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REVIEW ARTICLE

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Chondrogenic cell differentiation from membrane bone periostea

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Abstract Most craniofacial membrane bones are derived from neural crest (NC) cells. Interaction between NC cells and epithelium, and cellular condensation, are two major events that lead NC cells to become osteoblasts that deposit membrane bone. Unlike endochondral bone, membrane bone formation is not preceded by cartilage formation in normal development. However, chondrogenic potential in membrane bone is evidenced by several cartilage-associated phenomena in vivo. Furthermore, in vitro, periosteal cells of some membrane bones express cartilage phenotype gene products and even differentiate into chondrocytes. Hence, membrane bone periosteal cells can undergo chondrogenic differentiation. The precursor of chondrogenic cells in membrane bone is not clear: chondrocytes were proposed to arise from unipotential chondroprogenitor cells, bi- or multipotential progenitor cells, or differentiated osteogenic cells. There is experimental support for each, but studies on clonal and cell cultures provided more support for a common precursor of both chondro- and osteogenic cells. Moreover, in periostea, chondrogenesis probably arises from a differentiated cell type. Membrane bone formation in periostea may include a transient cell stage that is able to undergo both osteo- and chondrogenesis. Osteogenesis would be the normal pathway, but chondrogenesis can be evoked in certain microenvironments. It is not known whether microenvironmental factors trigger chondrogenesis through a universal molecular mechanism, nor is the molecule that triggers chondrogenesis known. Expression of neural cell adhesion molecule (NCAM) is down-regulated during commitment of periostal cells for secondary chondrogenesis, suggesting a possible regulatory role for NCAM in the alternative differentiation pathways of periosteal cells.

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Introduction

Higher vertebrate skeletons are formed by either endochondral or intramembranous bones. Endochondral bones include most of the axial and appendicular skeleton as well as some bones at the base of the skull. They form through endochondral ossification in which mesenchymal cells first develop into a cartilaginous template through precartilaginous condensation. The cartilage hypertrophies, is destroyed by vascular invasion and replaced by trabecular bone and bone marrow. Periosteum replaces the perichondrium adjacent to hypertrophic cartilage and osteogenesis is initiated to form lamina bone in the diaphyses of long bones (Scott-Savage and Hall 1980). Intramembranous bones, also called membrane bones, are flat and mostly seen in the cranial vault and facial region. They develop directly from mesenchyme without an intermediate cartilaginous phase in normal development.

The outer layer of the periosteum is fibrous, containing fibroblasts and abundant collagen fibers. It provides attachment for bone to tendons, ligaments and muscles. Its inner cambial layer contains cells at different stages of osteogenesis (Scott-Savage and Hall 1980). Osteogenic differentiation in a periosteum is a multi-step process during which cells progressively increase expression of osteogenic markers, and decrease proliferation (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, making 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 with type I collagen as 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 embedded in bony matrix. Hence, the periosteum is highly heterogeneous and contains cells in all transitional stages of osteogenic differentiation, as well as fibroblasts.

Because osteogenic markers are expressed in osteogenic cells sequentially, 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. All the osteogenic markers listed above have been reported in hypertrophic chondrocytes (Rodan and Noda 1991; Roach 1992). Thus, different stages of osteogenic cells are difficult to distinguish, especially early in differentiation.

Although cell differentiation in periostea has been extensively studied, many questions remain. One is the chondrogenic capacity of membrane bone periostea. It is well known that in endochondral bone, such as the tibia and ribs, chondrocytes can arise from periostea. For instance, tibia periosteal cells can differentiate into both cartilage and bone in high-density culture (Nakahara et al. 1990a, 1991, 1992; Nakata et al. 1992), or in diffusion chambers (Nakahara et al. 1990b, 1992). It is suggested that periostea contain stem cells for both osteogenesis and chondrogenesis (Caplan 1990), which could explain chondrogenesis in endochondral bone periostea, since mesenchyme has undergone precartilaginous condensation, a prerequisite for commitment of chondrogenic cells. In contrast, membrane bones arise directly from mesenchyme that never undergoes precartilaginous condensation or cartilage formation in normal development. For these reasons, several authors have claimed that membrane bones do not have chondrogenic potential (see review by Beresford 1981). However, chondrogenic capacity does exist in membrane bone. In fact, chondrogenic differentiation is important for growth, fracture repair, and articular cartilage formation in membrane bone. During the past several years, research on chondrogenic phenotypes in membrane bone has made remarkable progress and provided us with a better understanding of membrane bone. In this paper, we review recent progress in chondrogenic differentiation from membrane bone and discuss the precursor(s) of chondrogenic cells, relationships between osteogenic and chondrogenic cell lineages, and regulation of chondrogenesis in membrane bone.

Embryonic origin and commitment of membrane bone cells

Most of the craniofacial skeleton is of neural crest (NC) cell origin (Le Lièvre and Le Douarin 1975; Noden 1975, 1978; Le Lièvre 1978; Couly et al. 1993; Le Douarin et al. 1993). The NC is a group of cells located at the boundary between neural plate and epidermis in neurula stage embryos. NC cells 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 et al. 1993).

All facial membrane bones, including the mandible, the quadratojugal (QJ) and their secondary cartilage, are derived from NC (Noden 1975, 1978, 1988; Le Lièvre 1978; Couly et al. 1993; Le Douarin et al. 1993; Köntges and Lumsden 1996). NC also contributes to the skull. The frontal and parietal bones of the skull, previously believed to be of both NC and mesodermal origin (Noden 1975, 1978, 1988; Le Lièvre 1978), were recently found to arise entirely from NC cells (Couly et al. 1993; Le Douarin et al. 1993).

NC cells from different regions along the anterior-posterior axis of the neural tube migrate along characteristic pathways and reach specific sites, where they differentiate into distinct cell types. For instance, to form the mandibular skeleton of the embryonic chick, the presumptive mandible NC cells detach from epithelium of the mesencephalon at HH (Hamburger and Hamilton 1951) stage 8.5 (28 h of incubation) and reach the mandibular arch at HH stage 15 (52 h of incubation). They accumulate in the mandibular arch and differentiate into either Meckel's cartilage at HH stage 25–26, or into membrane bones at stage 31 (Tyler and Hall 1977; Hall 1978). In order to generate a skeletal element, NC cells undergo two major events: tissue interaction (Bee and Thorogood 1980; Hall 1991, 1992; Le Douarin et al. 1993) and skeletal condensation (Hall and Miyake 1992, 1995).

Interaction of NC with epithelium is crucial for commitment to a skeletal cell fate (Langille 1994). Isolated 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). The mechanism of tissue interaction is not fully 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 cells in vitro, while TGF-β1, another member of the same superfamily, promotes smooth muscle differentiation (Shah et al. 1996). During skeletogenesis, a number of molecules have been suggested as putative signalling molecules sent from epithelium to mesenchyme during tissue interaction. These include epithelial growth factor (EGF; Hall 1992), BMP-2 and -4 (Bennett et al. 1995; Ekanayake and Hall 1997), transforming growth factor-α (TGF-α; Huang et al. 1996), ET-1 protein (Richman and Mitchell 1996), and serotonin (Moiseiwitsch and Lauder 1997).

After NC cells arrive at the presumptive sites of a skeletal element, they form 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 both a basic cellular process and a unit of skeletal morphogenesis through which the overall pattern of a particular 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 the loss, ectopic addition, or change in morphology of a particular skeletal element (Erlebacher et al. 1995; Richman and Mitchell 1996).

There are two kinds of skeletal condensations. *Precartilaginous* condensations develop into primary cartilage. *Membrane bone* condensations develop into membrane bones (Hall and Miyake 1992, 1995; Dunlop and Hall 1995). Membrane bone condensation occurs in mesenchyme, followed by the appearance of an ossification center. In chick mandibles, membrane bone condensation can be visualized with peanut agglutinin lectin at HH stage 26 (5.75 days; Dunlop and Hall 1995). In the chick quadratojugal, condensation occurs by day 7 (Murray 1963). Condensation is critical for overall patterning of a membrane bone, but osteogenic cell differentiation does not depend on condensation. In mandibles, mesenchymal cells express APase – i.e., they become preosteoblasts – *prior to* condensation (Dunlop and Hall 1995). Hence, in mandibular membrane bone, preosteoblasts and not undifferentiated mesenchymal cells undergo cellular condensation. Following condensation, cells further differentiate into osteoblasts, which produce bony matrix and can be identified histologically as an ossification center. Finally a membrane bone is formed with a bony core surrounded by a periosteum.

Evidence for chondrogenic potential of membrane bones

Chondrogenic phenotype expression from membrane bone in vivo

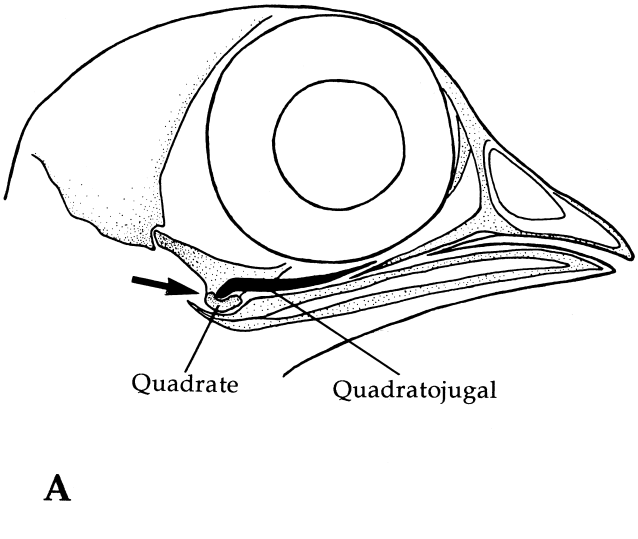
Cartilage or the cartilaginous phenotype is seen in membrane bone in vivo under certain circumstances, which include formation of secondary cartilage in some membrane bones, callus cartilage during fracture repairing, chondroid bone in developing skull, and cartilaginous tissue in calcium-deficient embryos.

Secondary cartilage formation

In normal embryonic development, cartilage develops in some membrane bones. Unlike primary cartilages, which form from mesenchymal condensation, chondrocytes in secondary cartilage are derived from periosteal cells. Because this cartilage appears after bone is established, it is termed secondary cartilage to distinguish it from primary cartilage. Secondary cartilage is also called accessory, adventitious, or embryonic cartilage (Murray 1963; Beresford 1981). Secondary cartilages are found in mammals, birds, and fish. In mammals, the mandible develops secondary cartilages in several locations, but the number of secondary cartilage sites varies among species. In mouse and rat mandibles, secondary cartilage is seen in the condylar, angular, and coronoid processes (Beresford 1981; Vinkka 1982). 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 extensively studied and characterized.

Since secondary cartilage develops from the periostea of membrane bones, its cell differentiation differs from primary cartilage. *Before* committing to secondary chondrogenesis, the periosteum where secondary cartilage will develop is morphologically identical to other regions of the periosteum and is undergoing intramembranous bone formation. However, *when* commitment to chondrogenesis occurs, the periosteum ceases bone formation and young chondroblasts arise from the periosteum, which is now a perichondrium (Hall 1979; Fang and Hall 1995). As chondrogenesis continues, hyaline cartilage appears between the perichondrium and membrane bone. In chicks, the switch from osteo- to chondrogenesis is dependent upon biomechanical stimulation generated by embryonic movement. Secondary chondrogenesis fails to occur if embryos are paralyzed before cartilage formation is triggered (Murray and Smiles 1965; Hall 1972, 1979, 1986; Fang and Hall 1995). Therefore, secondary cartilage formation is a response of membrane bone periostea to the local mechanical environment. In general, secondary cartilage functions as: (1) a growth center of bone, (2) an articular cartilage.

The chick quadratojugal is a membrane bone in the upper jaw with a slender shaft and a posterior hook (Fig. 1A). The QJ hook articulates with the quadrate (Fig. 1). During normal development before day 10, the QJ hook contains a bony core and surrounding periosteum. At day 11, triggered by biomechanical forces between the QJ and quadrate, the periosteum of the QJ hook on the posterior and anterior sides (not laterally, where force is not exerted) becomes committed to chondrogenesis (Hall 1972, 1979; Thorogood 1979; Fang and Hall 1995). The periosteum ceases bone formation, its cells gradually become more rounded, and then produce alcian-blue-positive extracellular matrix (ECM). Chondroblasts become distinguishable in embryos after day 12 (Fig. 1; Fang and Hall 1995). In embryos between days 12 and 14, secondary cartilage grows rapidly and some chondrocytes become hypertrophic. From day 15 to 17, blood vessels invade the hook and secondary cartilage is destroyed by osteoclasts. Meanwhile, new bony tissue forms via endochondral bone formation in the hook. Most secondary cartilage is replaced by bone via endochondral ossification similar to the situation in long bones. However, some secondary cartilage seems to be transformed directly into bone (Hall 1972). Consequently, secondary cartilage formation followed by endochondral bone formation greatly enlarges the QJ hook. Chondrogenesis in



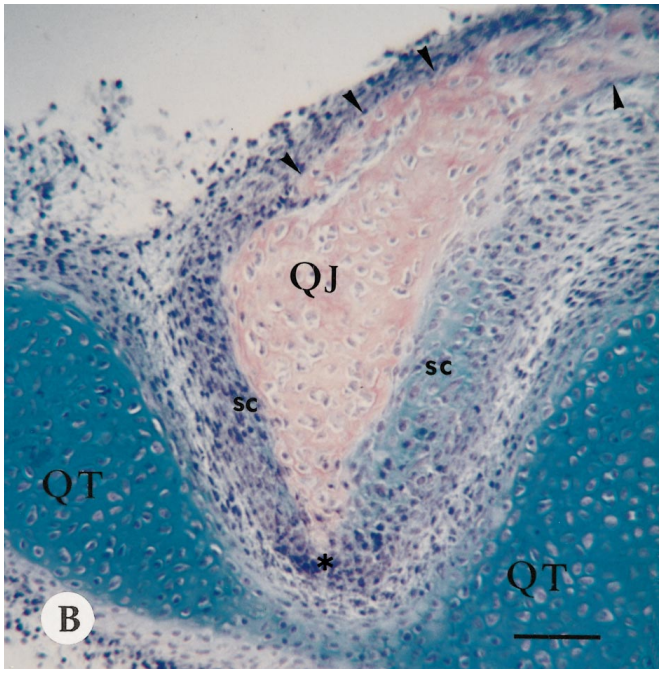


Fig. 1A, B Secondary cartilage in the avian quadratojugal. **A** An illustration of the chick head showing the position of the quadratojugal and its articulation with the quadrate. *Arrow* indicates the joint of the quadratojugal hook and the quadrate. **B** Histological section of the quadratojugal and quadrate joint in a 13-day chick embryo. Secondary cartilage (*sc*) forms at anterior and posterior sides of the quadratojugal (*QJ*) hook. The QJ hook articulates with the quadrate (*QT*), which appears as a primary cartilage at this developmental stage. Osteogenesis continues in the QJ shaft (*arrowheads*) and at the tip of the hook (*asterisk*). Section was stained with HBQ (Hall and Brunt's quadruple stain). Cartilage is blue and bone red. *Bar* 100 µm

the QJ ceases around day 15 but resumes after day 17 as a second phase of secondary chondrogenesis. The cartilage from the second phase is distributed along the articular surface of the hook (Fang and Hall 1995) to becomes the fibrous articular cartilage in the QJ-quadrate joint.

In mouse mandibular condyle, secondary cartilage arises from the periosteum in embryos at day 16 in utero, contributing to elongation of the condylar process through endochondral bone formation and to the articular cartilage in the squamosomandibular joint (Livne and Silbermann 1990). In the condyle of a neonatal mouse or in other mammals, several distinct cell layers exist. From the articular surface inwards they are: (1) fibrous cells, (2) progenitor cells, (3) chondroblasts, (4) chondrocytes, and (5) hypertrophic chondrocytes. The progenitor cell layer is mesenchyme-like and undergoes active cell proliferation (Livne et al. 1990; Livne and Silbermann 1990), which is enhanced by mechanical stimulation (Kantomaa et al. 1994). From progenitor cells to hypertrophic chondrocytes is a dynamic process of secondary chondrogenesis, during which cells gradually enlarge and become hypertrophic (Landesberg et al. 1995). The hypertrophic chondrocytes undergo resorption and endochondral ossification. Secondary cartilage formation in the condyle gradually ceases as the animal matures. A thin layer of secondary cartilage remains in the adult condyle as fibrous articular cartilage (Livne and Silbermann 1990).

Unlike avian secondary cartilage, cartilage formation in rodent mandibular processes can be *initiated* without biomechanical stimuli (Glasstone 1971; Herring and Lakars 1981; Vinkka-Puhakka and Thesleff 1993). However, *maintenance* of secondary cartilage in rodents requires mechanical stimulation. When fetal condyles are grown in organ culture, removed from mechanical stimuli, already-formed secondary cartilage gradually disappears and the progenitor cell layer, which previously gave rise to chondroblasts, switches to osteogenesis (Strauss et al. 1990; Ben-Ami et al. 1993). Finally the secondary cartilage is replaced by bone in cultured condyles. The contractile forces generated by masticatory muscles seem to be a major biomechanical stimulus exerted on the condyle (Takahashi 1991; Takahashi et al. 1995). Hyperactivity of the lateral pterygoid muscle leads to the disappearance of secondary cartilage in condyles of young rats. Instead, intramembranous bone formation takes place from the progenitor layer of the periosteum (Takahashi 1991; Takahashi et al. 1995).

Cartilage formation during fracture healing

In certain circumstances, chondrogenesis is evoked in membrane bone where cartilage development is normally not seen. One such situation is during fracture healing. It is well known that repairing fractured long bones involves the formation of cartilage that bridges the gap at the fracture site (McKibbin 1978). The cartilage is then replaced by bony tissue through endochondral ossification. Several investigators have reported the absence of

cartilage during membrane bone fracture healing (Richany et al. 1963; Radden and Fullmer 1969; Alberius and Johnell 1991), but cartilage was observed by many 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 fracture sites. Favorable conditions may include poor vascular supply or low oxygen tension (Girgis and Pretchard 1958; Henricson et al. 1987; Alberius and Johnell 1991). Interestingly, in the fracture site where chondrogenesis was not found, some cartilaginous macromolecules were detected (Alberius and Johnel 1991).

Callus cartilage cells in membrane bone repair may arise from periosteal cells. If the periosteum is removed before fracture occurs, cartilage nodules fail to form in the blastema, suggesting the periosteal origin of these chondrocytes (Hall and Jacobson 1975). Periosteal cells at the fracture site divide to form the blastema and differentiate into chondroblasts and chondrocytes to form cartilage nodules. They become hypertrophic and are replaced by bone through endochondral ossification later.

Condroid bone

Another cartilaginous phenotype is chondroid bone in some developing membrane bones. Chondroid bone refers to tissues which have characteristics of both bone and cartilage. The earliest description of such tissue was by Schaffer in 1888, but its definition is sometimes confusing because of the variety of histological features and terminology used (Beresford 1981; Taylor et al. 1994). Typically, chondroid bone was described as intermediate between bone and cartilage (Hall 1978).

Chondroid bone was 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; Lengelé et al. 1990, 1996). This tissue has cartilaginous cells that are larger than osteogenic cells and which express some chondrogenic markers such as type II collagen (Goret-Nicaise 1984), but its ECM appears bone-like with methylene blue staining (Lengelé et al. 1990, 1996) and contains type I collagen.

Chondroid bone often appears when membrane bones are undergoing fast growth (Lengelé et al. 1990). It does not become a genuine cartilage and is absent from adults. It remains unclear whether chondroid bone transforms to a bony tissue, is replaced by membrane bone, or persists. Goret-Nicaise (1984) claimed that chondroid bone is not transformed into bone and believed that it was replaced by membrane bone. However, replacement must involve resorption of chondroid bone, followed by osteogenesis. Evidence for resorption remains to be seen.

Since intramembranous bone is not preceded by cartilage, chondroid bone apparently does not represent a tissue transforming from cartilage to bone. Lengelé et al. (1996) considered it a distinct tissue type and an initial modality of skeletogenesis in membrane bones. Hence, chondroid bone may represent a special differentiation status during membrane bone development in which cells express both cartilaginous and bony characteristics. The significance of condroid bone is not well understood. The presence of this tissue in many fast growth sites of the fetal skull, and its later disappearance, indicate an adaptation to the rapid growth of membrane bone.

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 resulted 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 was 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 demonstrate that calvarial bones do have chondrogenic potential that is inhibited in normal embryos but can be induced. Furthermore, since cartilage was found in organ cultures of calvaria after its periosteum was removed, it seems that chondrogenic potential exists in the cells in the center of the membrane bone.

Expression of chondrogenic markers in developing intramembranous bones

Absence of cartilage formation is a significant characteristic of intramembranous bone formation. 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 bone formation actually involves expression of some genes associated with the cartilaginous phenotype. There are two kinds of procollagen II mRNA from differential splicing during transcription (Sandell et al. 1991). They differ in either including (type IIA) or excluding (type IIB) exon 2 of α1(II) gene.

Type IIA is seen in prechondrocytes as well as in some non-cartilage cells during development, such as in mesenchymal cells, notochord, and spinal ganglion, while type IIB is found only in mature chondrocytes (Sandell et al. 1991; Sandell 1994; Hughes et al. 1995). It remains to be demonstrated whether both types, or only type IIA, are expressed in membrane bone. Similarly, type IX collagen has two kinds of mRNA. Membrane bone expresses only the truncated form of α1(IX), which lacks exon 1–7, while hyaline cartilage expresses the entire α1(IX) (Ting et al. 1993). The significance of type II and IX expression in membrane bone is not clear. Since α1(IX) is expressed only in preosteoblasts, Ting et al. (1993) considered expression of type II and IX collagen as an early phenotypic feature of osteoblast differentiation and postulated that a switch in two kinds of α1(IX) mRNA transcription may be associated with the phenotypic switch between osteogenesis and chondrogenesis.

Chondrogenic phenotype of membrane bone periosteal cells cultured in vitro

The above in vivo data provide evidence of chondrogenic potential in membrane bones, but chondrogenesis occurs only in certain circumstances. Can calls isolated from membrane bone undergo chondrogenic differentiation? In vitro cell culture not only provides insights into osteo- and chondrogenic cell differentiation that would illuminate their in vivo counterparts, but also allows manipulation of cells by various cellular and molecular techniques.

Expression of cartilage phenotypic genes in cultured calvarial cells

Periosteal cells sequentially digested from calvaria provide 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 that include fibroblasts, osteoprogenitor cells, preosteoblasts, osteoblasts, and young and mature osteocytes. When plated in monolayer culture, 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). However, studies on collagen synthesis in chick calvarial periosteal cells show that, at the beginning of culture, the predominant collagens are osteogenic (type I and V collagens). After 14 days, type II and X collagens are expressed at high levels (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), it seems that calvarial cells can express some chondrogenic markers in monolayer culture. However, further expression of the chondrogenic phenotype seems to be limited by the more predominant osteogenic pathway in cultured calvarial cells and, therefore, they do not further differentiate to chondrocytes.

Chondrogenesis from membrane bone periosteal cells in vitro

Calvarial cells do not differentiate into morphologically recognizable chondrocytes in monolayer culture. The only exception seems to be the calvarial cells from rat parietal bone and sutural areas. Those cells can form cartilage nodules in vitro in the presence of dexamethasone (Bellows et al. 1989). However, as rat parietal bone and sutural areas contain cartilage tissue, progenitor cells for chondrocytes may arise from the cartilage (Bellows et al. 1989).

To achieve chondrogenesis from membrane bone in vitro, various culture conditions and cell subpopulations of periosteal cells have been tested. The favorite 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) and quadratojugal (J. Fang, B.K. Hall, unpublished observation) can differentiate into chondrocytes. 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 heterogeneous, it was postulated that certain subpopulations may have higher chondrogenic potential. By separating cell subpopulations of calvarial cells, Wong and Tuan (1992, 1995) achieved 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 by 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 while 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 and 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.

Overt chondrogenesis was not previously obtained in unfractionated calvarial cells in monolayer culture, but recently it was achieved in cell culture of QJ periosteal cells (Fang and Hall 1996b). The shaft of the quadratojugal is membrane bone. Secondary cartilage only appears

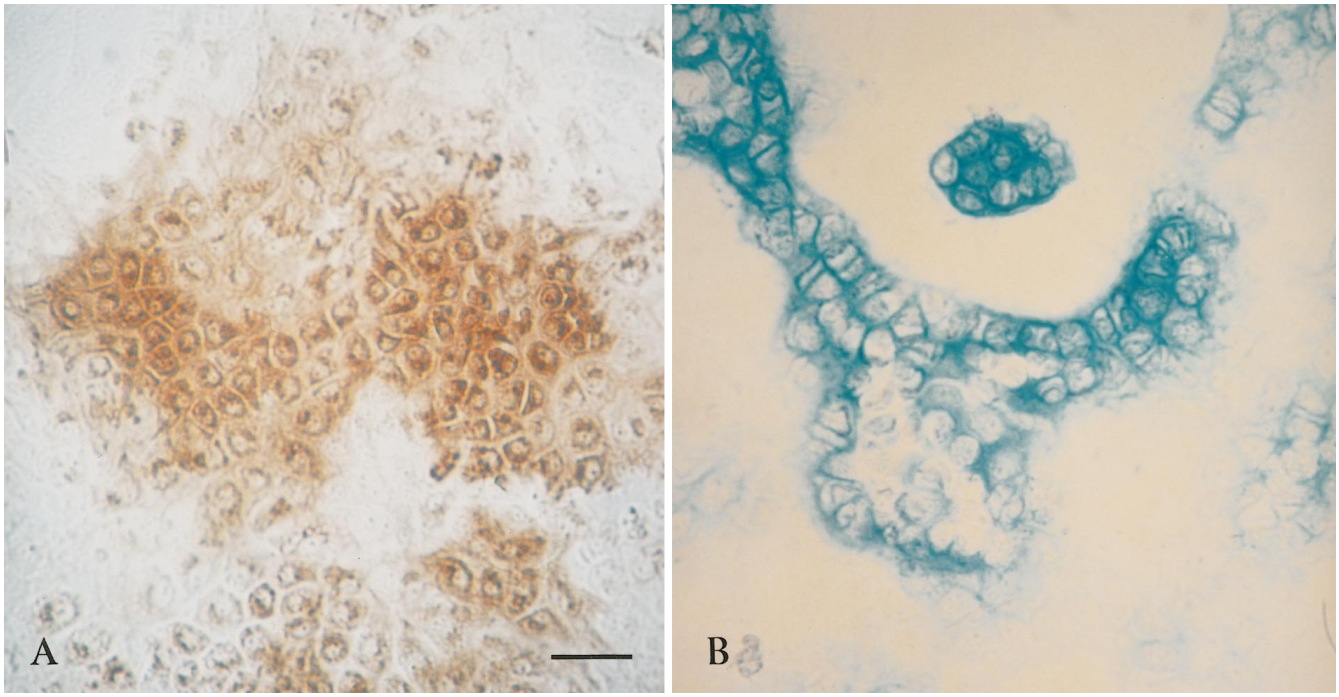


Fig. 2A, B Chondrogenic phenotype in cultured quadratojugal periosteal cells. The periosteal cells were enzymatically released from the QJ shafts and plated in low density monolayer culture (1×10^4 cells/ml). The cells were cultured for 14 days in Ham's F-12 and BGJb (3:1) medium, containing 10% fetal bovine serum and 150 µg/ml ascorbic acid. **A** Immunohistochemical staining of type II collagen; type II collagen was localized in the cytoplasm and extracellular matrix (ECM) of chondrocytes. **B** Alcian blue staining, showing positive staining in ECM of chondrocytes. *Bar* 40 µm

in the QJ hook. However, if the intact shaft is cultured submerged, chondrocytes differentiate from periosteal cells (Thorogood 1979), indicating the chondrogenic capacity of QJ periosteum. Recently, we demonstrated that enzymatically released QJ periosteal cells have high chondrogenic potential (Fang and Hall 1996b). QJ periosteal cells were released from the QJ shafts with collagenase and trypsin and plated at various densities. Chondrocyte colonies appeared in day 7–8 cultures in low density. These cells are typical chondrocytes in morphology (rounded shape and refractile ECM) and express chondrogenic markers such as type II collagen and sulfated proteoglycan (Fig. 2). Thus, chondrocytes can be achieved from membrane bone periosteal cells in primary monolayer culture.

Conclusions

Evidence from in vivo and in vitro studies demonstrates that membrane bone periosteal cells undergo both osteogenic and chondrogenic differentiation. In most circumstances, osteogenesis is the predominating pathway, and chondrogenesis is inhibited. However, chondrogenesis can be evoked under certain conditions and cartilage formation seems to be an adaptive response of membrane bone to local microenvironmental stimulation, such as articular movement for secondary cartilage formation, bone fracture for callus cartilage, or rapid growth for chondroid bone development. Cartilage is the only skeletal tissue that has active cell division, while osteoblasts deposit bony ECM but do not divide in vivo (Nijweide et al. 1988; McCulloch et al. 1990). Accordingly, in the sites where chondrogenic phenotypes appear – secondary cartilage, chondroid bone, and fracture healing – rapid skeletal growth is always required. Based on this, the significance of chondrogenesis in membrane bones may be that chondrogenic differentiation achieves a larger volume of skeletal tissue within a shorter period than does osteogenesis. This would explain why only small bones, especially flat bones, can form through intramembranous bone formation, while most of the skeleton, especially long bones, goes through endochondral bone formation. Intramembranous bone formation retains chondrogenic potential to meet certain circumstances when rapid increase of bone volume is demanded.

Progenitor cells and their differentiation

To understand chondrogenesis in membrane bones, it is necessary to explore the origin of the chondrogenic cells. One may wonder whether the blood stream provides progenitor cells from marrow stroma since membrane bones are vascularized and bone marrow stroma contains chondroprogentor cells (Berry and Grant 1992). However, secondary cartilage in the QJ and mandible is derived from neural crest, not from mesoderm, which gives rise to bone marrow stroma. Furthermore, that chondrogenesis occurs in cultured QJ periosteal cells (Fang and Hall

1996b) and in intact periosteum (Thorogood 1979) also indicates a local origin of cartilage cells in periostea. Hence, for periostea, bone marrow is unlikely to provide chondroprogenitor cells via the blood stream.

Cells of osteogenic and chondrogenic lineages arise from mesenchymal stem cells (Marks and Popoff 1988), but it is unclear whether membrane bone 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 periostea? Chondrogenic cells may arise from: (1) a restricted chondroprogenitor cell population, (2) bipotential or multipotential stem cells, or (3) osteogenic cells (Fig. 3). These three possibilities are discussed and reviewed below.

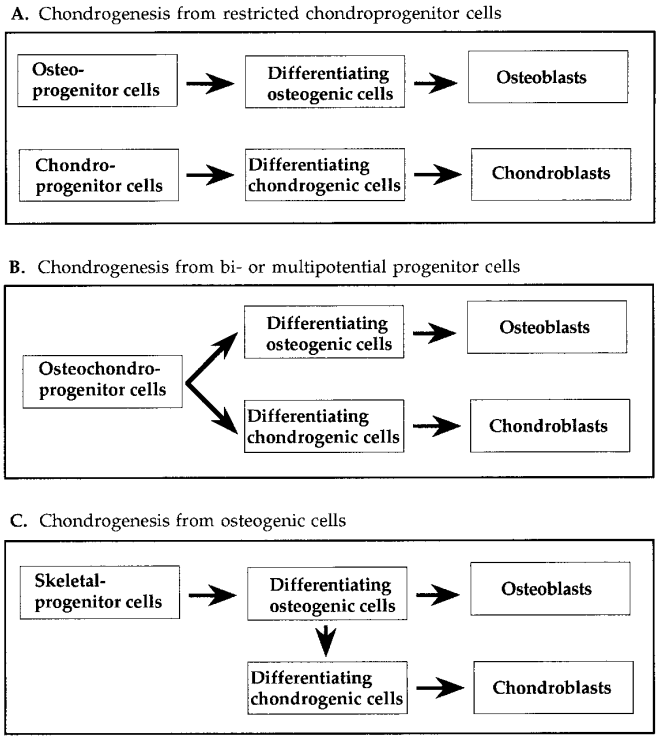


Fig. 3A–C Three possible origins of chondrogenic cells in membrane bone. See text for details. The terms "differentiating osteogenic cells" or "differentiating chondrogenic cells" cover all stages of differentiation between progenitor cells and osteo- or chondroblasts

Restricted progenitor cells (Fig. 3A)

One possibility is that progenitor cells from neural crest may become committed to two restricted cell subpopulations in periostea: osteoprogenitor and chondroprogenitor cells. These two subpopulations coexist in the germinal layer of a periosteum, each with a restricted pathway of differentiation. During intramembranous bone formation, only osteoprogenitor cells proliferate and differentiate to form bone. Chondroprogenitor cells are inhibited. However, 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 calvaria cells form only osteocyte or chondrocyte colonies, indicating a unipotential property of the progenitor cells (Bellows et al. 1989).

If both osteogenic and chondrogenic cell lineages are separated at their stem cell stage, each progenitor cell should give rise to only one cell type in clonal culture (Fig. 3A). However, clonal culture confirmed the multipotential nature of periosteal cells, contradicting the unipotential hypothesis (Fig. 3B, and below). Furthermore, if osteo- and chondroprogenitor cells are separate before expression of phenotypic markers, differentiated cells should only yield one phenotype: osteogenic or chondrogenic. This is challenged by observation that chondrogenic cells formed in membrane bones are often associated with the osteogenic phenotype (see below) and by chondroid bone.

Bi- or multipotential progenitor cells (Fig. 3B)

Another possibility is that progenitor cells are bipotential for both differentiation pathways, or multipotential for osteo- and 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). In membrane bone, bi- or multipotentiality of progenitor has been proposed, based on observation at the tissue level that periosteum gives rise to both bone and secondary cartilage (Hall 1979). While there is enough evidence to say that the progenitor layers are bipotential, it is not adequate to state 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.

Strong evidence supporting existence of a common precursor of both osteo- and chondrogenic lines in periostea comes from clonal cultures. Grigoriadis et al. (1988) reported a clonal cell line from rat calvarial periosteal cells. This cell line can give rise to cartilage, bone, muscle and fat in culture medium in the presence of dexamethasone. Their studies demonstrated the existence of multipotential progenitors in periostea. In QJ periosteal cells, both chondrogenic and osteogenic cells were found in the same colony in low density and clonal cultures, suggesting a common precursor for both lineages (Fang and Hall 1996a, b). Therefore, it seems that osteogenic and chondrogenic cells share the same precursor in periostea.

Osteogenic cells (Fig. 3C)

If a common precursor provides both osteo- and chondrogenic cells in membrane bone, are chondrogenic cells committed from the mesenchymal stem cell stage or from the later differentiated osteogenic cells? If stem cells directly undergo either osteo- or chondrogenesis, the two lineages separate before phenotypic expression and therefore a mixed phenotype should not be present during their differentiation. If chondrogenic cells arise from an osteogenic lineage, chondrogenesis must involve transdifferentiation, or dedifferentiation, following by redifferentiation. Transdifferentiation involves a direct phenotype modifiction and indicates the existence of a transient stage between osteo- and chondrogenic cell differentiation. Indeed, the following results from morphological, biochemical, and molecular biological studies suggest the existence of such transient cell type(s) and a close relationship between osteogenic and chondrogenic cells.

First, chondrogenic cells from membrane bones often express dual characteristics of bone and cartilage. For instance, chondrocytes in secondary cartilage express not only cartilage markers such as type II collagen and sulfated proteoglycan, but also osteogenic markers such as type I collagen and APase (Silbermann et al. 1987; Silbermann and von der Mark 1990; Mizoguchi et al. 1992a, b; Landesberg et al. 1995). In contrast, bony markers are not expressed in chondrogenic cells in primary cartilage. Chondroid bone (Beresford 1981; Lengelé et al. 1996) shows dual characteristics not only in gene expression but also in histological staining. At the mRNA level, chondrogenic genes are even detected in normal intramembranous bones (Ting et al. 1993; McDonald and Tuan 1989), indicating that dual characteristics exist widely in membrane bones in vivo. These dual characteristics can be interpreted as evidence for a transient stage during intramembranous bone formation (Tuan and Lynch 1983; Ting et al. 1993; Jacenko et al. 1995; Lengelé et al. 1996).

Second, two clonal cell lines, SM1/9 and SM25/3, isolated from the mandibular condyle, confirmed the existence of such transient cell types (Bhalerao et al. 1995). these two cell lines have characteristics that are intermediate between bony and cartilaginous cells. They show some osteogenic characteristics such as APase, 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 osteogenic cells, or as chondrocytes according to their phenotypic markers.

Third, studies on low-density and clonal cultures of QJ periosteal cells suggest that secondary chondrocytes may be phenotypically modified from osteogenic cells (Fang and Hall 1996a, b). Chondrocytes often appear from polygonal cells colonies, but not from fibroblast-like cell colonies. Polygonal cells can express APase and type I collagen, but not type II collagen. Therefore these cells are typical osteoblast-like in culture. Chondrogenesis from those APase-positive polygonal cells suggests that these osteogenic cells have the potential to undergo chondrogenesis. Interestingly, the chondrocytes generated from periosteal cells express some osteogenic markers (APase and type I collagen) from the beginning of chondrogenesis, indicating their close relationship with the osteogenic lineage (Fang and Hall 1996a, b).

That chondrocytes may arise from a differentiated cell type is supported by 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. The low proliferation rate indicates a relatively differentiated status since cell differentiation of periosteal cells is inversely related to proliferation in cultured periosteal cells (Owen et al. 1990; Stein et al. 1990, 1996).

Normal cell differentiation in membrane bone may therefore include a transient stage, with cells expressing osteogenic markers, such as APase, and so belonging to the osteogenic lineage. However, they also have some chondrogenic characteristics. In the normal process of membrane bone formation, they only express some chondrogenic markers at trace levels that shut down quickly during further differentiation. However, in some circumstances, as when switching to secondary cartilage formation or fracture healing, chondrogenic gene expression is enhanced, and the cells undergo chondrogenesis. Alternatively, these cells may switch partially to chondrogenesis and form an intermediate tissue between bone and cartilage as in chondroid bone.

Hence, it is likely that the transient stage cells in osteogenic lineages can provide precursors to chondrogenesis in membrane bone periostea. However, in some situations, contributions of stem cells to chondrogenesis cannot be completely ruled out. There is evidence that the bony core of calvaria has chondrogenic potential in organ culture after its periosteum has been removed (Jacenko and Tuan 1995), although contamination of periosteal cells is still possible in the organ cultures. Further research is needed.

Triggering chondrogenesis

As addressed above, local environmental factors trigger chondrogenic differentiation in membrane bones. The triggering signals are quite diverse, including 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 have to be

converted into an internal molecular signal. Then a cascade of change in gene expression must 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, and inorganic components such as calcium and oxygen concentrations. Among these, transcription factors such as homebox gene products contain information to pattern skeletal elements (Elrebacher et al. 1995) and play an important role in condensation and pattern formation (Richman and Mitchell 1996). They may be regulated by a signal from the epithelium during epithelium-mesenchyme interaction (Takahashi et al. 1991). Hormones, such as growth hormone and glucocorticoids, have systematic effects on all of the skeleton. Growth factors are important for paracrine and autocrine systems. They regulate proliferation, differentiation, and metabolism of skeletal cells by binding to receptors on the plasma membrane. For instance, TGF-β and basic fibroblast growth factor (bFGF) elicit chondrogenesis in otic capsule mesenchyme in vitro (Frenz et al. 1994). TGF-β1 enhances chondrogenesis in tibial periosteal cells in vitro (Iwasaki et al. 1993) and in rat parietal bone periosteum in vivo (Taniguchi et al. 1993). Several BMPs have been suggested to play important roles in osteogenic and chondrogenic differentiation (Langille 1994; Bennett et al. 1995; Ekanayake and Hall 1997). Other growth factors, such as EGF, acidic fibroblast growth factor (aFGF), and insulin-like growth factor-1 (IGF-1) also play various roles in osteo- and chondrogenesis (Hall and Ekanayake 1991; Erlebacher 1995). However, so far there is no evidence that any of those factors is a trigger molecule for chondrogenesis in membrane bone. In calvaria, mechanical loading can stimulate release of TGF-β (Klein-Nulend et al. 1995), but this does not lead to initiation of chondrogenesis.

Cell adhesion molecules (CAMs), a group of cell surface proteins mediating cell-cell adhesion, are potent regulators of cell differentiation. CAMs can function as morphoregulators in embryogenesis (Edelman and Crossin 1991; Edelman 1992, 1993). For chondrogenesis, neural cell adhesion molecule (NCAM) and N-cadherin play an important role in precartilaginous condensation in mesenchyme (Widelitz et al. 1993; Oberlender and Tuan 1994; Tavella et al. 1994). In secondary cartilage formation, NCAM is particularly interesting. NCAM is expressed when the periosteum undergoes osteogenesis. However, NCAM expression is shut down when periostea switch to secondary cartilage formation. The timing of NCAM down-regulation is *coincident* with the commitment to secondary chondrogenesis, and *earlier* than morphological initiation of catilage formation (Fang and Hall 1995). In paralyzed embryos in which secondary cartilage does not form, down-regulation of NCAM does not occur and the periosteum remains osteogenic. Hence, NCAM is a molecule whose down-regulation is correlated with embryonic movement and secondary cartilage formation.

Down-regulation of NCAM in the switch from osteogenesis to chondrogenesis suggests that it plays a role in secondary chondrogenesis. Structurally, NCAM contains several domains, such as those that bind to type I collagen (Probstmeier et al. 1992) and heparin sulfate proteoglycan (Kallapur and Akeson 1992), which provide interactions between cells and ECM. These interactions may further influence signal transduction pathways and therefore regulate cell differentiation (Schuch et al. 1989; Fagotto and Gumbiner 1996; Sastry and Horwitz 1996). An example of such regulation is that NCAM stimulates neurite extension through the aFGF receptor (Williams et al. 1994; Fagotto and Gumbiner 1996).

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

Recent evidence shows that the NCAM gene is a downstream target of *Hox* genes. The upstream region of the NCAM gene contains a promotor and a regulatory element (Hirsch et al. 1990, 1991). The regulatory element contains homeodomain binding sites of *Hox* genes. Activity of the *NCAM* 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 NCAM upstream region. *Cux* strongly inhibits the *NCAM* promoter but this inhibition can be prevented by *phox* 2 (Valarché et al. 1993). In situ hybridization shows that *phox* 2 expression is restricted to the areas where NCAM is expressed, while *cux* is expressed in many NCAM-negative areas (Valarché et al. 1993).

Therefore, NCAM is a possible regulator of osteo- and chondrogenic differentiation. It is not known whether environmental factors influence NCAM expression directly or through other genes. Further investigation is needed. NCAM may play different roles between early (condensation) and late differentiation stages of skeletal development. NCAM is required in bone and cartilage condensation in mesenchyme (Widelitz et al. 1993; Hall and Miyake 1995). However, after condensation, NCAM expression remained in osteogenesis but not in chondrogenesis. Hence, differential expression of NCAM after cellular condensation may provide a mechanism controlling alternative osteo- and chondrogenic differentiation pathways. Further investigation is required to explore the exact role of NCAM on chondrogenesis from membrane bones since NCAM may be downstream in the cascade.

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References

Alberius P, Johnell O (1991) Repair of intra-membranous bone fractures and defects in rats. J Craniomaxillofac Surg 19: 15–20

Aubin JE, Turksen K, Heersche JNM (1993) Osteoblastic cell lineage. In: Noda M (ed) Cellular and molecular biology of bone. Academic Press, San Diego, pp 1–45

Bee J, Thorogood P (1980) The role of tissue interactions in the skeletogenic differention of avian neural crest cells. Dev Biol 78:47–62

Bellows CG, Aubin JE (1989) Determination of numbers of osteoprogenitors present in isolated fetal rat calvaria cells *in vitro*. Dev Biol 133:8–13

Bellows CG, Aubin JE, Heersche JNM, Antosz ME (1986) Mineralized bone nodules formed in vitro from enzymatically-released rat calvaria cell populations. Calcif Tissue Int 38: 143–154

Bellows CG, Heersche JNM, Aubin JE (1989) Effects of deamethasone on expression and maintenance of cartilage in serum-containing cultures of calvaria cells. Cell Tissue Res 256:145–151

Ben-Ami Y, Mark K von der, Franzen A, Bernard B de, Lunazzi GC, Silbermann M (1993) Transformation of fetal secondary cartilage into embryonic bone in organ cultures of human mandibular condyles. Cell Tissue Res 271:317–322

Bennett JH, Hunt P, Thorogood P (1995) Bone morphogenetic protein-2 and -4 expression during murine orofacial development. Arch Oral Biol 40:847–854

Benya PD, Shaffer JD (1982) Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 30:215–224

Beresford WA (1981) Chondroid bone, secondary cartilage and metaplasia. Urban & Schwarzenberg, Baltimore

Berry L, Grant ME (1992) Bone-marrow-derived chondrogenesis in vitro. J Cell Sci 101:333–342

Berry L, Shuttleworth CA (1989) Expression of the chondrogenic phenotype by mineralizing cultures of embryonic chick calvarial bone cells. Bone Miner 7:31–45

Bhalerao J, Bogers J, Marck EV, Merregaert J (1995) Establishment and characterization of two clonal cell lines derived from murine mandibular condyles. Tissue Cell 27:369–382

Bhargava U, Bar-Lev M, Bellows CG, Aubin JE (1988) Ultrastructural analysis of bone nodules formed in vitro by isolated fetal rat calvaria cells. Bone 9:155–163

Bronner-Fraser M (1993) Segregation of cell lineage in the neural crest. Curr Opin Genet Dev 3:641–647

Bruder SP, Caplan AI (1990) Osteogenic cell lineage analysis is facilitated by organ cultures of embryonic chick periosteum. Dev Biol 141:319–329

Caplan AI (1990) Cartilage begets bone versus endochondral myelopoiesis. Clin Orthop 261:257–267

Couly GF, Coltey PM, Le Douarin NM (1993) The triple origin of skull in higher vertebrates: a study in quail-chick chimeras. Development 117:409–429

Craft PD, Mani MM, Pazel J, Masters FM (1974) Experimental study of healing in fractures of membrane bone. Plast Reconstr Surg 53:321–325

Dunlop L-LT, Hall BK (1995) Relationships between cellular condensation, preosteoblast formation and epithelial-mesenchymal interactions in initiation of osteogenesis. Int J Dev Biol 39:357–371

Ede DA (1983) Cellular condensations and chondrogenesis. In: Hall BK (ed) Cartilage, vol 2. Development, differentiation and growth. Academic Press, New York, pp 143–185

Edelman GM (1992) Morphoregulation. Dev Dyn 193:2–10

Edelman GM (1993) A golden age for adhesion. Cell Adhes Commun 1:1–7

Edelman GM, Crossin KL (1991) Cell adhesion molecules: implications for a molecular histology. Annu Rev Biochem 60: 155–190

Ekanayake S, Hall BK (1997) The *in vivo* and *in vitro* effects of bone morphogenetic protein-2 on the development of the chick mandible. Int J Dev Biol 41:67–81

Erlebacher A, Filvaroff EH, Gitelman SE, Derynck R (1995) Toward a molecular understanding of skeletal development. Cell 80:371–378

Fagotto F, Gumbiner BM (1996) Cell contact-dependent signaling. Dev Biol 180:445–454

Fang J, Hall BK (1995) Differential expression of neural cell adhesion molecule (NCAM) during osteogenesis and secondary chondrogenesis in embryonic chick. Int J Dev Biol 39: 519–528

Fang J, Hall BK (1996a) *In vitro* chondrogenesis from periosteal cells of secondary cartilage-forming membrane bone in chick embryos. Mol Biol Cell 7:583a

Fang J, Hall BK (1996b) *In vitro* differentiation potential of the periosteal cells from a membrane bone, the quadratojugal of the embryonic chick. Dev Biol 180:701–712

Frenz DA, Liu W, Williams JD, Hatcher V, Galinovic-Schwartz V, Flanders KC, Van De Water TR (1994) Induction of chondrogenesis: requirement for synergistic interaction of basic fibroblast growth factor and transforming growth factor-beta. Development 120:415–424

Fyfe DM, Hall BK (1983) The origin of the ectomesenchymal condensations which precede the development of the bony scleral ossicles in the eyes of embryonic chicks. J Embryol Exp Morphol 73:69–86

Gibson GJ, Flint MH (1985) Type X collagen synthesis by chick sternal cartilage and its relationship to endochondral bone development. J Cell Biol 101:277–284

Girgis FG, Pritchard JJ (1958) Experimental production of cartilage during the repair of fracture of the skull vault in rats. J Bone Joint Surg [Br] 40:274–281

Glasstone S (1971) Differentiation of the mouse embryonic mandible and squamo-mandibular joint in organ culture. Arch Oral Biol 16:723–730

Goret-Nicaise M (1984) Identification of collagen type I and type II in chondroid tissue. Calcif Tissue Int 36:682–689

Goret-Nicaise M, Dhem A (1982) Presence of chondroid tissue in the symphyseal region of the growth human mandible. Acta Anat 113:189–195

Goret-Nicaise M, Manzanares MC, Bulpa P, Nolmans E, Dhem A (1988) Calcified tissues involved in the ontogenesis of the cranial vault. Anat Embryol 178:399–406

Granström G, Nilsson LP (1987) Experimental mandibular fracture: studies on bone repair and remodellation. Scand J Plast Reconstr Surg 21:159–165

Grigoriadis AE, Heersche JNM, Aubin JE (1988) Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal population: effect of dexamethasone. J Cell Biol 106:2139–2151

Guenther HL, Hofstetter W, Stutzer A, Mühlbauer R, Fleisch H (1989) Evidence for heterogeneity of the osteoblastic phenotype determined with clonal rat bone cells established from transforming growth factor-β-induced cell clones growth anchorage independently in semisolid medium. Endocrinology 125:2092–2102

Hall BK (1972) Immobilization and cartilage transformation into bone in the embryonic chick. Anat Rec 173:391–404

Hall BK (1978) Developmental and cellular skeletal biology. Academic Press, New York

Hall BK (1979) Selective proliferation and accumulation of chondroprogenitor cells as the mode of action of biomechanical factors during secondary chondrogenesis. Teratology 20:81–92

Hall BK (1986) The role of movement and tissue interactions in the development and growth of bone and secondary cartilage in the clavicle of the embryonic chick. J Embryol Exp Morphol 93:133–152

Hall BK (1991) Cellular interactions during cartilage and bone development. J Craniofac Genet Dev Biol 11:238–250

Hall BK (1992) Cell-cell interactions in craniofacial growth and development. In: Davidovitch Z (ed) Biological mechanisms of tooth movement and craniofacial adaptation. Ohio State University, Coloumbus, pp 11–17

Hall BK, Ekanayake S (1991) Effects of growth factors on the differentiation of neural crest cells and neural crest cell-derivatives. Int J Dev Biol 35:376–387

Hall BK, Jacobson HN (1975) The repair of fractured membrane bones in the newly hatched chick. Anat Rec 181:55–70

Hall BK, Miyake T (1992) The membranous skeleton: the role of cell condensations in vertebrate skeletogenesis. Anat Embryol 186:107–124

Hall BK, Miyake T (1995) Divide, accumulate, differentiate: cell condensation in skeletal development revisited. Int J Dev Biol 39:881–893

Hamburger V, Hamilton HL (1951) A series of normal stages in development of the chick embryo. J Morphol 88:49–92

Henricson A, Hulth A, Johnell O (1987) The cartilaginous fracture callus in rats. Acta Orthop Scand 58:244–248

Herring SW, Lakars TC (1981) Craniofacial development in the absence of muscle contraction. J Craniofac Genet Dev Biol 1:341–375

Hirsch M-R, Gaugler L, Deagostini-Bazin H, Bally-Cuif L, Goridis C (1990) Identification of positive and negative regulatory elements governing cell-type-specific expression of the neural cell adhesion molecule gene. Mol Cell Biol 10:1959–1968

Hirsch M-R, Valarché I, Deagostini-Bazin H, Pernelle C, Joliot A, Goridis C (1991) An upstream regulatory element of the NCAM promoter contains a binding site for homeodomain. FEBS Lett 287:197–202

Huang L, Solursh M, Sandra A (1996) The role of transforming growth factor alpha in rat craniofacial development and chondrogenesis. J Anat 189:73–86

Hughes SS, Hicks DG, O'Keefe RJ, Hurwitz SR, Crabb ID, Krasinskas AM, Loveys L, Puzas JE, Rosier RN (1995) Shared phenotypic expression of osteoblasts and chondrocytes in fracture callus. J Bone Miner Res 10:533–544

Iwasaki M, Nakata K, Nakahara H, Nakase T, Kimura T, Kimata K, Caplan AI, Ono K (1993) Transforming growth factor-β1 stimulates chondrogenesis and inhibits osteogenesis in high density culture of periosteum-derived cells. Endocrinology 132:1603–1608

Jacenko O, Tuan RS (1986) Calcium deficiency induces expression of cartilage-like phenotype in chick embryonic calvaria. Dev Biol 115:215–232

Jacenko O, Tuan RS (1995) Chondrogenetic potential of chick embryonic calvaria. I. Low calcium permits cartilage differentiation. Dev Dyn 202:13–26

Jacenko O, San Antonio JD, Tuan RS (1995) Chondrogenetic potential of chick embryonic calvaria. II. Matrix calcium may repress cartilage differentiation. Dev Dyn 202:27–41

Jones FS, Prediger EA, Bittner DA, De Robertis EM, Edelman GM (1992) Cell adhesion molecules as targets for Hox genes: neural cell adhesion molecule promoter activity is modulated by cotransfection with hox-2.5 and -2.4. Proc Natl Acad Sci USA 89:2086–2090

Kallapur SG, Akeson RA (1992) The neural cell adhesion molecule (NCAM) heparin binding domain binds to cell surface heparin sulfate proteoglycans. J Neurosci Res 33:538–548

Kantomaa T, Tuominen M, Pirttiniemi P (1994) Effect of mechanical forces on chondrocyte maturation and differentiation in the mandibular condyle of the rat. J Dent Res 73: 1150–1156

Klein-Nulend J, Roelofsen J, Sterck JGH, Semeins CM, Burger EH (1995) Mechanical loading stimulates the release of transformation growth factor-β activity by cultured mouse calvariae and periosteal cells. J Cell Physiol 163:115–119

Köntges G, Lumsden A (1996) Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. Development 122:3229–3242

Landesberg R, Proctor RL, Rosier RN, Puzas JE (1995) The mandibular condyle growth center: separation and characterization of the cellular elements. Calcif Tissue Int 56:71–77

Langille RM (1994) Differentiation of craniofacial mesenchyme. In: Hall BK (ed) Bone, vol 9. Differentiation and morphogenesis of bone. CRC Press, Boca Raton, pp 1–63

Le Douarin NM, Ziller C, Couly GF (1993) Patterning of neural crest derivatives in the avian embryo: *in vivo* and *in vitro* studies. Dev Biol 159:24–49

Le Lièvre C (1978) Participation of neural crest-derived cells in the genesis of the skull in birds. J Embryol Exp Morphol 47:17–37

Le Lièvre C, Le Douarin NM (1975) Mesenchymal derivatives of the neural crest: analysis of chimaeric quail and chick embryos. J Embryol Exp Morphol 34:125–154

Lengelé B, Dhem A, Schowing J (1990) Early development of the primitive cranial vault in the chick embryo. J Craniofac Genet Dev Biol 10:103–112

Lengelé B, Schowing J, Dhem A (1996) Chondroid tissue in the early facial morphogenesis of the chick embryo. Anat Embryol 193:505–513

Liu F, Malaval L, Gupta AK, Aubin JE (1994) Simultaneous detection of multiple bone-related mRNAs and protein expression during osteoblast differentiation: polymerase chain reaction and immunocytochemical studies at the single cell level. Dev Biol 166:220–234

Livne E, Silbermann M (1990) The mouse mandibular condyle: an investigative model in developmental biology. J Craniofac Genet Dev Biol 10:95–98

Livne E, Lewinson D, Dickson G, Silbermann M (1990) Histochemical study of matrical components in the mandibular condyle of the neonatal mouse. Acta Anat 138:32–41

Lyles JM, Amin W, Bock E, Weill CL (1993) Regulation of NCAM by growth factors in serum-free myotube culture. J Neurosci Res 34:273–286

Marks SC, Popoff SN (1988) Bone cell biology: the regulation of development, structure, and function in the skeleton. Am J Anat 183:1–44

McCulloch CAG, Fair CA, Tenenbaum HC, Limeback H, Homareau R (1990) Clonal distribution of osteoprogenitor cells in cultured chick periostea: functional relationship to bone formation. Dev Biol 140:352–361

McDonald SA, Tuan RS (1989) Expression of collagen type transcripts in chick embryonic bone detected by *in situ* cDNA-mRNA hybridization. Dev Biol 133:221–234

McKibbin B (1978) The biology of fracture healing in long bones. J Bone Joint Surg [Br] 60:150–162

Mizoguchi I, Nakamura M, Takahashi I, Kagayama M, Mitani H (1992a) An immunohistochemical study of localization of type I and type II collagens in mandibular condylar cartilage compared with tibial growth plate. Histochemistry 93:593–599

Mizoguchi I, Nakamura M, Takahashi I, Kagayama M, Mitani H (1992b) A comparison of the immunohistochemical localization of type I and type II collagens in craniofacial cartilage of the rat. Acta Anat 144:59–64

Moiseiwitsch JRD, Lauder LM (1997) Regulation of gene expression in cultured embryonic mouse mandibular mesenchyme by serotonin antagonists. Anat Embryol 195:71–78

Murray PDF (1963) Adventitious (secondary) cartilage in the chick embryo, and the development of certain bones and articulations in the chick skull. Aust J Zool 11:368–430

Murray PDF, Smiles M (1965) Factors in the evocation of adventitious (secondary) cartilage in the chick embryo. Aust J Zool 13:351–381

Nakahara H, Bruder SP, Goldberg VM, Caplan AI (1990a) *In vitro* osteochondrogenic potential of cultured cells derived from the periosteum. Clin Orthop 259:223–232

Nakahara H, Bruder SP, Haynesworth SE, Holecek JJ, Baber MA, Goldberg VM, Caplan AI (1990b) Bone and cartilage formation in diffusion chamber by subcultured cells derived from the periosteum. Bone 11:181–188

Nakahara H, Dennis JE, Bruder SP, Heaynesworth SE, Lennon DP, Caplan AI (1991) *In vitro* differentiation of bone and hypertrophic cartilage from periosteal-derived cells. Exp Cell Res 195:492–503

Nakahara H, Goldberg VM, Caplan AI (1992) Culture-expanded human periosteal-derived cells exhibit osteochondral potential *in vivo*. J Orthop Res 9:465–476

Nakata K, Nakahara H, Kimura T, Kojima A, Iwasaki M, Caplan AI, Ono K (1992) Collagen gene expression during chondrogenesis from chick periosteum-derived cells. FEBS Lett 299: 278–282

Nefussi J-R, Boy-Lefevre ML, Boulekbache H, Forest N (1985) Mineralization *in vitro* of matrix formed by osteoblasts isolated by collagenase digestion. Differentiation 29:160–168

Nijweide PJ, Plas A van der, Olthof AA (1988) Osteoblastic differentiation. In: Cell and molecular biology of vertebrate bone tissue. Ciba Found Symp 136:61–77

Noden DM (1975) An analysis of the migratory behavior of avian cephalic neural crest cells. Dev Biol 42:106–130

Noden DM (1978) The control of avian cephalic neural crest cytodifferentiation. I. Skeletal and connective tissues. Dev Biol 67: 296–312

Noden DM (1988) Interactions and fates of avain craniofacial mesenchyme. Development [Suppl] 103:121–140

Oberlender SA, Tuan RS (1994) Expression and functional involvement of N-cadherin in embryonic limb chondrogenesis. Development 120:177–187

Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB, Stein GS (1990) Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol 143:420–430

Perides G, Safran RM, Rueger DC, Charness ME (1992) Induction of the neural cell adhesion molecule and neuronal aggregation by osteogenic protein 1. Proc Natl Acad Sci USA 89: 10326–10330

Perides G, Safran RM, Downing LA, Charness ME (1994) Regulation of neural cell adhesion molecule and L1 by the transforming growth factor-β superfamily. J Biol Chem 269: 765–770

Precious DS, Hall BK (1994) Repair of fractured membrane bone. In: Hall BK (ed) Bone, vol 9. Differentiation and morphogenesis of bone. CRC Press, Boca Raton, pp 145–163

Probstmeier R, Fahrig T, Spiess E, Schachner M (1992) Interactions of the neural cell adhesion molecule and the myelin-associated glycoprotein with collagen type I: involvement in fibrillogenesis. J Cell Biol 116:1063–1070

Radden BG, Fullmer HM (1969) Morphological and histochemical studies of bone repair in the rat. Arch Oral Biol 14: 1243–1252

Richany SF, Bast TH, Sprinz H (1963) The repair of bone and fate of autogenous bone grafts in the skull. Acta Neurochir (Wien) 11:61–82

Richman J, Mitchell PJ (1996) Craniofacial development: knockout mice take one on the chin. Curr Biol 6:364–367

Roach HI (1992) Trans-differentiation of hypertrophic chondrocytes into cells capable of producing a mineralized bone matrix. Bone Miner 19:1–20

Rodan GA, Noda M (1991) Gene expression in osteoblastic cells. Crit Rev Eukaryot Gene Expr 1:85–98

Roubin R, Deagostini-Bazin H, Hirsch M-R, Goridis C (1993) Modulation of NCAM expression by transforming growth factor-beta, serum, and autocrine factors. J Cel Biol 111:673–684

Sandell LJ (1994) In situ expression of collagen and proteoglycan genes in notochord and during skeletal development and growth. Microsc Res Rech 28:470–482

Sandell LJ, Morris N, Robbins JR, Goldring MB (1991) Alternative spliced type II procollagen mRNAs define distinct populations of cells during vertebral development: differential expression of the amino-propeptide. J Cell Biol 114:1307–1319

Sastry SK, Horwitz AF (1996) Adhesion-growth factor interactions during differentiation: an integrated biological response. Dev Biol 180:455–467

Schmid TM, Linsenmayer TF (1985a) Immunohistochemical blocalization of short chain cartilage collagen (type X) in avian tissues. J Cell Biol 100:598–605

Schmid TM, Linsenmayer TF (1985b) Development acquisition of type X collagen in the embryonic chick tibiotarsus. Dev Biol 107:373–381

Schuch U, Lohse MJ, Schachner M (1989) Neural cell adhesion molecules influence secondary messenger systems. Neuron 3: 13–20

Scott-Savage P, Hall BK (1980) Differentiative ability of the tibial periosteum from the embryonic chick. Acta Anat 106:129–140

Shah NM, Groves AK, Anderson DJ (1996) Alternative neural crest cell fates are instructively promoted by TGFβ superfamily members. Cell 85:331–343

Silbermann M, Mark K von der (1990) An immunohistochemical study of the distribution of matrical proteins in the mandibule condyle of neonatal mice. I. Collagens. J Anat 170:11–22

Silbermann M, Reddi AH, Hand AR, Leapman RD, Mark K von der, Franzen A (1987) Further characterization of the extracellular matrix in the mandibular condyle in neonatal mice. J Anat 151:169–188

Stein GS, Lian JB, Owen TA (1990) Bone cell differentiation: a functionally coupled relationship between expression of cell-growth- and tissue-specific genes. Curr Opin Dev Biol 2: 1018–1027

Stein GS, Lian JB, Stein JL, Van Wijnen AJ, Montecino M (1996) Transcriptional control of osteoblast growth and differentiation. Physiol Rev 76:593–629

Strauss PG, Closs EI, Schmidt J, Erfle V (1990) Gene expression during osteogenic differentiation in mandibular condyles in vitro. J Cell Biol 110:1369–1378

Takahashi I (1991) Histological study of the effect of the lateral pterygoid muslce activity on the growth of rat mandibular condly cartilae. Jpn J Orthop Soc 50:368–382

Takahashi I, Mizoguchi I, Nakamura M, Kagayama M, Mitani H (1995) Effects of lateral pterygoid muscle hyperactivity on differentiation of mandibular condyles in rats. Anat Rec 241: 328–336

Takahashi Y, Bontoux M, Le Douarin NM (1991) Epithelio-mesenchymal interactions are critical for *Quox* 7 expression and membrane bone differentiation in the neural crest derived mandibular mesenchyme. EMBO J 10:2387–2393

Taniguchi Y, Tanaka T, Gotoh K Satoh R, Inazu M (1993) Transformation growth factor β1-induced cellular heterogeneity in the periosteum of rat parietal bones. Calcif Tissue Int 53: 122–126

Tavella S, Raffo P, Tacchetti C, Cancedda R, Castagnola P (1994) N-CAM and N-Cadherin expression during *in vitro* chondrogenesis. Exp Cell Res 215:354–362

Taylor LH, Hall BK, Miyake T, Cone DK (1994) Ectopic ossicles associated with metacercariae of *Apophallus brevis* (trematoda) in yellow perch, *Perca flavescens* (Teleostei): development and identification of bone and chondroid bone. Anat Embryol 190:29–46

Thorogood P (1979) *In vitro* studies on skeletogenic potential of membrane bone periosteal cells. J Embryol Exp Morphol 54: 185–207

Thorogood P, Hinchliffe JR (1975) An analysis of the condensation process during chondrogenesis in the embryonic chick limb. J Embryol Exp Morphol 33:581–606

Ting K, Petropulos LA, Iwatsuki M, Nishimura I (1993) Altered cartilage phenotype expressed during intramembranous bone formation. J Bone Miner Res 8:1377–1387

Tuan T, Lynch MH (1983) Effect of experimentally induced calcium deficiency on the development expression of collagen types in chick embryonic skeleton. Dev Biol 100:374–386

Tyler MS, Hall BK (1977) Epithelial influences on skeletogenesis in the mandible of the embryonic chick. Anat Rec 188: 229–240

Valarché I, Tissier-Seta J-P, Hirsch M-R, Martinez S, Goridis C, Brunet J-F (1993) The mouse homeodomain protein Phox2 regulates *Ncam* promoter activity in concert with Cux/CDP and is a putative determinant of neurotransmitter phenotype. Development 119:881–896

Villanueva JE, Nishimoto SK, Nimni E (1989) Cells isolated from fetal rat calvaria by isopycnic separation express different collagen phenotypes. Matrix 9:40–48

Vinkka H (1982) Secondary cartilages in the facial skeleton of the rat. Proc Finn Dent Soc 78 [Suppl VII]:1–137

Vinkka-Puhakka H, Thesleff I (1993) Initiation of secondary cartilage in the mandible of the Syrian hamster in the absence of muscle function. Arch Oral Biol 38:49–54

Widelitz RB, Jiang T-X, Murray BA, Chuong C-M (1993) Adhesion molecules in skeletogenesis: II. Neural cell adhesion molecules mediate precartilaginous mesenchymal condensations and enhance chondrogenesis. J Cell Physiol 156:399–411

Williams EJ, Furness J, Walsh FS, Doherty P (1994) Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin. Neuron 13:583–594

Wong G, Cohn DV (1974) Separation of parathyroid hormone and calcitonin-sensitive cells from non-responsive bone cells. Nature 252:713–715

Wong M, Tuan RS (1992) In vitro chondrogenic potential of embryonic chick calvarial cell subpopulations. Mol Biol Cell 3:229a

Wong M, Tuan RS (1995) Interactive cellular modulation of chondrogenic differentiation *in vitro* by subpopulations of chick embryonic calvarial cells. Dev Biol 167:130–147

Young HE, Ceballos EM, Smith JC, Mancini ML, Wright RP, Ragan BL, Bushell I, Lucas PA (1993) Pluripotent mesenchymal stem cells reside within avian connective tissue matrices. In Vitro Cell Dev Biol 29A:723–736