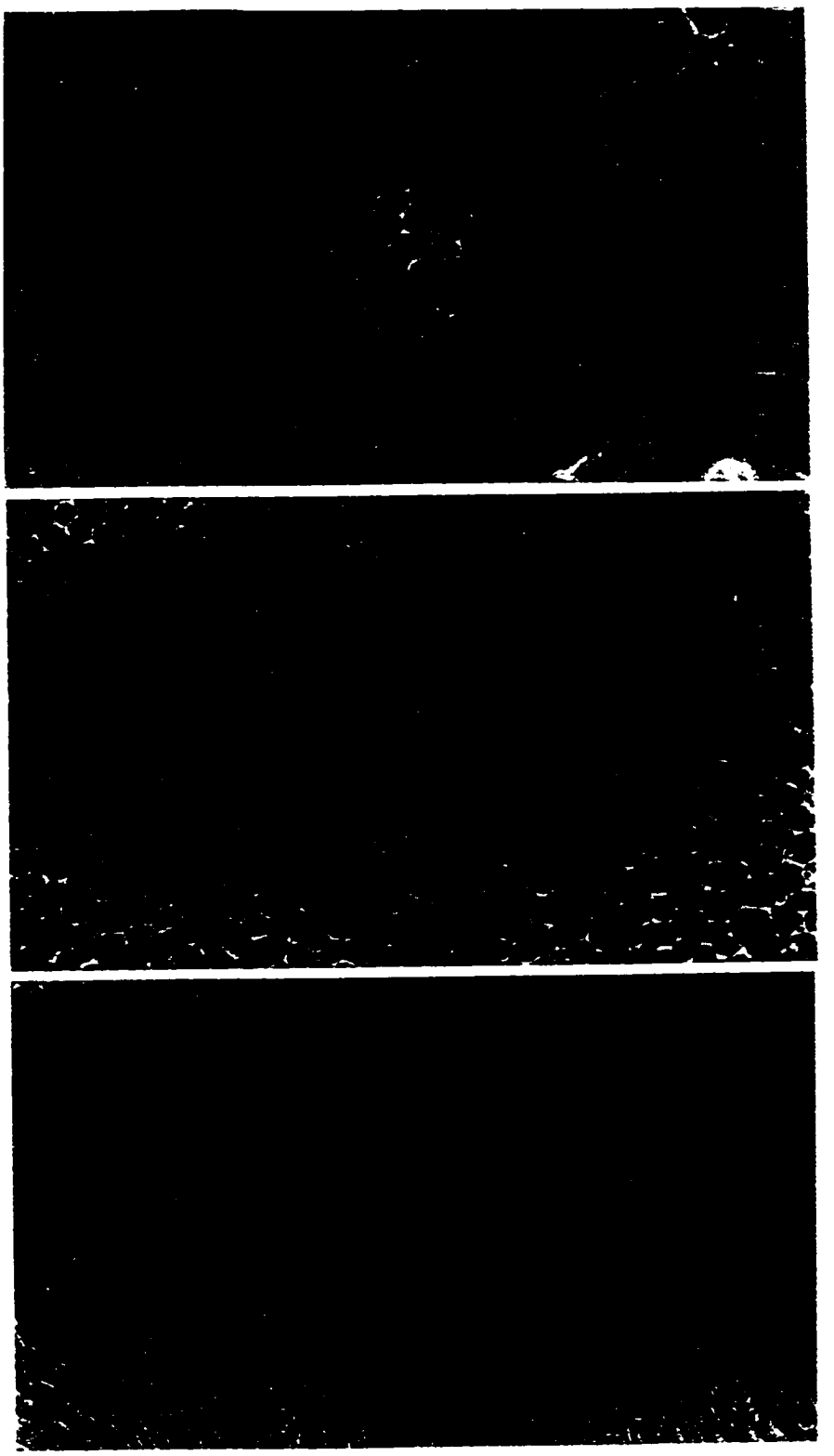


Figure 14

Table 3. Chondrocyte phenotypic expression in day 14 monolayer cultures of QJ periosteal cells in different cell densities (cells/ml)

	$1x10^3$	$2.5x10^3$	$5x10^3$	$1x10^4$	$2.5x10^4$	$5x10^4$	$1x10^5$
Pop. 1	4	4	3	0	0	0	0
Pop. 2	4	4	4	3	0	0	0
Pop. 3	4	4	4	4	4	2	0

Notes: Periosteal cells were enzymatically released from the periostea and three cell populations were plated at different densities as indicated. Each cell density was tested in 4 wells in 24 well culture plates. Each well received 0.5 ml cell suspension. Chondrocyte phenotype was identified according to type II collagen expression. **Pop. 1**: population 1, cells from the first digestion. **Pop. 2**: population 2, cells from the second digestion. **Pop. 3**: population 3, cells from the third digestion. Results are expressed as: **4**: chondrocytes appeared in all 4 wells. **3**: chondrocytes appeared in 3 out of 4 wells. **2**: chondrocytes appeared in 2 out of 4 wells. **0**: no chondrocyte colony formed.

or among polygonal cells. These cells divided rapidly and formed colonies by day 14 (Fig. 14A, B). Within a colony, the refractile cells might round up, become multilayered and detach from the colony.

The chondrocyte phenotype of these pericellular refractile cells was confirmed by immunostaining and histochemical methods. They expressed type II collagen (Fig. 15A) and the ECM stained with alcian blue (Fig. 15B). Polygonal with no pericellular refractile matrix and fibroblast-like cells did not. Because of the very low culture density, their development could be followed clonally in some cases. Chondrogenesis occurred in cultures with or without ascorbic acid.

At moderate density monolayer cultures, i.e. $>1 \times 10^4$ in population 1, $>2.5 \times 10^4$ in population 2, and $>1 \times 10^5$ in population 3, chondrogenesis failed to occur (Table 3). At these densities, the cultures had reached or were close to confluence by day 7-8. Before confluence, they contained fibroblast-like and polygonal cells. After confluence, fibroblast-like cells continued to divide rapidly, while polygonal cells gradually modified their morphology to become fibroblast-like so that no polygonal cells were seen under phase contrast microscopy in later stage cultures (Fig. 14C). No bone nodules were observed in day 14 cultures and thereafter.

Cell density is critical for chondrogenesis, which cannot be initiated at a local density higher than confluence. An important factor contributing to cell density was different growth rates in the three cell populations. Fig. 16 shows the growth curves of populations initially plated at 5×10^4. Growth is highest in population 1 and lowest in population 3.

Fig. 15. Chondrogenic phenotype of the QJ periosteal cells in low density monolayer culture. The periosteal cells were plated at $1x10^4$ cells/ml. The cells were cultured for 14 days in medium with supplement of ascorbic acid.

(A) Immunohistochemical staining of type II collagen. Type II collagen is localized in the cytoplasm and ECM of chondrocytes.

(B) Alcian blue staining, showing positive staining in ECM of chondrocytes.

Bar: (A, for A and B) 40μm.

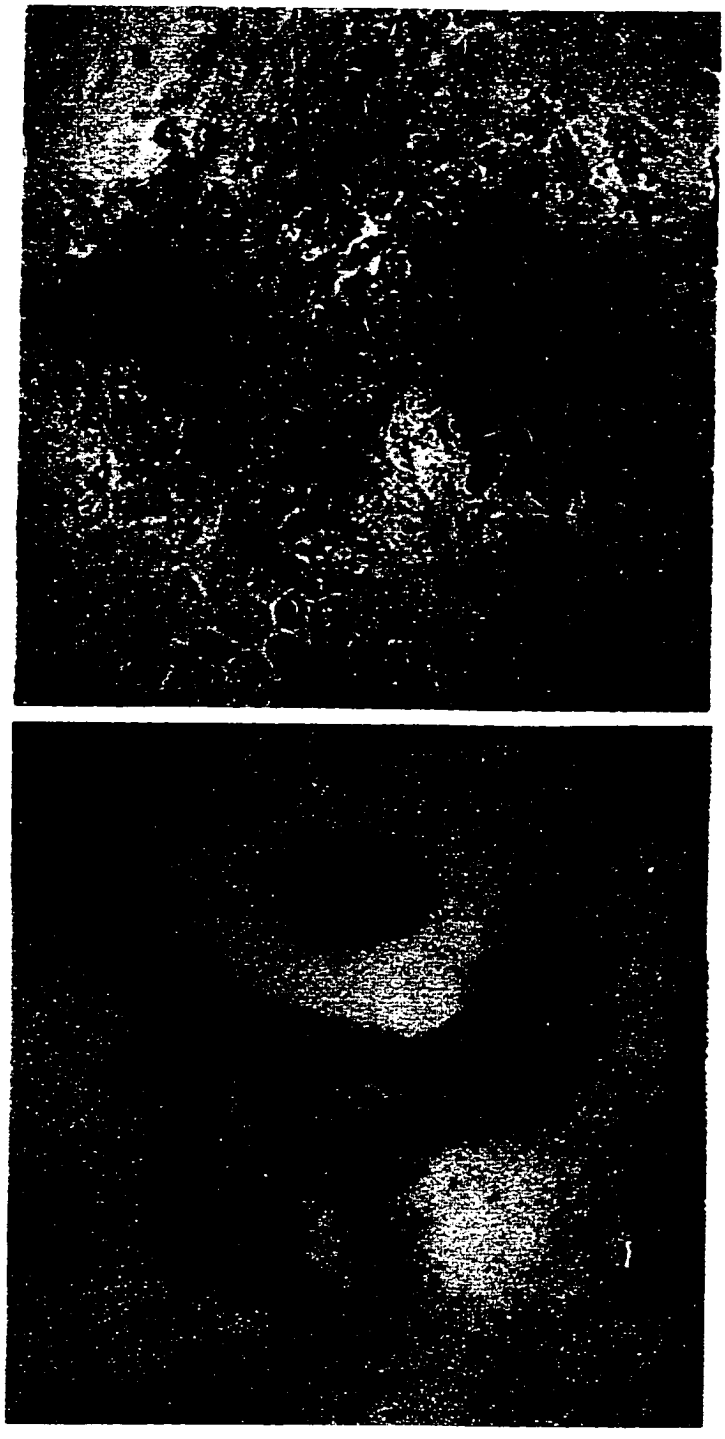


Figure 15

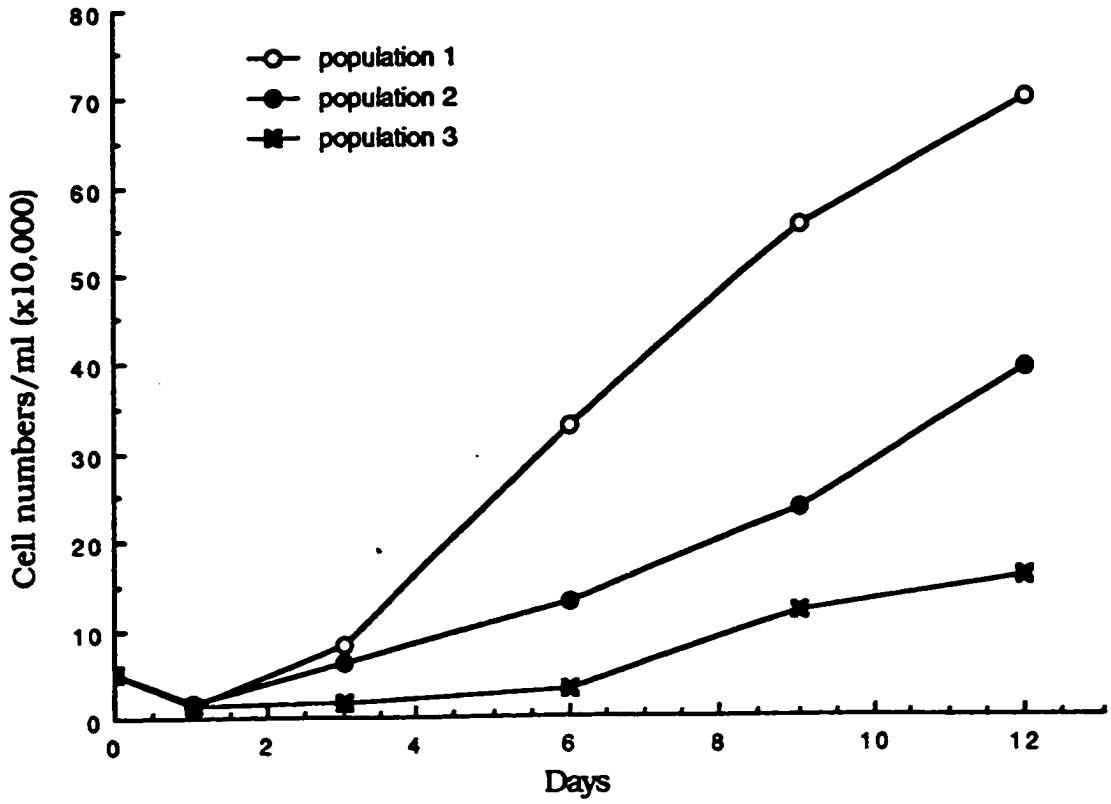


Figure 16

Fig. 16. Growth curves of three cell populations. Periosteal cells were enzymatically released from the QJ shafts and plated at 5 x 10^4 cell/ml. Cell numbers were counted at day 1, 3, 6, 9, and 12.

Relative potential for chondrogenesis in the three cell populations was evaluated by counting chondrocyte colony number from 4 wells in day 14 cultures initially plated at 1 x 10^3. Of 500 cells plated in each well, about 125 would survive according to the growth curves in Fig. 16. Population 2 had the highest chondrogenic potential and developed 40 colonies from 4 culture wells. Populations 1 and 3 had a relatively low chondrogenic potential (Fig. 17).

(B).Type II collagen expression and alcian blue staining

To show chondrocyte phenotype, type II collagen expression was revealed with immunostaining. In cultures supplemented with ascorbic acid, type II collagen was localized in both cytoplasm and ECM of the refractile cells (see Fig. 15A), while in cultures without ascorbic acid type II collagen was located only in the cytoplasm (see Fig. 18B, D). These refractile cells had alcian blue positive ECM (Fig. 15B). However, more cells were collagen type II positive than were alcian blue positive. It seems that alcian blue was seen only in relatively mature chondrocytes. In contrast, polygonal cells with no pericellular refractile matrix and fibroblast-like cells did not express type II collagen or show alcian blue staining.

(C). Alkaline phosphatase expression

In low density cultures, only a few polygonal cells appeared APase-positive at day 6. In cultures after days 7-8, APase-positive polygonal cells increased in numbers. However, they rarely formed purely polygonal cell colonies. In fact, many APase-positive

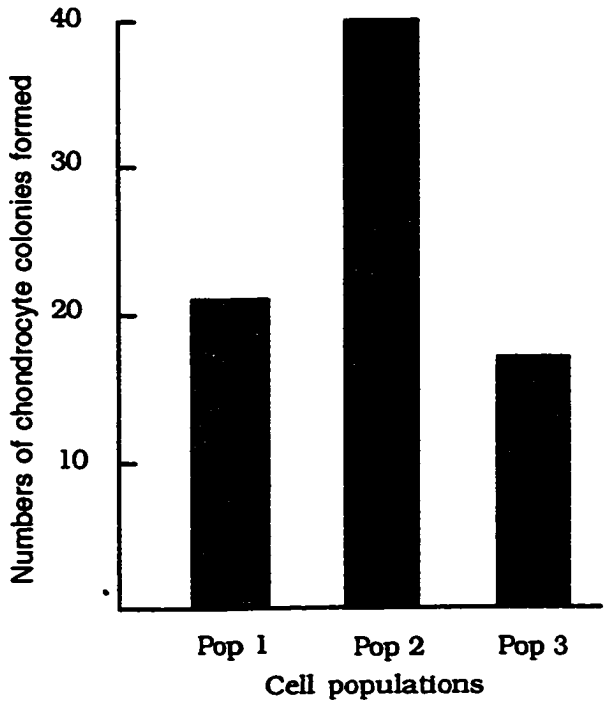


Figure 17

Fig. 17. Numbers of chondrocyte colonies formed in day 14 monolayer cultures of the three cell populations. The periosteal cells were plated at 1 x 10^3 cells/ml. Each well received 0.5 ml cell suspension (500 cells) in which about 25% (125 cells) survived. Chondrocyte colonies were identified on the basis of type II collagen immunostaining. Numbers represent the total colonies in 4 wells in 24-well culture plates for each cell population.

Fig. 18. Collagen and APase expression in periosteal cells in monolayer culture.

(A) Type II and APase-double staining in an early chondrocyte colony in a day 8 culture at low density (1 x 10^3 cells/ml). Four kinds of cells can be seen: APase negative polygonal cells (1), APase-positive polygonal cells (2), APase/type II collagen double-positive chondrocytes (3), type II collagen-positive chondrocytes (4).

(B) The same field as in (A), processed only for type II collagen immunostaining. Type II collagen is expressed in pericellular refractile cells but not in polygonal cells.

(C) Type II collagen and APase double-staining in a chondrocyte colony in a day 10 culture of low density. APase expression appeared in many chondrocytes, including small chondrocytes. As in (A), the four kinds of cells (1-4) can be seen.

(D) The same field as in (C), processed only for type II collagen expression.

(E) Type II collagen and APase-double staining in a day 14 culture in moderate density (1 x 10^5 cells/ml). All cells are fibroblast-like. Neither APase nor type II collagen expression can be seen.

(F) Type I collagen expression in fibroblast-like cells (f), polygonal cell (p), and chondrocytes (c) in a day 14 culture of low density (1 x 10^3 cells/ml).

Bar: (A, for A, B, C, and D) 40 μm, (E, for E and F) 100 μm.

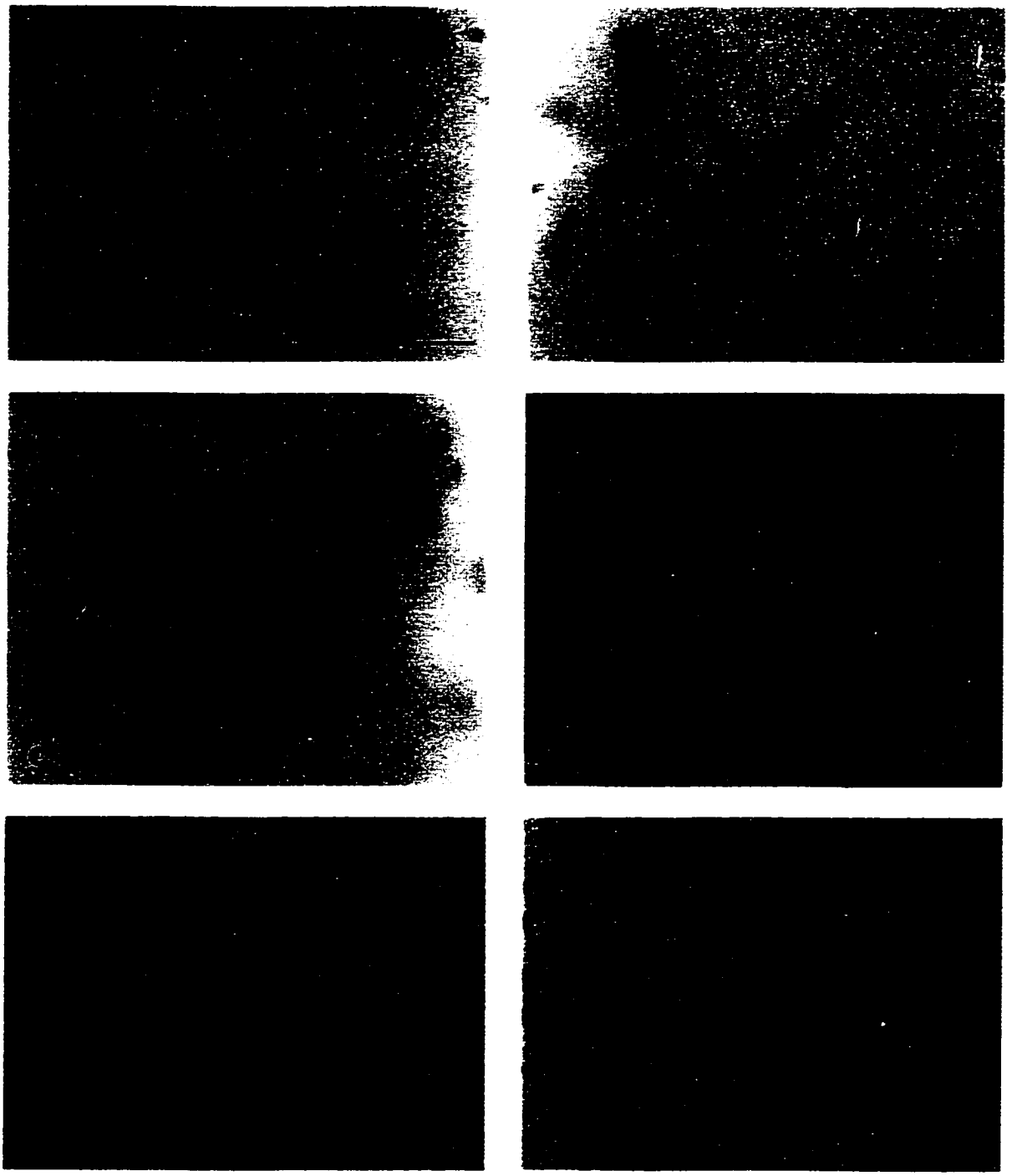


Figure 18

polygonal cells were located within or surrounding a chondrocyte colony.

A surprising observation was that many chondrocytes also appeared APase positive. To further investigate the properties of APase-positive cells in chondrocyte colonies, a double staining procedure for type II collagen and APase was designed. This method demonstrated that almost all chondrocyte colonies at all stages contain some chondrocytes that express both APase and type II collagen. In day 8 cultures, when chondrogenesis had just been initiated, some cells — the frequency differs among colonies — were already both type II collagen- and APase-positive (Fig. 18A, B). These double-positive chondrocytes are not hypertrophic in morphology. In fact, APase expression exists in chondrocytes of all sizes, from relatively small to relatively large (Fig. 18C, D). By day 14, APase expression remained in many chondrocytes. The earliest chondrocytes often appeared at locations close to polygonal cells which do not express type II collagen, but might be APase-positive (Fig. 18A, B). On the other hand, some polygonal cells and some chondrocytes did not express APase. Therefore, in terms of APase and type II collagen expression, four kinds of cells exist within a chondrocyte colony:

(1) APase-negative polygonal cells,

(2) APase-positive polygonal cells,

(3) APase/type II collagen double-positive chondrocyte cells, and

(4) type II collagen-positive chondrocyte cells (Fig. 18A, B, C, D).

In monolayer cultures with densities higher than confluence, polygonal cells modified their morphology to fibroblasts and the

numbers of APase-positive cells decreased quickly. There were almost no APase positive cells in later cultures, which were dominated by fibroblast-like cells (Fig. 18E).

(D).Type I collagen expression

Collagen type I expression was observed in polygonal cells, fibroblasts and most chondrocytes. All polygonal cells had uniform distribution of type I procollagen in their cytoplasm. Some fibroblast-like cells stained similarly to polygonal cells, while others had weak staining. Most chondrocytes were type I collagen-positive (Fig. 18F).

4.3.3. Chondrogenesis in clonal culture

To further examine differentiation of the periosteal cells, clonal culture was performed. Single cells were marked two hours after plating and their differentiation was followed by daily observation with an inverted microscope. Most cells died during the first week in culture. Most of those surviving for two weeks gave rise to fibroblast-like cell colonies and did not express a chondrocyte phenotype. Some cells gave rise to polygonal cells. Chondrocytes developed from about 5% of the clones. Chondrocytes often appeared from polygonal cell clones and were often mixed with APase-positive polygonal cells (Fig. 19A)

4.3.4. Chondrogenesis in suspension culture

Some QJ periosteal cells differentiated and developed into chondrocyte colonies in agarose culture. After culture in agarose for

two days, some cells became large and rounded and started to secrete alcian blue-positive ECM and undergo cell division. The earliest chondrocyte nodules appeared in 4 day cultures. The numbers of chondrocyte nodules gradually increased over time. Two types of colonies were seen (Fig. 19B).

(1). Chondrocyte nodules. These nodules contained rounded chondrocytes embedded in alcian blue-positive extracellular matrix. The nodules could range from small, with a single chondrocyte, to large, containing more than one hundred cells.

(2). Compacted cell nodules. These nodules contain compacted cells, which were smaller than chondrocytes and were not surrounded by ECM. This type of nodule was negative for alcian blue staining and therefore was not chondrogenic.

Fig. 19. Chondrogenesis of the QJ periosteal cells in clonal and suspension cultures.

(A). A clone which was double-stained with type II collagen immunostaining and APase histochemical staining. Chondrocytes were identified by type II collagen expression. Both chondrocytes (1) and APase-positive polygonal cells (3) coexisted in the clone. Many chondrocytes were type II collagen/APase double-positive (2).

(B). Chondrocyte nodules in suspension culture in agarose gel. Alcian blue staining. Chondrocyte nodules have large amount of ECM, stained with alcian blue. The chondrocyte nodules varied in size from single cell (1) to mutiple cells (2). Compacted nodules were alcian blue-negative (3).

Bar: (A) 100 μm, (B) 40 μm.

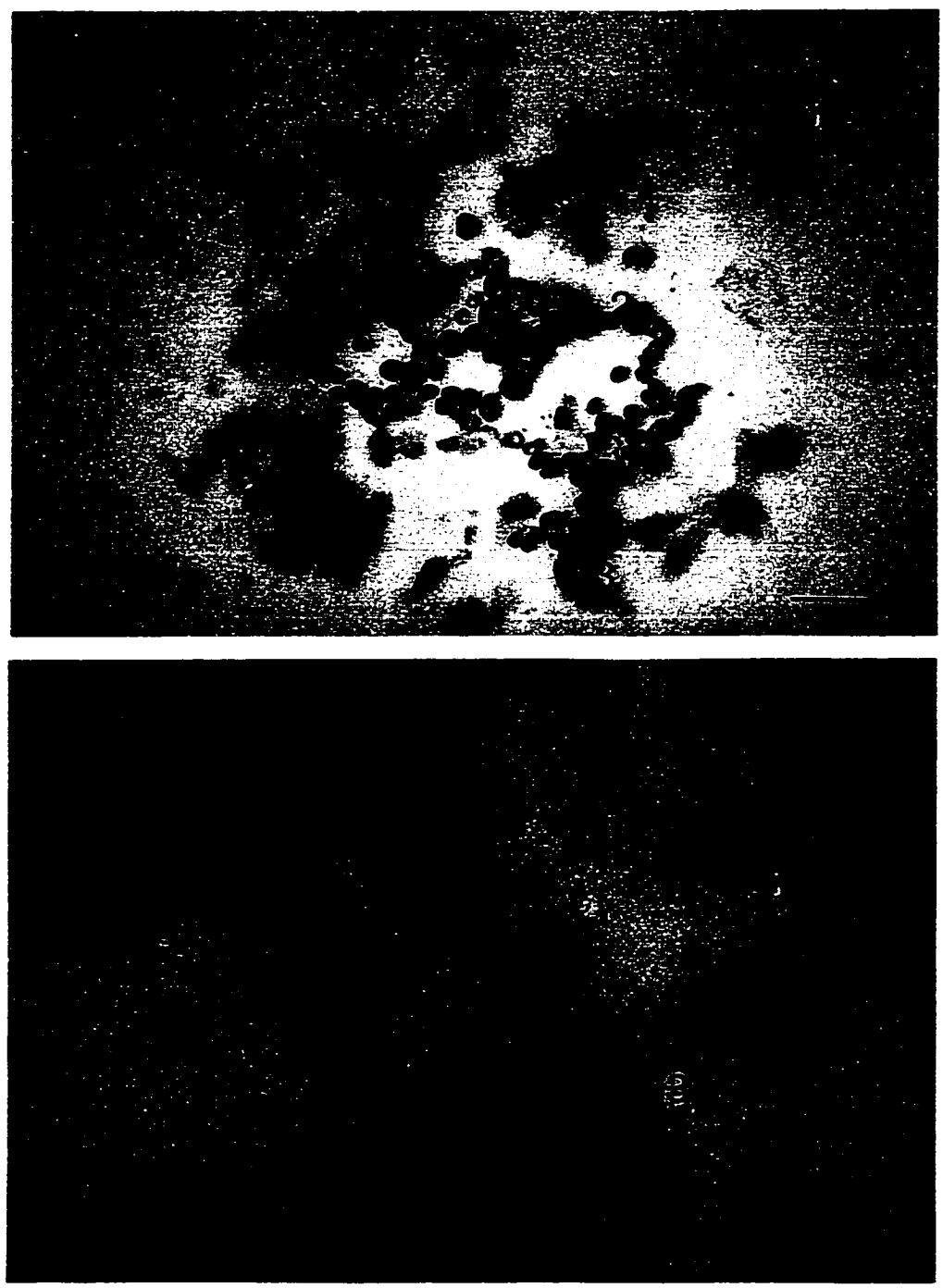


Figure 19

4.4. Discussion

These studies seek cell culture conditions appropriate for studying chondrogenesis in QJ periosteal cells. Since it had been demonstrated previously that higher cell density favors chondrogenesis from limb bud and mandible mesenchymal cells (Ahrens et al., 1977; Solursh and Meier, 1978; Ekanayake and Hall, 1994), as well as from periosteal cells of tibiae (Nakahara et al., 1990a, b, 1991), the cell culture study started with micromass culture. However, QJ periosteal cells did not initiate chondrogenesis under this culture condition. Instead, a layer of bony tissue formed at the top of the micromass. Therefore, periosteal cells maintain their osteogenic differentiation pathway in high density culture.

To further explore chondrogenesis *in vitro*, periosteal cells were cultivated in standard monolayer culture. It is worth noting that much time was spent testing chondrogenesis in monolayer culture at regular densities, but all failed to give rise to chondrocytes. Finally, it was found that chondrocytes arose from cultures in exceptionally low densities. In such conditions, QJ periosteal cells express chondrogenic markers, such as type II collagen and sulfated proteoglycans, as well as assuming a typical chondrocyte morphology. These data demonstrate for the first time that a fully expressed chondrogenic phenotype can be achieved from membrane bone periosteal cells in primary monolayer culture. Therefore periosteal cells can undergo both osteogenesis and chondrogenesis after being enzymatically released from the QJ shafts and subjected to cell culture. The differentiation pathway is highly influenced by cell density:

osteogenesis is favored at high density and chondrogenesis at low density.

How cell density influences differentiation pathways is unknown. A major difference between the two culture conditions is that cells contact each other three-dimensionally in micromass culture but are isolated from one another in low density culture. It seems that such cell-cell contact is required for osteogenesis from the periosteal cells. The observation in this and other studies (Nakahara et al., 1990a) that osteoblasts decrease APase expression in monolayer culture may be interpreted as the result of lack of cell-cell contact. On the other hand, cell-cell contact is not favorable for chondrogenesis. A similar situation also exists *in vivo*: osteocytes always remain in contact with each other through their filopodial processes, but chondrocytes are always isolated by their ECM.

It should be noted that cell-cell contact required for osteogenesis does not necessarily mean only physical contact. It more likely involves interaction of cell surface signal(s). Since two major cell types — fibroblast-like and polygonal cells — sort out after plating in micromass culture, it is apparent that there is cell-cell recognition and interaction in periosteal cells. One signal molecule possibly involved in the interaction is N-CAM. From the previous chapter, it has been demonstrated that N-CAM is down-regulated during the switch from osteogenesis to secondary chondrogenesis in the periosteum-perichondrium of the QJ hook. It is possible that such signals as N-CAM may mediate cell-cell interaction between periosteal cells and affect differentiation. Hence, differentiation pathways of the periosteal

cells may be regulated by altering expression of signal molecules *in vivo*. Micromass culture may facilitate interactions of signal molecules between periosteal cells, while low density culture fails to support such interactions. It would be interesting to further examine how cell density affects N-CAM expression.

A cell density lower than confluence is critical for initiation of chondrogenesis from periosteal cells. It is unclear why chondrogenesis fails to occur in a confluent culture. From this experiment, it seems that overgrowth of fibroblast-like cells is an important factor influencing chondrogenesis. Population 1, which contains the most fibroblast-like cells and reaches confluence soonest, requires a much lower initial cell density to elicit chondrogenesis than population 3, which contains the fewest fibroblast-like cells and is the slowest to reach confluence. Fibroblast-like cells appear to inhibit chondrogenic phenotype expression in this system as in the co-culture of chondrogenic limb mesenchymal cells and nonchondrogenic tendon fibroblasts (Cottrill et al., 1987).

Since chondrogenesis was initiated at low densities and inhibited at high density, it is apparent that chondrogenesis from periosteal cells does not require a cellular condensation stage, which is a prerequisite for primary chondrogenesis in limb bud and mandible mesenchyme (Ede 1983; Thorogood and Hinchliffe, 1975; Hall and Miyake, 1992, 1995). This result is strengthened by clonal culture, in which a single periosteal cell was demonstrated to differentiate as a chondrocyte colony. Hence, QJ periosteal cells can undergo chondrogenesis without condensation. This result is contrary to the

suggestion from morphological observations that secondary chondrogenesis involves cellular condensation (Mizoguchi et al., 1992a). Hence, based on this study, initiation of secondary chondrogenesis differs from primary cartilage.

From which cell type do chondrogenic cells arise? In Chapter 1, I stated three possible precursors of chondrogenic cells: (i) separate unipotential osteo- or chondrogenic progenitor cells, (ii) bi- or multi-potential progenitor cells, or (iii) osteogenic cells. In this chapter, by following chondrogenesis at an exceptionally low density, it was found that almost all chondrocyte colonies contained both chondrocytes and APase-positive polygonal cells. Since an APase-positive polygonal cell is a typical osteogenic cell type, it is likely that secondary cartilage cells share the same precursor with osteogenic cells. This observation was further supported by clonal culture, in which both type II collagen-positive chondrocytes and APase-positive polygonal cells arose from a single cell.

A further consideration is whether chondrocytes differentiate from stem cells in periosteum or from cell types of the osteogenic lineage. A stem cell origin for chondrogenesis would mean a complete separation between osteogenesis and chondrogenesis, while the osteogenic origin of chondrogenesis indicates a transdifferentiation from osteogenic to chondrogenic pathways, implying the existence of transient cell types and a close relationship between the two lineages. There is evidence suggesting a close relationship between chondrogenesis and osteogenesis in membrane bone.

First, chondrocytes in low density culture have characteristics distinct from those of primary cartilage: they express APase and type I collagen, but are not hypertrophic morphologically. These characteristics are consistent with *in vivo* studies, which showed that non-hypertrophied secondary cartilage expressed APase and type I collagen in the QJ hook, as well as in mammalian mandibular condyles (Silbermann et al., 1987; Silbermann and von der Mark, 1990; Mizoguchi et al., 1992a, b). Since APase and type I collagen are considered osteogenic markers (Rodan and Noda, 1991; Aubin et al., 1993; Liu et al., 1994), secondary cartilage has dual characteristics, i.e. it expresses both chondrogenic and osteogenic markers. The significance of this distinct characteristic is unclear. In primary cartilage, it was demonstrated that hypertrophic chondrocytes underwent further transdifferentiation into a cell type that expresses bony markers (Cancedda et al., 1992; Roach, 1992; Kirsch et al., 1992; Ishizeki et al., 1996a, b, 1997); i.e. they are convertible from chondrogenic to osteogenic cells through modulating gene expression. Dual phenotypic markers are seen in the cells undergoing the conversion to hypertrophic cells (Roach, 1992; Ishizeki, 1997). Considering the presence of dual markers in secondary chondrocytes, the origin of secondary chondrocytes from osteogenic cells may explain their expression of osteogenic markers.

Second, this study of low density and clonal cultures showed that chondrocytes often appear from polygonal cell colonies, rather than from fibroblast-like cell colonies. The polygonal cells express APase and type I collagen but not type II collagen and are typically

osteoblast-like in culture. Chondrocytes could be phenotypically modified from the osteogenic polygonal cells. This idea is strengthened by the fact that the earliest chondrocytes often appear APase-positive in low density culture.

Third, studies on condylar secondary cartilage provided evidence for existence of transient cell types. Two clonal cell lines, SM1/9 and SM25/3, isolated from mandibular condyles, have intermediate characteristics between bony and cartilaginous cells (Bhalerao et al., 1995). They show some osteogenic characteristics such as APase expression, but also produce cartilage ECM components such as sulfated proteoglycans and type II collagen. They are not mesenchymal stem cells since they express many phenotypic markers, but they cannot be categorized as either osteogenic or chondrogenic cells according to their phenotypic markers.

Furthermore, that chondrocytes may arise from an osteogenic cell type draws further support from other experiments. With fractionation to separate subpopulations of calvarial cells, Wong and Tuan (1995) found that a polygonal (but not fibroblast-like) cell subpopulation has chondrogenic capacity *in vitro*. The properties of this subpopulation are more like those of osteoblasts than fibroblast-like stem cells. They have a polygonal morphology and slow proliferation rate. Low proliferation rate indicates a relatively differentiated status since cell differentiation is inversely related to proliferation in cultured periosteal cells (Owen et al., 1990; Stein et al., 1990, 1996).

On this basis, cell differentiation in membrane bone may include a transient stage that expresses osteogenic markers such as APase, and therefore belongs to the osteogenic lineage. However, they also have chondrogenic potential and can modify their gene expression to undergo chondrogenesis under certain microenvironmental condition as in secondary cartilage formation.

Comparison with two other well-studied cell culture models — periosteal cells from tibia and calvaria — reveals that QJ periosteal cells have their own characteristic cell differentiation *in vitro*. In high density, the periosteal cells of chick tibiae form a bony layer and a well developed hypertrophic chondrocyte layer (Nakahara et al., 1990a, b, 1991; Nakata et al., 1992; Iwasaki et al., 1993), whereas QJ periosteal cells form a bony cell layer and a fibroblast-like cell layer. In low density culture, QJ periosteal cells give rise to chondrocytes, whereas tibial periosteal cells do not (Nakahara et al 1991).

QJ periosteum cells also behave differently from the periosteal cells of calvaria. In monolayer culture, although type II collagen is detectable in the culture medium (Berry and Shuttleworth, 1989) and in some cells (Jacenko et al., 1995), chick calvarial periosteal cells express a full chondrocyte phenotype (including molecular markers and typical morphology) only after being fractionated by Percoll gradient isopycnic centrifugation (Wong and Tuan, 1992, 1995). In contrast, QJ periosteal cells spontaneously chondrify in low density monolayer culture. In densities higher than confluence, calvarial periosteal cells increasingly express osteogenic markers, including APase in chicks (Gerstenfeld, et al., 1987) and form bony nodules in

rats (Nefussi et al., 1985; Bellows et al., 1986; Bellows and Aubin, 1989; for review see Aubin et al., 1993). In the chick QJ, APase expression barely exists and bone nodules were never observed in confluent monolayer cultures.

These differences in cell differentiation *in vitro* may reflect different *in vivo* properties of periostea in the three skeletons. Tibiae are long bones and develop from a cartilage template through endochondral bone formation. Both the calvarium and the QJ are membrane bones. However, the QJ can form secondary cartilage *in vivo*, whereas major components of the calvaria do not. It seems that QJ periosteal cells have more chondrogenic potential than calvarial periosteal cells.

In addition, chondrogenesis was tested in agarose culture. In this condition, the periosteal cells round up, a condition that is very favorable for chondrocyte phenotype expression (Solursh, 1988). Cartilage nodules were achieved from all three populations of QJ periosteal cells cultured in agarose. This result further supports an isolated environment and rounded cell shape as important for chondrogenesis.

Chapter 5

N-CAM Expression during Osteogenesis and Chondrogenesis *in Vitro*.

5.1. Introduction

In Chapter 3, I discussed immunohistochemical data to show that N-CAM is expressed persistently throughout osteogenic differentiation, from osteoprogenitor cells to osteoblasts in the QJ *in vivo*. N-CAM expression disappears only in mature osteocytes when they are embedded in extracellular matrix. On the other hand, N-CAM is not detectable in chondrogenic cells in primary or secondary cartilage *in vivo*. In primary cartilages, such as the quadrate, only the outer fibrous layer of the perichondrium has weak N-CAM staining; all other chondrogenic cells, from chondroprogenitor cells to chondrocytes, are N-CAM-negative. In secondary cartilage, N-CAM is not expressed in the perichondrium or in cartilage cells. N-CAM expression is shut down in the germinal cell layer when a periosteum transforms to a perichondrium. These *in vivo* data suggest a general pattern of N-CAM expression in skeleton tissues: N-CAM expression correlates with osteogenic differentiation, but not with chondrogenic differentiation.

Is this pattern only a correlation or a substantial molecular mechanism? Further information on a relationship between N-CAM

expression and osteogenic and chondrogenic differentiation is required. An alternative way to examine this relationship is to examine bone and cartilage formations *in vitro*.

So far, only a few studies have been done on N-CAM expression in skeletal tissues *in vitro*; N-CAM expression in chondrogenesis of chick limb bud mesenchyme in micromass culture (Widelitz et al., 1993; Chuong et al., 1993), and chondrogenic differentiation of the dedifferentiated tibial chondrocytes in monolayer culture (Tavella et al., 1994). Both of them are typical primary cartilages derived from mesoderm and chondrogenesis occurs after cellular condensation. N-CAM expression is required during precartilaginous condensation in these cases. No *in vitro* information is available for N-CAM expression in NC-derived ectomesenchyme from which secondary cartilage and most membrane bones arise. No data are available to show whether N-CAM expression persists in osteogenic differentiation *in vitro* as it does *in vivo*. Therefore, in order to further characterize N-CAM expression patterns in bone and cartilage formation and to examine its physiological roles in skeletal tissues, it is important to examine expression *in vitro*.

Several research models are available for *in vitro* chondrogenesis and osteogenesis from ectomesenchyme. For chondrogenesis, the previous chapter has shown that QJ periosteal cells give rise to chondrocyte colonies in low density monolayer and in suspension culture in agarose. For osteogenesis, micromass culture of QJ periosteal cells is a useful *in vitro* model. In addition, chondrogenesis and osteogenesis can take place in organ culture. For example,

secondary cartilage can form in intact QJs in organ culture when maintained at the air/medium interface (Thorogood, 1979). Both membrane bone and cartilage (Meckel's cartilage) develop in organ culture of whole mandibles (Tyler and Hall, 1977). These model systems should facilitate research on N-CAM expression in skeletogenesis *in vitro*.

In this chapter, I report that N-CAM expression was studied with various *in vitro* approaches in which osteogenesis and/or chondrogenesis take place. It is found that N-CAM is present in osteogenic cells but absent in chondrogenic differentiation *in vitro*, demonstrating that the N-CAM expression pattern is consistent both *in vitro* and *in vivo*.

5.2. Materials and Methods

5.2.1. Monolayer culture of QJ periosteal cells

Monolayer cultures of QJ periosteal cells were set up in 24-well culture plates as described in Chapter 4. Only cells from population 2 of day 13 embryos were used. The periosteal cells were initially plated at a density of 1 x 10^4 cells/ml in F-12 + BGJb (3:1) medium containing 10% FBS and 1 mM L-glutamine. The day in which the cultures were set up was counted as day 0. The cultures were fixed on days 1, 3, 7 and 14 with 70% ethanol or PLP fixative for 10 minutes. Then, the cells were rinsed with PBS and culture plates were stored in 4° C for immunohistochemistry.

5.2.2. Agarose culture of QJ periosteal cells

Agarose culture was set up as described in Chapter 4. Briefly, cells from the second digestion of day 13 QJ shafts were suspended in 2 x DMEM and mixed with 1% low gelling temperature agarose to yield a final concentration of 1 x 10^5 cells/ml. The agarose mixture was plated as 50 µl drops on 35 or 60 mm culture dishes precoated with high gelling temperature agarose and flooded with 1 x DMEM containing 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. The cultures were maintained in a 5% CO_2 incubator at 37° C for 14 days and then fixed with cold 70% ethanol overnight.

5.2.3. Micromass culture of QJ periosteal cells

Micromass cultures were set up based on the protocol in Chapter 4. QJ periosteal cells from population 2 were pipetted as 10 µl drops of 2 x 10^7 cells/ml onto 30 mm culture dishes. The cultures were maintained for 14 days and then fixed with PLP fixative for histology and immunohistochemistry.

5.2.4. Organ culture of intact quadratojugal and mandibles

To set up organ cultures, the mandibular processes of chick embryos at H.H. stage 23-24 were aseptically dissected using two #4 forceps under a dissecting microscope, and intact QJs were dissected from day 11 embryos using the same technique. Then the mandibles or QJs were rinsed with PBS twice and placed on black Millipore filters which had been cut into about 0.5 x 0.5 cm pieces. The black filters were sterilized by soaking in 70% ethanol for 30 minutes, followed by rinsing in PBS three times each for 10 minutes. The intact mandibles or QJs on the black filters were cultured at the air/medium interface on stainless steel grids in 30 mm tissue culture dishes in a 5% CO_2 incubator. Each dish contained 2 µl medium of Ham's F-12 and BGJb (3:1) containing 10% FBS, 150 µg/ml ascorbic acid, 1 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. The medium was changed once at day 4. After being maintained in culture for 7 days, the quadratojugals or mandibles were fixed in 70% ethanol or PLP fixative overnight and processed for histology and immunohistochemistry.

5.2.5. Histology and immunohistochemistry

The micromass culture, intact QJs, and mandibles were processed for histology sections as described in Chapters 2 and 4. Monoclonal antibody 5e was used to detect N-CAM in cultured cells or in histological sections of micromass cultures, the intact QJs, and the mandibles. For revealing bone and cartilage phenotypes, APase histochemistry and HBQ staining were applied as in Chapters 2 and 4.

5.3. Results

5.3.1. N-CAM expression during chondrogenesis *in vitro*

To assess change in N-CAM expression in periosteal cells after plating in monolayer culture, N-CAM immunohistochemical staining was carried out on cultured cells fixed at days 1, 3, 7, and 14 after plating. In cells fixed on day 1 after initial plating, immunohistochemistry showed that all cells were N-CAM-negative (Fig. 20A). Although the culture consisted of morphologically heterogeneous cell types, including fibroblast-like and polygonal cells, all cells were N-CAM-negative. N-CAM remained negative in monolayer cultures fixed in 3, 7 and 14 days. In those cultures with growing chondrocyte colonies, N-CAM was also absent from the entire culture, including chondrocyte colonies (Fig. 20B). Thus, in monolayer culture, N-CAM expression was shut down during the first day of culture and it was not expressed throughout the culture period.

In agarose culture, after 4 days of cultivation, some periosteal cells started to differentiate into chondrocyte nodules, which were characterized by cartilaginous ECM with alcian blue staining. Immunostaining revealed that chondrocytes in agarose culture were N-CAM-negative (Fig. 20C). There were no N-CAM-positive cells surrounding the chondrocytes in nodules. In the chondrocyte nodules containing a single chondrocyte, that chondrocyte was also N-CAM negative (Fig. 20C). Only the compacted cell nodules, which did not exhibit cartilage phenotype, expressed N-CAM (Fig. 20C).

Fig. 20. N-CAM expression in chondrogenesis *in vitro*.

(A). QJ periosteal cells in monolayer culture. The cells were fixed 24 hours after plating and processed for N-CAM immunolocalization. N-CAM was undetectable in these cells.

(B). N-CAM immunolocalization in a chondrocyte colony in a day 14 monolayer culture of QJ periosteal cells. N-CAM was not expressed in any cell types.

(C). Suspension culture of QJ periosteal cells in agarose. The culture was fixed on day 7 and double-stained for N-CAM by immunohistochemistry and by alcian blue staining. N-CAM was absent from chondrocyte nodules which had single (upper-left) or multiple (middle) chondrocytes with abundant alcian blue-positive ECM. N-CAM was expressed in the compacted cell nodules which were not stained by alcian blue (bottom-right).

Bar: (A, for A, B, and C) 40 μm.

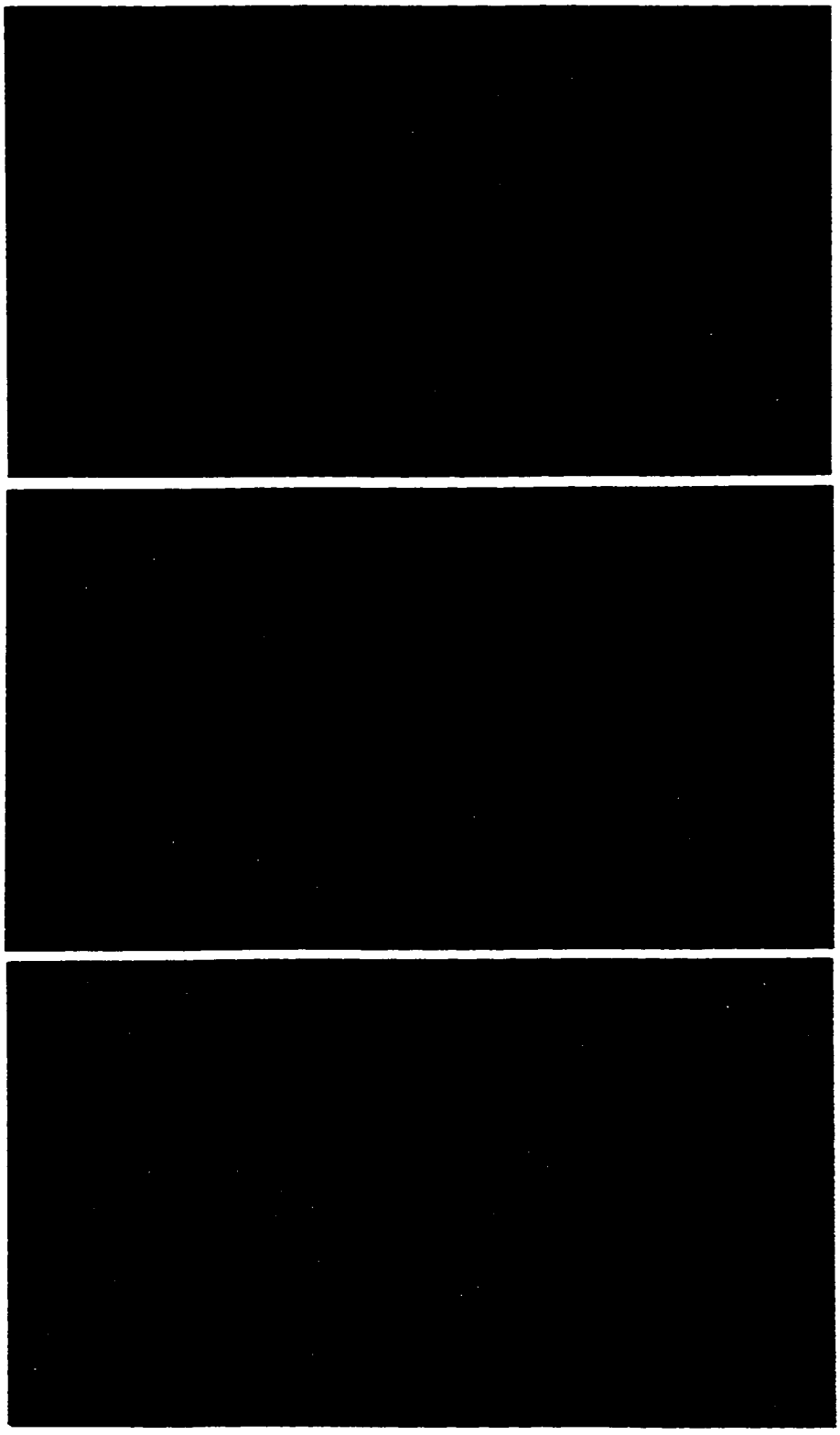


Figure 20

When the intact QJs from day 11 to 14 embryos were cultured at the air/medium interface as organ cultures, secondary cartilage developed from the periostea of the QJ hooks, as they did *in vivo*. This secondary cartilage was visible in day 7 cultures with HBQ stain in histological sections (Fig. 21A). Immunostaining revealed that N-CAM was absent from secondary cartilage in the QJ hook (Fig. 21B). In contrast, N-CAM was positive in bone formation sites (Fig. 21B). Thus, secondary cartilage formation *in vitro* is associated with down-regulation of N-CAM expression.

Chondrogenesis is seen in organ cultures of mandibular processes. When the intact mandibles were maintained at the air/medium interface for 7 days, cartilage was found in histological sections with positive alcian blue staining (Fig. 22A). This cartilage had an overall rod-like shape, that was similar to its *in vivo* counterpart, Meckel's cartilage. *In vitro*, this Meckel's cartilage was surrounded by a well developed perichondrium. Like the perichondrium of the quadrate *in vivo*, this perichondrium consisted of two cell layers, a fibrous outer layer and a cambium inner layer. N-CAM was weakly positive in the outer fibrous layer of the perichondrium. All other cell types in Meckel's cartilage, from progenitor cells in the cambial layer to mature chondrocytes, were N-CAM-negative (Fig. 22B).

5.3.2. N-CAM expression in osteogenesis *in vitro*

In order to determine if N-CAM expression remained during osteogenesis *in vitro*, QJ periosteal cells were plated in micromass

Fig. 21. N-CAM expression in the quadratojugal in organ culture.

(A). Organ culture of QJ from a day 13 embryo. The QJ was cultured for 7 days, sectioned and stained for HBQ staining. Secondary cartilage (s) was seen in the hook and bone formation (b) seen in the shaft.

(B). N-CAM immunolocalization in the section adjacent to (A). N-CAM was negative in secondary cartilage but was expressed in the areas (arrowheads) where bone formation was continuing.

Bar: (A, for A and B) 100 μm.

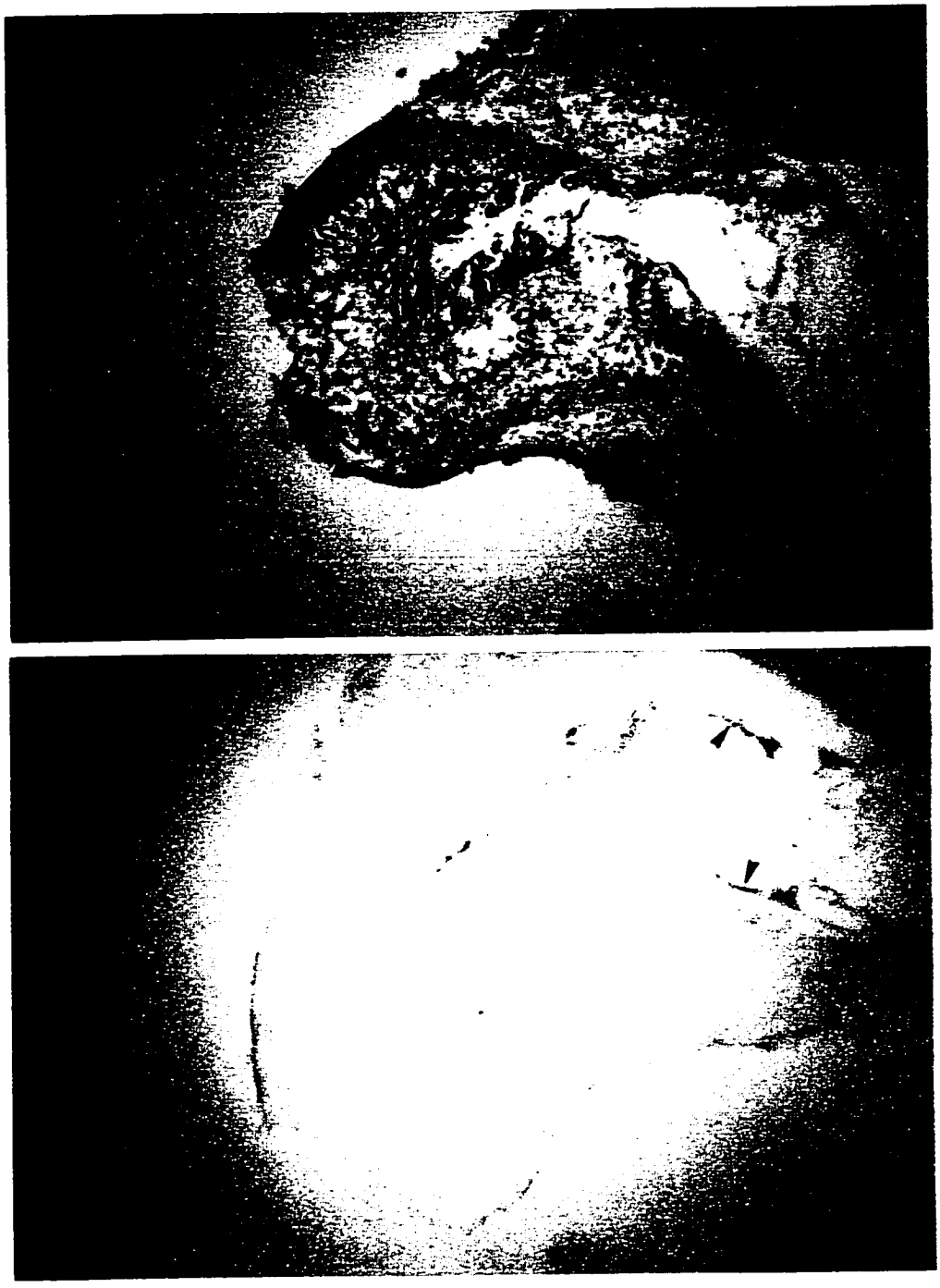


Figure 21

Fig. 22. Mandibular organ culture.

(A). Meckel's cartilage (Mc) and membrane bone (mb) in organ culture of the mandible. stained with HBQ staining. In the membrane bone region, most cells have not produced bony matrix. The arrowhead indicates the ossification center, which has bony matrix.

(B). N-CAM immunolocalization. N-CAM was negative in chondrocytes and the inner cell layer of the perichondrium, and was weakly positive in the outer cell layer of the perichondrium in Meckel's cartilage. N-CAM was expressed in the area where membrane bone was forming. N-CAM was localized to the cell surface (arrow).

Bar: (A, for A and B) 60 μm.

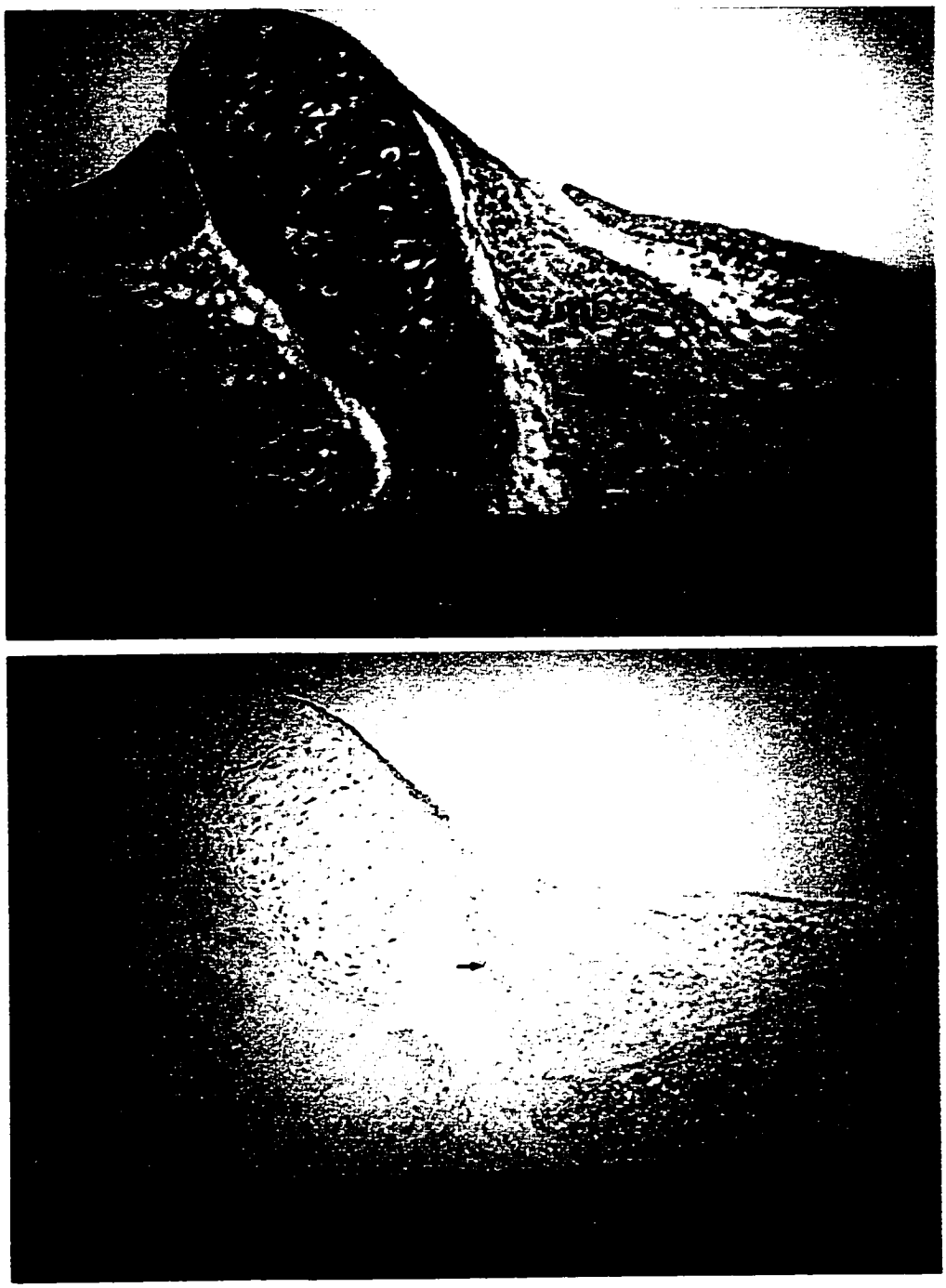


Figure 22

culture and N-CAM immunostaining was conducted in histological sections fixed on day 14. As addressed in Chapter 4, QJ periosteal cells sort out to form two cell layers in micromass culture. One, at the top, expresses APase. The upper portion of this APase-positive layer is stained red by HBQ. Another layer was fibroblast-like cells, which did not express APase and were not stained red by HBQ stain (see Fig. 13 in Chapter 4). Immunostaining revealed that N-CAM expression was retained in the periosteal cells in micromass culture. N-CAM appeared weakly positive in the fibroblast-like cell layer and remained positive in the lower part of the APase-positive cell layer (Fig. 23A). N-CAM was negative in most cells at the upper portion of the micromass which was stained red by HBQ stain.

When cultured at the air/medium interface, the QJ generally retained its overall shape and was surrounded by a periosteum. Immunostaining showed that N-CAM was expressed in the periosteum *in vitro* (Fig. 21B). The expression pattern was very similar to that seen in the periosteum *in vivo*.

Osteogenesis occurred in mandibles cultured in the air/medium interface. In histological sections, membrane bone was identified by HBQ stain and located beside Meckel's cartilage (Fig. 22A). Unlike Meckel's cartilage, membrane bone did not form a rod shape in culture. Moreover, there was no periosteum surrounding membrane bone at this stage. Instead, bony extracellular matrix was deposited by polygonal cells which were differentiated from surrounding mesenchyme-like cells. This process was very similar to intramembranous ossification centers *in vivo*. Immunostaining showed

Fig. 23. N-CAM expression in micromass culture of QJ periosteal cells.

(A). N-CAM immunolocalization in micromass culture. N-CAM is expressed in both top and bottom cell layers except the upper most region (arrowheads).

(B). Negative control for N-CAM immunostaining in the section adjacent to (A). No positive stain was seen.

Bar: (A, for A and B) 30 μm.

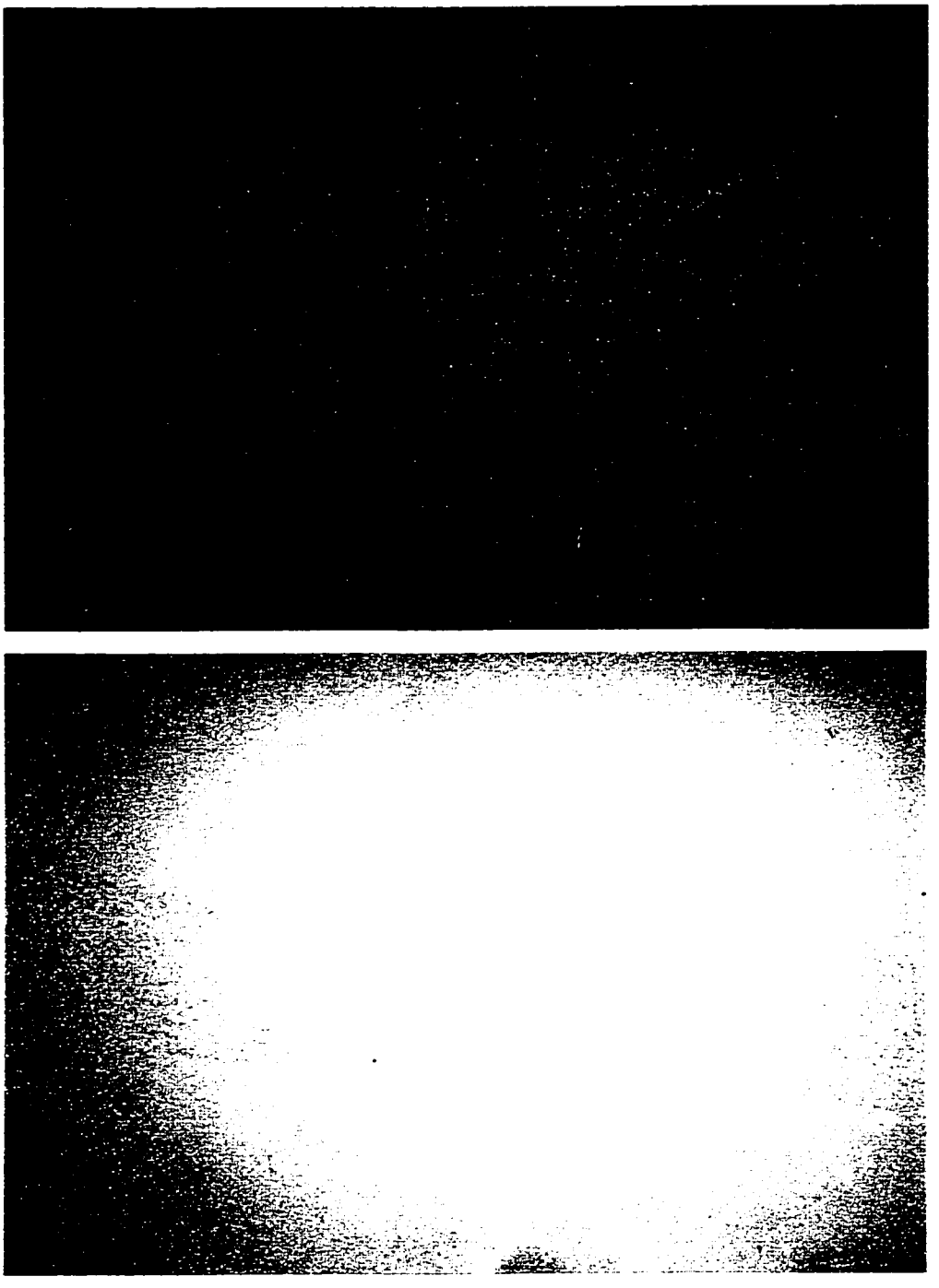


Figure 23

that N-CAM was expressed in membrane bone regions (Fig. 22B). Cell surface localization of N-CAM was very clear in some cells (Fig. 22B). The entire mandible was covered by epithelium which was N-CAM-negative.

5.4. Discussion

In this chapter, I have reported N-CAM expression in several *in vitro* osteogenic and chondrogenic cultures. N-CAM expression was detected in cultures which supported osteogenesis, including micromass culture of QJ periosteal cells, periosteum of the quadratojugals, and membrane bone in mandibles in organ culture. By contrast, N-CAM was not detectable in the situations where cells were undergoing chondrogenesis *in vitro*, including monolayer culture, chondrocyte colonies in agarose culture, secondary cartilage in the intact quadratojugals, and Meckel's cartilage in whole mandibles. These results, taken together with the *in vivo* studies in Chapter 3, demonstrated that N-CAM was expressed in osteogenic differentiation but not in chondrogenic differentiation.

This N-CAM expression pattern is conserved over the skeletal tissues of various embryonic origins, (e.g., neural crest and mesoderm derivatives), and developmental processes, (e.g., through intramembranous and endochondral bone formations). This pattern indicates an intrinsic linkage of N-CAM expression to the osteogenic lineage and suggests a role of this molecule in regulation of osteogenic and chondrogenic differentiation. Osteogenic and chondrogenic cells are very closely related. In the previous chapter, it was shown that osteogenic and chondrogenic cells may share the same periosteal precursor. It would be very interesting to see whether the differential expression of N-CAM is relevant to determination of the alternative

(osteo- and chondrogenic) differentiation pathways of the periosteal cells.

In Chapter 3, I demonstrated that N-CAM expression is down-regulated in secondary chondrogenesis *in vivo*. In this chapter, secondary chondrogenesis was achieved in QJ organ culture, consistence with previous reports (Thorogood, 1979). Moreover, immunostaining revealed that transformation from a periosteum to a perichondrium is also accompanied by down-regulation of N-CAM in organ cultures of the QJ hooks. Therefore N-CAM down-regulation occurs in secondary chondrogenesis both *in vivo* and *in vitro* and may reflect an important molecular event during the switch from osteogenesis to chondrogenesis in the germinal cells.

An interesting finding is that N-CAM expression quickly disappears when the periosteal cells are plated in monolayer culture. N-CAM is not expressed throughout the 2-week period of monolayer culture, in which chondrocyte colonies emerge after day 7 or 8. This result indicates that chondrogenesis can be initiated without an N-CAM signal. N-CAM is expressed in precartilaginous condensation, which is a critical phase in primary chondrogenesis from mesenchyme (Hall and Miyake, 1992, 1995). In micromass culture of limb bud mesenchyme cells, numbers and sizes of cartilage nodules are reduced by antibodies against N-CAM and increased by overexpression of N-CAM (Widelitz et al., 1993). It has been hypothesized that N-CAM may enhance precartilaginous condensation and chondrogenesis via increasing cell-cell adhesion (Chuong et al., 1993).

The interactions between cell-cell and cell-matrix provided by N-CAM and other molecules such as N-cadherin, fibronectin, tenascin, and integrins (Chuong et al., 1993; Oberlender and Tuan, 1994; Hall and Miyake, 1995) may account for the fundamental molecular events in condensation; perturbation or overexpression of those molecules would either reduce or augment primary cartilage formation. However, it is not clear whether N-CAM is required merely for cellular recruitment and aggregation in condensation or whether it is involved in signal transduction required for cellular commitment to chondrogenic differentiation (Widelitz et al., 1993). In Chapter 4, it was demonstrated that chondrogenesis occurs in monolayer culture of periosteal cells in the absence of the condensation process, indicating that chondrogenic differentiation can be independent from precartilaginous condensation. Chondrocyte phenotype expression in periostea cells in agarose culture also supports this finding. Hence, condensation is probably mainly responsible for the overall patterning of the skeletal element.

Recent evidence shows that patterning and cell differentiation in skeletal tissue are achieved through different molecular pathways (Ducy et al., 1997). Thus, molecules which lead to precartilaginous condensation are not necessarily required for initiation of chondrogenic differentiation. Since chondrogenesis can take place without N-CAM participation in monolayer culture, N-CAM seems not to be a necessary signal for chondrogenic differentiation. Accordingly, N-CAM is involved in condensation per se rather than as a signal for chondrogenic differentiation.

The previous chapter showed that QJ periosteal cell differentiation was influenced by culture conditions. In micromass culture, the periosteal cells gave rise to a bony cell layer and a fibroblast-like cell layer, while, in low density monolayer cultures, chondrogenesis was elicited. It has been speculated that cell-cell interaction may contribute to the outcome of differentiation pathways in the periosteal cells (Fang and Hall, 1996a, b). This chapter added evidence that such interaction may have a substantial basis in terms of molecular expression since N-CAM is present in micromass culture but not in monolayer culture. It would be very interesting to see if N-CAM expression is a prerequisite for osteogenesis in micromass culture. On the other hand, N-CAM expression is shut down when the periosteal cells are plated in monolayer culture. It is also of interest to see if termination of N-CAM expression is required for initiation of chondrogenesis in periosteal cells.

Chapter 6

Effects of N-CAM Overexpression and Antibody Perturbation on Cell Differentiation of Periosteal Cells

6.1. Introduction

In Chapter 4, I showed that secondary chondrogenesis might involve transdifferentiation from an osteogenic to a chondrogenic pathway. Both *in vivo* and *in vitro* studies revealed that N-CAM was persistently expressed in osteogenic cells but not in chondrogenic cells. Alteration of N-CAM expression correlates with the switch of differentiation pathways. Thus, it is of interest to ask how important down-regulation of N-CAM expression is for the switch, and what the specific role of N-CAM in chondrogenesis and osteogenesis is?

N-CAM expression is associated with various types of osteogenesis, including endochondral, intramembranous, and endosteal bone formation *in vivo* and *in vitro*. This suggests a requirement for N-CAM in osteogenesis.

N-CAM participates in precartilaginous condensation during primary cartilage morphogenesis, as demonstrated by N-CAM overexpression and antibody perturbation assays (Chuong et al., 1993; Widelitz et al., 1993). On the other hand, that N-CAM is not the molecular signal necessary for chondrogenic differentiation is strongly suggested by the fact that chondrogenesis can be elicited in periosteal

cells without N-CAM expression (see previous chapter). However, several observations indicate that N-CAM may still play a role in chondrogenesis. This is particularly suggested by N-CAM expression in the NCL cells during secondary cartilage formation in the QJ hook. N-CAM is expressed in the NCL when secondary chondrogenesis ceases. The NCL cells are probably dedifferentiated from chondroblasts according to their morphological similarity to chondroblasts and location between perichondrium and cartilage. N-CAM expression correlates with cessation of chondrogenesis and disappears later when the NCL resumes chondrogenesis to undergo the second phase of secondary cartilage formation. The correlation of N-CAM expression with the cessation of chondrogenesis in the NCL suggests that N-CAM is probably a factor that prevents chondrogenic differentiation. This suggestion is supported by down-regulation of N-CAM in periosteum during commitment of secondary chondrogenesis and up-regulation of N-CAM in hypertrophied chondrocytes when transforming into an osteogenic phenotype.

Thus, N-CAM probably has opposite roles in osteogenesis and chondrogenesis in periosteal cells; enhancing osteogenesis and inhibiting chondrogenesis. In order to test this hypothesis, further experiments were conducted.

First, I tested whether enhancement of N-CAM expression can inhibit chondrogenesis in QJ periosteal cells. Enhancement of N-CAM expression can be achieved by transfection of plasmids containing the N-CAM gene into cultured periosteal cells. In order for an exogenous gene to be expressed in eukaryotic cells, the cDNA of the gene is

usually inserted into a vector after an appropriate promoter, which allows transcription of the inserted genes. In this experiment, a plasmid encoding a whole 140 kD N-CAM sequence under simian virus (SV) 40 promoter was used. Several methods are available to introduce exogenous vectors into eukaryotic cells, such as calcium-mediated transfection, electroporation, and liposome-mediated transfection (Ausubel et al., 1992). In this study, the liposomal method was used because of a number of advantages, including high transfection efficiency and low toxicity (Ausubel et al., 1992). The liposome method utilizes polycationic lipids which are mixed with plasmid DNA to form a DNA-liposome complex. The complex binds to plasma membranes and transports the plasmid into cells.

Second, I tested whether inactivation of N-CAM function can reduce osteogenesis. Monoclonal antibody 5e was used to inactivate the N-CAM. This antibody binds to the epitope in the heparin binding domain in the extracellular region of N-CAM and reduces homophilic interaction (Watanabe et al., 1986; Frelinger III and Rutishauser 1986).

This chapter reports the results of N-CAM overexpression and antibody perturbation assays on chondrogenesis and osteogenesis in QJ periosteal cells in monolayer and micromass cultures. Overexpression of N-CAM during initiation of secondary chondrogenesis reduced numbers of chondrocyte colonies in monolayer culture, suggesting an inhibitory role of N-CAM in chondrogenesis. Therefore, down-regulation of N-CAM in periosteum

is a necessary molecular event for the onset of chondrogenesis in the QJ hook.

6.2. Materials and Methods

6.2.1. Periosteal cells

For monolayer and micromass cell cultures, QJ periosteal cells of day 13 embryos were prepared as described in Chapter 4. Only the cell population from the second digestion was used, since this population appeared to have relatively higher differentiation potential.

6.2.2. Preparation of plasmid DNA

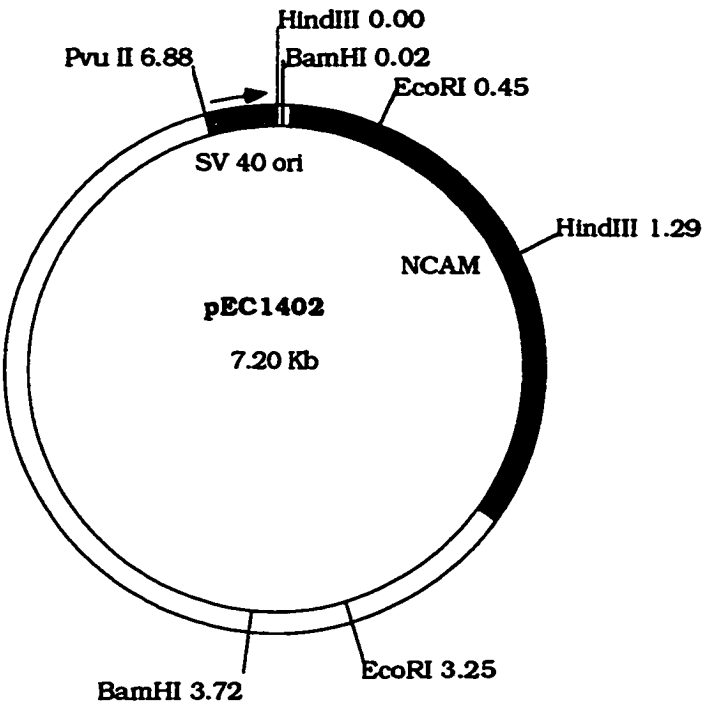
pEC 1402 plasmid was a gift from Dr. B. Murray at University of California, Irvine, CA. This plasmid contains a DNA sequence encoding the whole 140 kD N-CAM, a 325-bp DNA fragment containing the SV40 early promoter and a sequence from pBR 322 plasmid including an ampicillin resistant gene (Edelman et al., 1987, Fig. 24A). pEC 1402 plasmid DNA was transformed into *E. coli* with standard transformation protocols (Sambrook et al., 1989; Ausubel et al., 1992) and selected in LB agar plates in the presence of ampicillin. The plasmid DNA was purified and verified with Hind III and BamH I restriction endonuclease map analysis in a 1% agarose gel (Fig. 24B).

A control plasmid was constructed by deleting the N-CAM cDNA sequence from pEC 1402 plasmid. Briefly, pEC 1402 plasmid was digested with BamH I (GIBCO BRL, Burlington, ON). The 3.5 kb BamH I fragment was rejoined by T-4 DNA ligase (GIBCO BRL, Burlington, ON). This plasmid contains sequence derived from pBR322 and the SV40 promoter, but does not contain N-CAM cDNA (Fig. 25). The ligated plasmid was transformed into *E. coli* and positive clones were

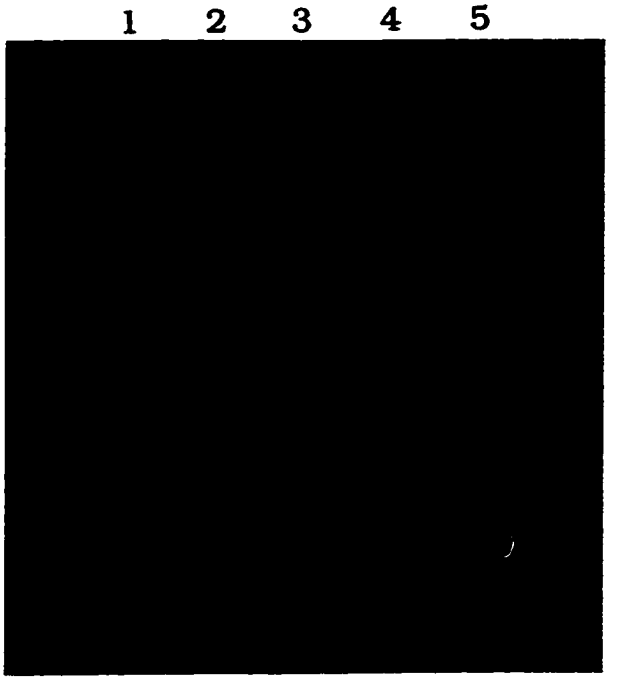
Fig. 24. Illustration and restriction endonuclease map of pEC 1402 plasmid.

(A). Illustration of pEC 1402 plasmid. This plasmid contains the entire sequence of 140 kD N-CAM under control of an SV 40 promoter.

(B). Restriction endonuclease map of pEC 1402 plasmid. Lanes 1 and 5: λ DNA Hind III marker; lane 2: uncut plasmid; lane 3: pEC 1402 digested with Hind III; lane 4: pEC 1402 digested with Hind III + BamH I.


HindIII 0.00
Pvu II 6.88
BamHI 0.02
EcoRI 0.45
SV 40 ori
HindIII 1.29
NCAM
pEC1402
7.20 Kb
BamHI 3.72
EcoRI 3.25

A


1
2
3
4
5

B

Figure 24

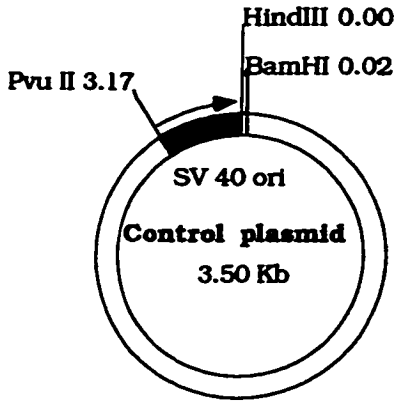


Figure 25

Fig. 25. Illustration of control plasmid. This plasmid was constructed by deleting the sequence encoding the 140-kD N-CAM in pEC 1402 plasmid.

selected in the LB plates with ampicillin. Then plasmid DNA was purified from *E coli* clones and verified by restriction map in 1% agarose gel.

Transfection requires a large amount of ultrapure plasmid DNA. A large amount of plasmid DNA was prepared using 2 x CsCl gradient ultracentrifugation. Briefly, *E. coli* containing either pEC 1402 or control plasmid was grown and harvested from 1000 ml LB medium. The bacteria were lysed with lysozyme and SDS, and plasmid DNA was precipitated with potassium acetate and then purified with phenol/chloroform. The plasmid DNA was spun in a CsCl-ethidium bromide gradient for 39 hours (Sambrook et al., 1989). The plasmid DNA was recovered and further purified by another centrifugation. RNA was removed by digesting plasmid DNA with RNase A between the two centrifugations. Ethidium bromide was removed with n-butanol after the second centrifugation. Finally the plasmid DNA was precipitated in cold 70% ethanol, dried, and resuspended in Tris-EDTA (TE) buffer. Alternatively, DNA was purified with a Qiagen (Santa Clarita, Ca) large-scale plasmid DNA purification kit, following the protocol from the manufacturer. Purity and concentration of plasmid DNA were determined with a spectrophotometer by measurement of OD_{260} and OD_{280}.

6.2.3. Transfection of N-CAM-encoding plasmid into cultured periosteal cells

Periosteal cells were plated in 6-well or 24-well culture plates at a density of 2 x 10^5 cells/ml. The cells were allowed to grow in

monolayer culture until they reached 60-80% confluence (about 48 hours). Liposomal transfection was performed using LIPOFECTAMINE reagents from GIBCO, BRL (Burlington, ON, CAT. No. 18324-012). Optimal transfection conditions, including concentration of plasmids and lipofect reagents, were predetermined based on the N-CAM expression revealed by immunostaining. Briefly, 2 μg of pEC1402 DNA or control plasmid DNA was mixed with 5 μl LIPOFECTAMINE Reagent in F-12 + BGJb (3:1) medium without FBS for 30 min. Then 800 μl of the same medium without FBS was added to make 1000 μl of transfection solution. The periosteal cells were rinsed with the FBS-free medium twice. Transfections were conducted by incubating the cells with the transfection solution (800 μl/well for 6-well plates and 200 μl/well for 24-well plates) for 6 hours. Then the same volume of medium containing 20% serum was added to each well, which then remained in culture overnight. The next morning, the medium in each well was replaced by fresh medium containing 10% FBS and cells were allowed to grow for another 24 hours.

To test the effect of N-CAM overexpression on chondrogenesis, the cells at 48 hours after initiation of transfection at optimal conditions were trypsinized and plated in monolayer culture in 5 x 10^3 cells/ml in 24-well culture plates. Culture medium was F-12 + BGJb without ascorbic acid as described in Chapter 4. The cultures were maintained for 2 weeks and fixed with PLP fixative for 30 min.

6.2.4. N-CAM transfection during chondrogenesis in low density monolayer culture.

To enhance N-CAM expression during initiation of chondrogenesis in monolayer culture, QJ periosteal cells were plated in 24-well culture plates at a density of 5 x 10^3 cells/ml and maintained for 6 days in F-12 + BGJb complete medium as described previously. The cells were then transfected with pEC1402 or control plasmid DNA. The DNA and lipofect reagent concentrations were as previously described. The transfection was carried out in the presence of 10% serum in the medium. After a 5 hour transfection, the culture medium was replaced with fresh complete medium. Some wells were left without transfection, serving as normal controls. The cells were maintained in culture for 4 days, fixed with PLP fixative, and stained immunohistochemically for type II collagen. Numbers of chondrocyte colonies in different groups were counted from three independent cultures based on type II collagen immunolocalization and data were analyzed with student t-test.

6.2.5. Micromass culture

Micromass cultures of periosteal cells were set in 24-well plates as described in Chapter 4. Two experiments were carried out.

i) To test the effects of N-CAM transfection, periosteal cells 48 hours after transfection with N-CAM plasmid in monolayer culture were trypsinized and set up for micromass culture. The cultures were incubated for 2 weeks. Some cells from monolayer culture without transfection were set up for micromass culture, serving as controls.

Cellular phenotype was examined by HBQ staining in histological sections as described in Chapter 4.

ii) To test the effects of N-CAM antibody perturbation, QJ periosteal cells were set up for micromass culture and purified 5e monoclonal antibody IgG1 was added to culture medium in a concentration of 0, 80, 160, and 260 μg/ml (for purification of IgG1, see page 70 in Chapter 3). The cultures were incubated for 14 days with a medium change and antibody addition every 3 days. Fixation, section and HBQ staining of the micromasses were as described in Chapter 4.

6.2.6. Immunohistochemistry

To verify N-CAM expression in transfected cells, 48 hours after transfection the cultures were fixed with PLP fixative for 30 min and processed for ABC immunohistochemical staining based on the protocol described in Chapters 2 and 3. Monoclonal antibody 5e was used to detect N-CAM expression. To count the percentage of N-CAM positive cells, some cultures were trypsinized after 48 hours of transfection and cells were smeared on slides and fixed in PLP for 30 min. The slides were then stained immunohistochemically for N-CAM and numbers of N-CAM-positive cells were counted from 600 randomly chosen cells in 3 slides.

6.2.7. Determination of chondrogenic and osteogenic phenotypes

For determination of chondrogenic phenotype of monolayer cultures, immunohistochemical staining for type II collagen and alcian

blue stain were carried out as in Chapter 4. To reveal the osteogenic phenotype in micromass culture, the micromasses were sectioned, and HBQ and APase histochemical stains were used as described in Chapter 2 and 4.

6.3. Results

6.3.1. N-CAM expression in transfected periosteal cells

In order to enhance N-CAM expression in monolayer culture, a plasmid encoding the whole chicken 140 kD N-CAM cDNA sequence under the control of an SV40 promoter was transfected into primary cultures of QJ periosteal cells using a liposomal transfection technique. At 48 hours after transfection, N-CAM protein was detected in cultures by immunohistochemistry. N-CAM was localized on the surface of the cells transfected with pEC1402 plasmid (Fig. 26A). By contrast, N-CAM was not detected in the cultured periosteal cells transfected with a control plasmid which lacked the N-CAM sequence (Fig. 26B). N-CAM expression levels varied among cells. Some cells had strong positive staining on the surface and weak cytoplasmic staining, while others had weak surface and cytoplasmic staining. The percentage of positive cells was counted in trypsinized cells smeared on slides. About 6% were strongly positive and 37% weakly positive. In cultures transfected with control plasmid, less than 2% of the cells appeared weakly positive from nonspecific reaction with the same criteria.

6.3.2. Effects of N-CAM overexpression on chondrogenesis

To test whether enhancement of N-CAM expression affects chondrogenic differentiation, the QJ periosteal cells were first transfected with the pEC1402 plasmid and then plated in low density monolayer culture. Control cells were transfected with the control

Fig. 26. Immunolocalization of N-CAM in monolayer culture of the periosteal cells transfected with pEC 1402 or control plasmids.

(A). Transfection of periosteal cells with pEC 1402 plasmid. Cells were fixed at 48 hours after initiation of transfection. N-CAM was expressed in some cells (arrowheads).

(B). Transfection of the periosteal cells with the control plasmid. Transfection and sample processing were identical to (A). No N-CAM was detected in this culture.

Bar: (A, for A and B) 40 μm.

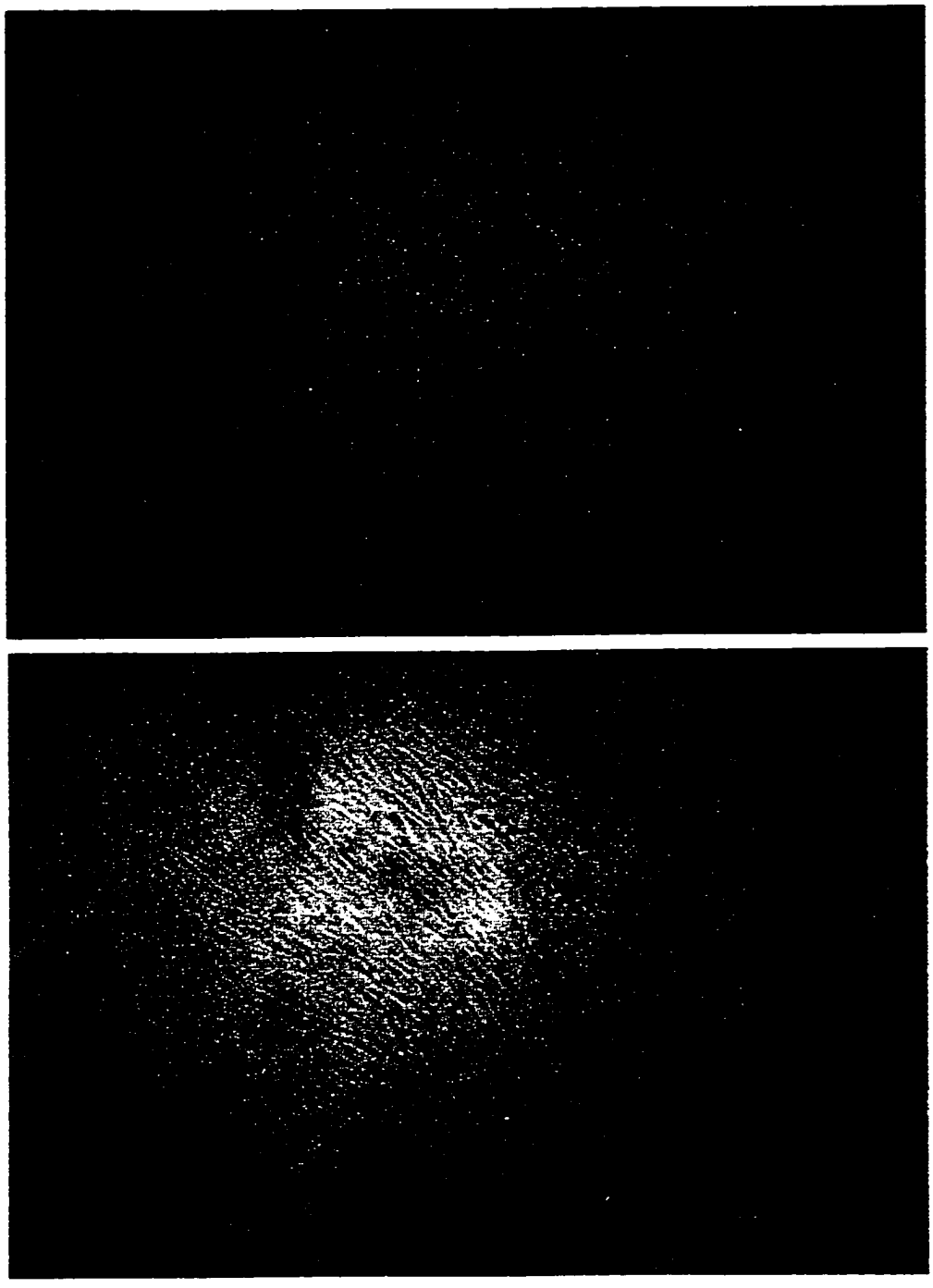


Figure 26

plasmid and plated in low density monolayer culture. Chondrogenesis occurred in both pEC 1402 and control plasmid-transfected cells, starting from day 7 or 8 in both cultures. Numbers of chondrocyte colonies were counted in day 14 cultures based on immunostaining of type II collagen, and no significant difference was found between experimental and control cultures.

To reveal N-CAM expression levels during initiation of chondrogenesis in transfected cells, immunostaining was carried out in day 7 cultures of cells transfected with pEC1402 plasmid. Immunostaining showed that N-CAM expression became undetectable by day 7, consistent with the N-CAM-encoded plasmid being only transiently transfected in culture and diluted by cell proliferation over time.

To minimize the dilution of N-CAM expression due to cell proliferation and examine the roles of N-CAM in chondrogenesis, transfection was carried out in low density monolayer cultures at day 6, the time immediately before initiation of chondrogenesis. The experiment was conducted in three culture groups: i) transfected with pEC1402 plasmid, ii) transfected with control plasmid, iii) not transfected. Chondrocyte colonies were counted in each group at day 10. Chondrocyte colonies were identified based on immunolocalization of type II collagen. Results are shown in Fig. 27. The average number of chondrocyte colonies was 8.4 ± 2.2 colonies/well in cultures transfected with pEC1402 plasmid, which was significantly lower than the 17.9 ± 4.3 colonies/well in cultures transfected with control plasmid, or 17.5 ± 1.1 colonies/well in non-transfected

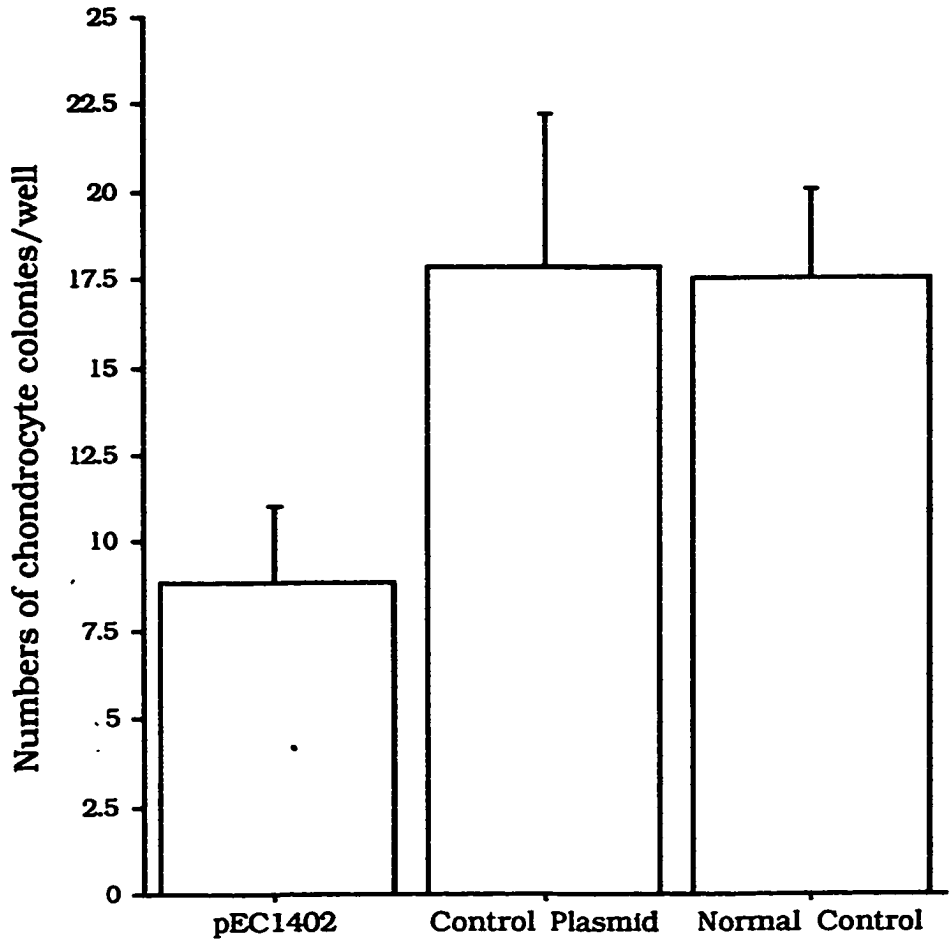


Figure 27

Fig. 27. Inhibitory effect of N-CAM overexpression on chondrogenesis. The periosteal cells were plated in low density monolayer culture (5 x 10^3 cells/ml). Transfections were conducted in day 6 cultures and the numbers of chondrocyte colonies (means ± SD) were counted in day 12 cultures based on type II collagen immunostaining in the three groups.

cultures. Statistically, the differences between group one and two, and between group one and three were significant ($p<0.01$), while difference between group two and three was not ($p>0.1$).

6.3.3. Effects of N-CAM overexpression and antibody perturbation on osteogenesis

To test if N-CAM overexpression enhanced osteogenesis, periosteal cells were transfected with pEC 1402 plasmid in subconfluent monolayer culture and plated in micromass. The effects were evaluated in histology sections with HBQ stain after 14 days of culture. Both cultures formed a fibroblast-like cell layer only. No consistent difference was observed between the cultures transfected with pEC-1402 and with control plasmid (Fig. 28). Therefore overexpression of N-CAM failed to affect osteogenesis in micromass culture of periosteal cells at the second passage.

To examine if osteogenesis is inhibited by blocking N-CAM activity, an antibody perturbation test was carried out in micromass culture. The monoclonal antibody 5e was added to the culture medium to block the function of N-CAM in the micromass culture. The cellular phenotype was evaluated in histological sections of 14 day cultures. Mcromass cultures with N-CAM antibody still formed osteogenic and fibroblast-like cell layers with similar thickness to those in the control cultures (see Fig. 13 in Chapter 4) where antibody was not added.

Fig. 28. Micromass culture of periosteal cells transfected with pEC 1402 or control plasmids.

(A). Micromass of periosteal cells transfected with pEC 1402 plasmid. The culture formed only a fibroblast-like cell layer.

(B). Micromass of periosteal cells transfected with control plasmid. The morphology was similar to (A).

Bar: (A, for A and B) 30 μm.

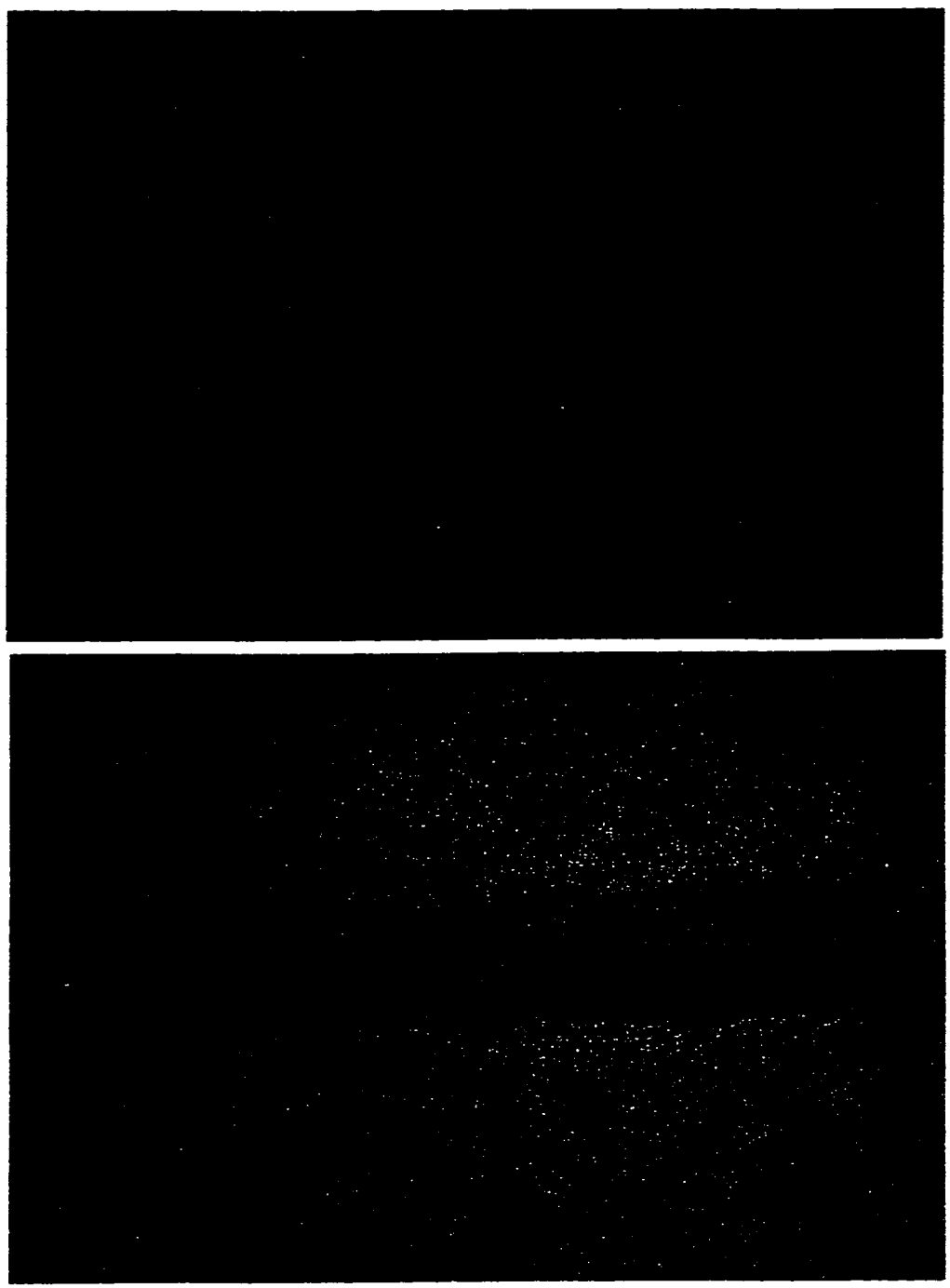


Figure 28

6.4. Discussion

In this study, N-CAM overexpression and antibody perturbation tests were carried out. With liposomal transfection technique, N-CAM encoded plasmid DNA was introduced into QJ periosteal cells in primary culture. Detection of N-CAM protein by immunohistochemistry confirms that the exogenous N-CAM gene was transcribed and subsequently translated into N-CAM protein in the periosteal cells. Immunolocalization of N-CAM on the plasma membrane confirms that N-CAM proteins were properly folded and embedded in the plasma membrane.

6.4.1. Role of N-CAM in chondrogenesis

The effects of N-CAM on chondrogenesis were assessed in low cell density monolayer culture of QJ periosteal cells. Overexpression of N-CAM immediately before initiation of chondrogenesis significantly reduces the number of chondrocyte colonies. Hence, N-CAM seems to play an inhibitory role on chondrogenic differentiation in QJ periosteal cells. This result is consistent with transient expression of N-CAM during secondary chondrogenesis in the QJ hook: down-regulation in transformation from a periosteum to a perichondrium at day 11, reappearance in the NCL at day 15, and disapearance in the NCL at day 19. Since N-CAM prevents chondrogenic differentiation, N-CAM disappearance is therefore critical when periosteal cells switch to chondrogenesis in membrane bones. Down-regulation of N-CAM

permits periosteal cells to express the chondrogenic phenotype and is therefore a prerequisite for secondary chondrogenesis.

Inhibitory effects of N-CAM on chondrogenesis were not observed in monolayer cultures which were transfected before plating. Because the periosteal cells were only transiently transfected, the number of plasmids per cell was reduced as cells proliferated and eventually lost from most cells. Immunohistochemistry confirmed that N-CAM was undetectable in culture 7 days after plating. Therefore, despite the initial enhancement of N-CAM expression by transfection, N-CAM was no longer expressed when chondrogenesis occurs in this culture. This explains why chondrogenesis was not affected in these cultures. Unfortunately, I could only achieve transient transfection of N-CAM in the periosteal cells due to the limitation of primary culture. This disadvantage could be overcome if established cell lines were used to obtain stable transfection in which a foreign gene integrates into the genome of the host cell. Stable transfection was not employed in this study because: i) no established cell lines from avian membrane bone periostea are currently available; 2) primary cells are closer to periosteal cells *in vivo* and therefore results better represent the *in vivo* situation.

6.4.2. Role of N-CAM in osteogenesis

In the previous chapters, N-CAM was shown to be present during osteogenesis, suggesting it is necessary for osteogenic differentiation. However, N-CAM overexpression and antibody perturbation assays failed to suggest a specific role of N-CAM on

osteogenesis. No difference was found in micromass culture between the N-CAM transfected and control cells when periosteal cells were transfected in monolayer culture and then plated in micromass culture. Two major problems were found during the study. First, QJ periosteal cells did not form two cell layer in micromass culture after being trypsinization from monolayer culture, even in cultures which were not transfected. The periosteal cells form only a thin cell layer dominated by fibroblast-like cells in both transfected and non-transfected cells. It seems that periosteal cells lose much of their proliferation and differentiation capacities in the subculture. Second, N-CAM expression in the transfected cells occurs only transiently and is eventually lost. It is therefore difficult to evaluate the roles of N-CAM in osteogenesis with this method.

Antibody perturbation test did not affect osteogenesis in micromass culture. Although N-CAM antibody appeared to inhibit proliferation of the periosteal cells in some micromass cultures, consistent morphological changes were not seen in histological sections between cultures with and without addition of N-CAM antibody. Two possibilities can be draw from this result. First, N-CAM is probably not essential for osteogenic differentiation and therefore perturbation of N-CAM would not affect osteogenesis. Second, antibody used in this experiment did not block the specific site necessary for the function of N-CAM in osteogenesis and therefore osteogenesis is not affected by the antibody. N-CAM contains multiple domains for cell-cell and cell-matrix interactions (See Chapter 1), but its specific molecular mechanism in cell differentiation is not known. It is

probably worthwhile to use polyclonal antibodies or other monoclonal antibodies against various N-CAM domains. Unfortunately, other antibodies against chick N-CAM are not readily available. Hence, my experiments do not confirm whether N-CAM is a sufficient or necessary factor for osteogenesis. Participation of N-CAM in osteogenesis cannot be ruled out and further investigation is still needed.

6.5. A Functional Model of N-CAM in Skeletal Development

Based on the findings reported in this thesis and published data related to N-CAM in skeletal tissues, it seems that N-CAM plays different roles between early and late stages of skeletal development. A model for how N-CAM functions during skeletal development is proposed below.

6.5.1. Mediating cellular condensation and pattern formation

N-CAM is expressed during cellular condensation during both cartilage and membrane bone development in embryos. Condensation is an essential cellular event in morphogenesis and N-CAM is expressed in condensation processes of many organs, as in the formation of feathers (Chuong and Edelman, 1985), kidney tubules (Crossin et al., 1985), muscle (Knudsen et al., 1990; Soler and Knudsen, 1991), etc. Condensation is regulated by pattern-regulatory genes such as *Hox* genes, which encode genetic information for position and shape of a particular organ element. The regulatory region of the N-CAM gene contains homeodomain binding sites (Hirsch et al., 1990, 1991) and N-CAM gene expression is regulated by some *Hox* genes (Jones et al., 1992; Valarche et al., 1992;). Thus, N-CAM is probably a downstream target of *Hox* genes and may directly cause cells to condense by enhancing cellular aggregation through homophilic binding.

N-CAM may play a similar role in cartilage and membrane bone formation at this stage. N-CAM probably increases both bone and

cartilage formation by driving the condensation process through aggregating and recruiting mesenchyme cells. This would explain why enhancement of N-CAM in mesenchyme increases cartilage formation and perturbation of N-CAM decreases cartilage formation, as demonstrated by Chuong's group (Widelitz et al., 1993; Chuong et al., 1993).

On the other hand, N-CAM is not the only molecule regulating cellular condensation. Some other molecules such as N-cadherin, fibronectin, and tenascin have a similar role. This is probably because condensation is so critical for organogenesis that animals adopted duplicate mechanisms to avoid lethality due to mutation of a single gene.

6.5.2. Mediating alternative cell differentiation pathways after condensation

After condensation, the cells in precartilaginous and membrane bone condensations undergo further differentiation to express osteogenic or chondrogenic phenotypes. N-CAM expression differs at this stage between the two skeletal tissues: N-CAM persists in osteogenesis but disappears in chondrogenesis. This pattern is demonstrated *in vivo* and *in vitro* in both primary and secondary cartilage. This raises the possibility that N-CAM plays a role in controlling further differentiation of skeletal cells. This possibility is further strengthened by transient expression of N-CAM during secondary cartilage development in the QJ hook. More importantly, my experiments demonstrate that N-CAM has an inhibitory role on

chondrogenesis in monolayer cultures of QJ periosteal cells. Thus, N-CAM regulates skeletal cell differentiation by preventing chondrocyte phenotype expression and promotes osteogenesis.

The intracellular signal pathway by which N-CAM influences chondrogenesis is not clear. As addressed before, cell shape has a great effect on chondrogenic phenotype expression. An isolated environment without cellular contact is a permissive condition for chondrogenesis, allowing cells to round up and activate chondrocyte phenotype genes as demonstrated in low density and agarose cultures. Thus, N-CAM may prevent chondrogenesis by blocking cell shape and cytoskeleton architectural changes necessary for chondrocytes. As an adhesion molecule, expression of N-CAM results in cell-cell homophilic binding. N-CAM also participates in the formation of cell junctions, such as gap junctions. Consequently, N-CAM may prevent the separation of cells by extracellular matrix *in vivo*. Furthermore, interactions between N-CAM and N-CAM or N-CAM and other molecules can influence intracellular second messenger systems, including increases of intracellular calcium, decrease of inositol phosphates IP2 and IP3, and decrease of intracellular pH (Schuch et al., 1989; Williams et al., 1994). An involvement of G-proteins and tyrosine kinases of in N-CAM signal transduction pathway has been suggested (Schuch et al., 1989; Williams et al., 1994). Both pathways are associated with proteins which influence the cytoskeleton. For instance, activation of G-protein coupled receptors and tyrosine kinase receptors can lead to protein kinase C activation which associates with

the cytoskeleton (Newton, 1997). Thus, an N-CAM signal may affect cytoskeletal architecture and influence cellular phenotypic expression.

In contrast to chondrogenesis, osteogenesis does not require an isolated microenvironment and cellular contact is always maintained throughout differentiation. This may be why down-regulation of N-CAM is not required for osteogenesis. In fact, although my overexpression and antibody perturbations assays failed to support a specific role of N-CAM in osteogenesis, it is possible, based on immunostaining data, that N-CAM enhances osteogenesis or secretion of bony matrix in osteoblasts.

In summary, N-CAM may play opposite roles in two development stages of chondrogenesis. In the early stage, i.e. during condensation, N-CAM stimulates chondrogenesis by promoting mesenchymal cell aggregation. After condensation, N-CAM inhibits chondrogenic phenotype expression by preventing cellular shape changes necessary for the chondrocyte phenotype. In contrast, in membrane bone, N-CAM probably has a promoting role in both early and late differentiation stages. N-CAM may regulate differentiation pathways of periosteal cells by differentially altering gene expression in chondrogenesis and in osteogenesis. Hence, for secondary chondrogenesis, down-regulation of N-CAM is a prerequisite when periosteum transforms to perichondrium.

Chapter 7

Conclusions

1. Secondary chondrogenesis in chick quadratojugal hooks is initiated between days 11 and 12 in most embryos. After initiation, secondary cartilage develops on the posterior and anterior sides of the QJ hook until 14 days. From 15 days, a new cell layer, which prevents further cartilage formation, appears between the existing cartilage and the perichondrium. Meanwhile, the already-formed cartilage in the hook undergoes endochondral ossification, resulting in significant enlargement of the hook. The location and morphology of the new cell layer suggests its association with the chondrogenic lineage and that these cells probably are dedifferentiated from chondroblasts. The NCL resumes chondrogenic differentiation at day 19 and gives rise to the second phase of secondary cartilage in the hook, which produces the cartilage that covers the entire hook and forms the articular cartilage in the QJ-quadrate joint.

2. Initiation of secondary chondrogenesis at days 11 and 19 in the hook is dependent on biomechanical stimulation generated by embryonic movement. Paralysis of embryos at day 9 prevents secondary cartilage from forming. Paralysis of embryos at day 13 prevents the second phase of secondary cartilage formation.

3. Five growth factors (TGF-β, aFGF, bFGF, EGF, and PDGF) are expressed both in bone and cartilage cells. Transient expression patterns of these growth factors in the switch from osteogenesis to

chondrogenesis were not seen and therefore they are unlikely to be factors triggering secondary chondrogenesis in the hook.

4. N-CAM exhibits a temporal and spatial pattern of expression during secondary chondrogenesis. N-CAM is expressed in the periosteum and is down-regulated when periosteum switches to perichondrium. Down-regulation of N-CAM in the hook occurs earlier than morphological initiation of secondary cartilage but is coincident with commitment for secondary chondrogenesis. Furthermore, N-CAM is up-regulated in the new cell layer when chondrogenic differentiation ceases in the hook around day 17 and is again down-regulated when the NCL resumes the second phase of chondrogenesis at day 19.

5. Down-regulation of N-CAM in the two phases of chondrogenesis in the QJ hook is dependent on biomechanical stimulation. In embryos paralyzed on day 9, N-CAM expression persists in the hook and the first phase of secondary cartilage is not seen. In embryos paralyzed on day 13, N-CAM expression remains in the NCL and the second phase of secondary chondrogenesis is prevented. Therefore, change of N-CAM expression is directly related to chondrogenesis and to embryonic movement, which suggests that N-CAM may mediate cell differentiation in the periosteum.

6. To further understand secondary chondrogenic differentiation, several cell culture approaches were developed with QJ periosteal cells enzymatically released from the shafts. Chondrogenesis was achieved in low density monolayer, clonal, and agarose suspension cultures. In monolayer culture of exceptionally low

density, the QJ periosteal cells differentiated into fully differentiated chondrocytes, characterized by a round shape, type II collagen expression, and pericellular refractile ECM positive for alcian blue staining. This is the first demonstration that fully differentiated chondrocytes can be achieved from membrane bone periosteal cells in primary monolayer culture.

7. Differentiation pathways of QJ periosteal cells *in vitro* are greatly influenced by culture conditions. In high cell density (micromass) culture, the periosteal cells give rise to a bony cell layer and a fibroblast-like cell layer. In low cell density culture, chondrocyte colonies formed. In moderate cell density culture, neither chondrogenesis nor osteogenesis occurred. It seems that chondrogenesis requires cells to be in an isolated microenvironmental condition. Cell-cell contact likely inhibits chondrogenesis, but probably promotes osteogenesis.

8. Both low cell density and clonal cultures show that chondrogenesis often occurs from osteoblast-like polygonal cell colonies which express APase and type I collagen. Moreover, some early chondrocytes also show APase and type I collagen expression. Since APase and type I collagen are expressed in osteogenic cells and are not seen in nonhypertrophied chondrocytes in primary cartilage, secondary chondrocytes are closely related to the osteogenic lineage. Secondary chondrocytes likely share a common precursor with osteogenic cells. Moreover, secondary chondrocytes may differentiate from certain cell types in the osteogenic lineage rather than from stem cells. Intramembranous differentiation may include a transient

stage in which cells undergo both osteo- and chondrogenesis. Osteogenesis would be the normal pathway, but chondrogenesis is evoked in certain microenvironments.

9. The condensation process is not involved in secondary chondrogenesis *in vitro*. Chondrogenesis in low density monolayer, clonal and suspension cultures without condensation indicates that cellular condensation is not a prerequisite for chondrogenic phenotype expression. It is probable that condensation and chondrogenic differentiation are two separate events regulated by different mechanisms.

10. To further characterize the expression pattern during skeletal differentiation, N-CAM was studied in several cell and organ culture models. The pattern of expression of N-CAM *in vitro* is consistent with that *in vivo*. N-CAM is expressed in micromass culture of QJ periosteal cells, and at sites of membrane bone formation of both mandibles and QJs in organ cultures. On the other hand, N-CAM is not expressed in situations where cells are undergoing chondrogenic differentiation, including monolayer and suspension cultures of QJ periosteal cells. Hence, both *in vivo* and *in vitro* data demonstrated that N-CAM expression is associated with osteogenesis but not chondrogenesis. Occurrence of the chondrogenic phenotype in monolayer and agarose cultures without N-CAM expression indicates that N-CAM is not a signal required for chondrogenic differentiation.

11. The role of N-CAM in regulating the switch between osteogenesis and chondrogenesis was tested by overexpression and perturbation of N-CAM *in vitro*. Overexpression of N-CAM was achieved

by transfection of a plasmid containing the N-CAM gene into periosteal cells in monolayer culture. Enhancement of N-CAM expression results in a significant reduction in chondrocyte colonies, indicating an inhibitory effect of N-CAM on chondrogenic differentiation from periosteal cells. Meanwhile, micromass culture with N-CAM overexpression and antibody perturbation did not show any significant effect on osteogenesis.

12. N-CAM appears to play two different roles in cartilage development. In the mesenchymal condensation stage, N-CAM enhances precartilaginous condensation by increasing cell aggregation and recruitment through homophilic binding and therefore promotes cartilage formation by increasing the numbers of potential cartilage cells. However, at the differentiation stage, N-CAM limits cartilage phenotypic gene expression and therefore inhibits chondrogenesis. In membrane bones, N-CAM is expressed in periosteal cells undergoing osteogenesis. Down-regulation of N-CAM as a response of bipotential periosteal cells to embryonic movement is a prerequisite for transformation of periostea to perichondria and initiation of secondary chondrogenesis.

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IMAGE EVALUATION
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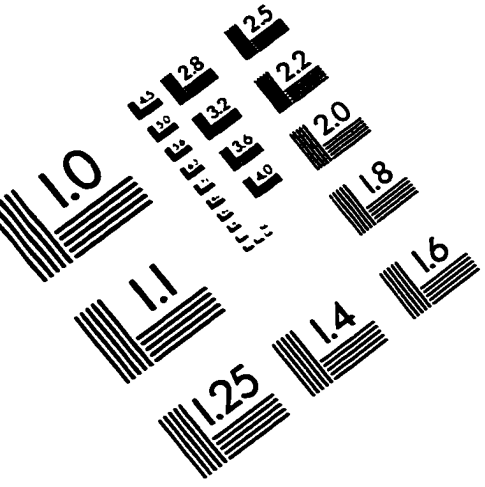

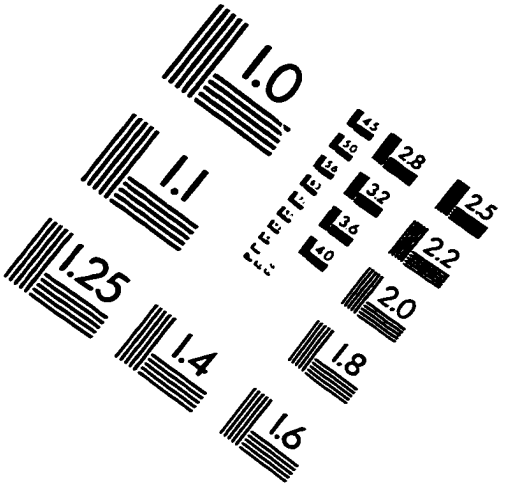

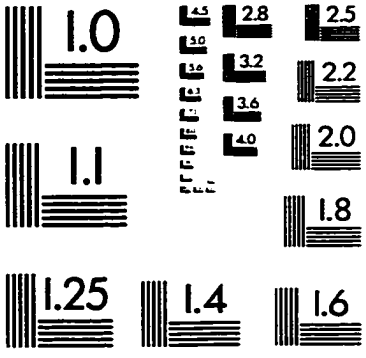

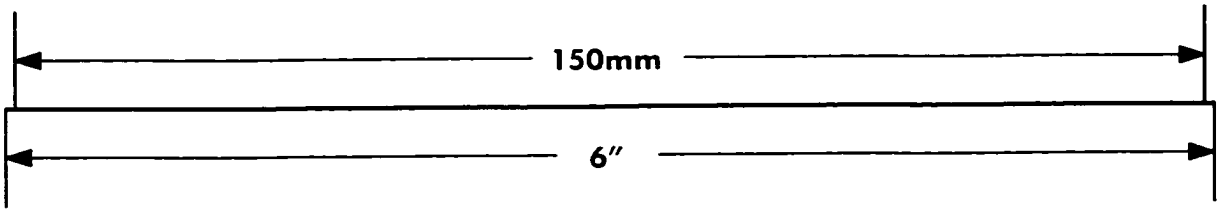

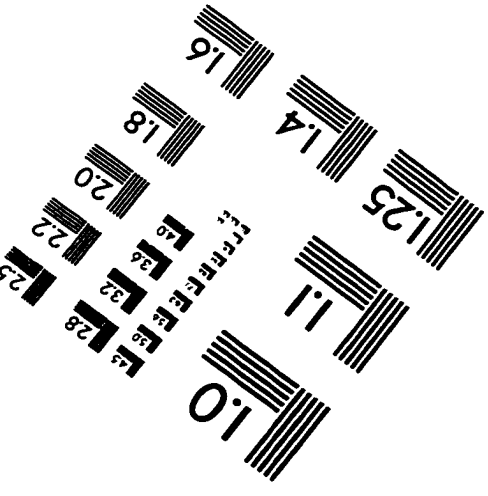

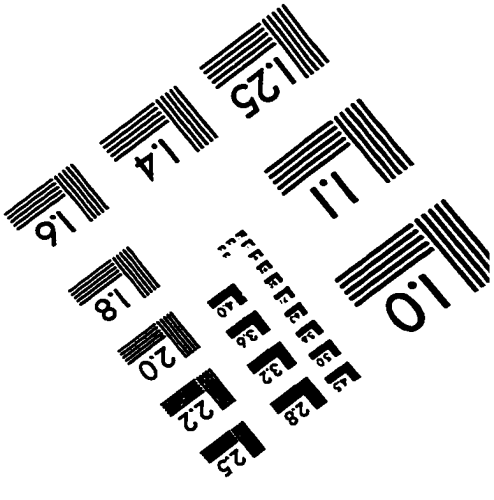

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