

Embryonic Origin and Fate of Chondroid Tissue and Secondary Cartilages in the Avian Skull

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ABSTRACT *Background:* Chondroid tissue is an intermediate calcified tissue, mainly involved in desmocranial morphogenesis. Often associated with secondary cartilages, it remained of unprecise embryonic origin.

Methods: The latter was studied by performing isotopic isochronic grafts of quail encephalon onto 30 chick embryos. The so-obtained chimeras were sacrificed at the 9th, 12th, and 14th day of incubation. The contribution of graft- and host-derived cells to the histogenesis of chondroid tissue, bone, and secondary cartilages was analyzed on both microradiographs of thick undecalcified sections and on classical histological sections after several DNA or ECM specific staining procedures.

Results: Chondroid tissue is deposited in the primitive anlage of all membranous bones of the avian skull. Also present on their sutural edges, it uniformly arises from the neural crest. In the face, bone and secondary cartilages share this mesectodermal origin. However, secondary cartilages located along the basal chondrocranium and bone formed on the chondroid primordium of the cranial vault, originate from the cephalic mesoderm.

Conclusions: These facts provide evidence that chondroid tissue arises from a specific differentiation of neural crest derived cells and that this original skeletogenic program differs from that of secondary chondrogenesis. Moreover, they obviously indicate that in membranous bone ontogenesis, chondroid tissue replaces functions devoted to mesodermal primary cartilages of the cranial base, and so corroborates at the tissue level, the dual embryonic and phyletic origin of the skull. © 1996 Wiley-Liss, Inc.

Key words: Chondroid tissue, Avian skull, Neural crest-derived cells

The skull of higher vertebrates consists of bones from both cartilaginous and membranous origin (Poirier, 1892; Sappey, 1889). Indeed, the cranial base is preceded by a primordium of hyaline cartilage which calcifies, then undergoes endochondral ossification following the basic histogenic program that occurs simultaneously in both axial and appendicular skeleton. In contrast, the membranous bones of the cranial vault and those of the facial desmocranium directly appear within the cephalic mesenchyme without the production of a preliminary cartilaginous anlage (Hall, 1971a; Gray, 1973).

In the evolution of species, this so-called "intramembranous ossification" represents the main and nearly unique alternative to endochondral osteogenesis (Hall, 1975) and has always been reported to be highly characteristic of the early development of the facial and calvarial skeleton. According to classical descriptions, it results from a direct and specific differentiation of the mesenchymal stem cells into osteoblasts and gives rise to the deposit of woven or lamellar bone (Bernard and Pease, 1969; Bloom and Fawcett, 1986).

Recently, however, we emphasized that each dermal

anlage of the avian skull was in fact firstly constituted of chondroid tissue (Lengelé et al., 1990a, 1996). Already mentioned as "Knorpel Knochen" by Schaffer (1888), this tissue is obviously evidenced in the growing skull by means of microradiographic analysis or by methylene blue staining of thick undecalcified sections (Goret-Nicaise and Dhem, 1982). It contains large rounded cells embedded in a heterogeneously calcified matrix and the presence of both collagen type I and type II mixed in its ground substance obviously allows to differentiate it from bone and cartilage although it exhibits some structural characteristics of both (Goret-Nicaise, 1984; Goret-Nicaise and Dhem, 1987). During desmocranial morphogenesis, it not only represents the initial modality of skeletogenic differentiation for membranous ossification, but it also strikingly acts as the main appositional support for sutural growth

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(Goret-Nicaise et al., 1988; Manzanares et al., 1988; Lengelé et al., 1990a).

Secondary chondrogenesis is another typical developmental feature associated with the growth of membranous bones. Largely documented in the avian skull (Gegenbaur, 1867; Gaupp, 1907; Murray, 1963; Murray and Smiles, 1965), this phenomenon has in addition the peculiar property to be followed by a progressive transformation of the cartilage into bone giving rise to the appearance of an intermediate tissue currently listed as "chondroid bone" (Hall, 1971b, 1972; Beresford, 1981). The same phenotypic changes were recorded in murine septal chondrocytes becoming embedded in a bone-like matrix under various experimental conditions (Silbermann et al., 1983; Tsao and Chuah, 1988). Diversely interpreted, all these observations lead to the opinion that chondroid tissue should correspond to the advanced stage of a "modulative" metaplasia through which a secondary cartilage slowly becomes woven bone (Hall, 1972; Beresford, 1981; Richman and Diewert, 1988; Mizoguchi et al., 1990, 1993). However, an alternative hypothesis about the origin of chondroid tissue is that it should directly derive from a specific differentiation of the skeletogenic precursor cells, as a full autonomous tissue entity.

In close relation with the latter hypothesis, we pointed out the fact that the ubiquitous presence of chondroid tissue in the avian desmocranium could be correlated, at least partially, with the neural induction areas of the cephalic skeleton (Schowing, 1968a,c) and with the wide distribution of the neural crest in the cranial derivatives (Hörstadius, 1950; Johnston, 1966; Lelièvre, 1974). We therefore suggested that chondroid tissue should originate from an original pattern of skeletogenic differentiation, specifically devoted to mesenchymal stem cells arising from the cephalic neural folds (Lengelé et al., 1990b; Schowing et al., 1991). However, an important objection could be raised against this opinion: the bones of the cranial vault which are deposited on a chondroid primer were indeed considered to be essentially derived from the cephalic mesoderm (Lelièvre, 1978; Noden, 1978; Le Douarin, 1982). According to Noden (1982), neural crest and mesodermal mesenchymal cell populations occupy completely separate regions of the head, and migrating neural crest cells never cross the well-defined interface between the two. Nevertheless, by performing isotopic microtransplantations of meso- or mesectodermal quail cells in the chick embryo at the late neurula stage, Couly et al. (1992, 1993) have recently demonstrated a larger contribution of the neural folds to the head morphogenesis.

The purpose of the present study is to determine the embryonic origin of chondroid tissue and to delineate its relationships with secondary chondrogenesis. Therefore, the fate of mesectodermal cells is followed in the chick-quail chimeric model (Le Douarin, 1971, 1973) and the attention is focused on the histogenic sequences leading, from their early morphogenesis, to the definitive shaping of membranous bones.

MATERIALS AND METHODS

Microsurgical Experiments

Chick embryos of Vedette strain (*Gallus Gallus*) were incubated in a humidified atmosphere at 38°C for

about 38 hours. In order to obtain isochronic grafts, Japanese quail embryos (*Coturnix Coturnix Japonica*) were prepared in similar conditions for 35 hours. The eggs were then opened and chimeras were constructed by replacing the encephalon of a stage 9 or 10 chick embryo (Hamburger and Hamilton, 1951) by the corresponding neural primordium of a synchronous quail embryo.

Microscalpels were used to carefully separate the neural tube of the chick from both optic vesicles and from the surrounding mesenchyme (Schowing, 1968a). The embryonic encephalon was so dissected from adjacent paraxial mesoderm, transected at the level of the first somite, then elevated from the underlying notochord and prechordal plate. The groove left by the host resected tissue was subsequently filled with a homologous fragment harvested from the quail embryo (Fig. 1). The latter consisted of the anterior portion of the neural tube, excised with the overlying neural folds and corresponding dorsal ectoderm. Medial paraxial mesoderm was not included within the graft (Fig. 1, insets).

The so-obtained chimeras were sealed with tape and maintained at 38°C in a water saturated environment for an additional period of at least 7 days. Thirty embryos, harmoniously and symmetrically developed, were retained for further analysis and killed, by groups of ten, after 9, 12, and 14 days of incubation. According to Rumpel (1962), the first ossification centers of the chick embryonic skull indeed appear during the 8th day of incubation. By the 14th day, all the bones are present and easy to identify.

In the present paper, they are listed according to the description of Romanoff (1960) for the early embryonic stages. In older specimens, the used nomenclature is that of the *Nomina Anatomica Avium* (Baumel, 1979).

Microradiographic Study

Two skulls at each stage of development were selected for microradiographic analysis on thick undecalcified sections. They were embedded in methyl methacrylate (Vincent, 1955) and cut into frontal and transverse serial sections by means of an automatic saw (Safag, Type 32, Bienne, Switzerland).

After reduction to a uniform thickness of 80 µm by manual grinding on a ground glass plate under methanol, the sections were placed on a fine grain film (Kodak, spectroscopic plate 649.0) and exposed to long wave length X-radiations produced by a Machlett tube (Baltograph, BF 50-20, Balteau, Liège, Belgium). The exposure lasted 15 minutes for a film-focus distance of 61 mm. The microradiographic plates were developed in a D 19-Kodak solution, fixed, washed under running water, and mounted using DPX (BDH Chemical Ltd, Poole, UK) as for classical histologic preparations.

After exposure, the sections were submitted to a further manual grinding to reach a uniform thickness of 30 µm. Thereafter, they were stained with a 1% aqueous solution of methylene blue buffered at pH 4.8 with 0.1 N potassium biphtalate, dehydrated with tertiary butylic alcohol, and mounted with Canada balsam.

Chimerism Analysis

The heads of the remaining chimeric embryos were fixed in formol or with Zenker's fluid and embedded in

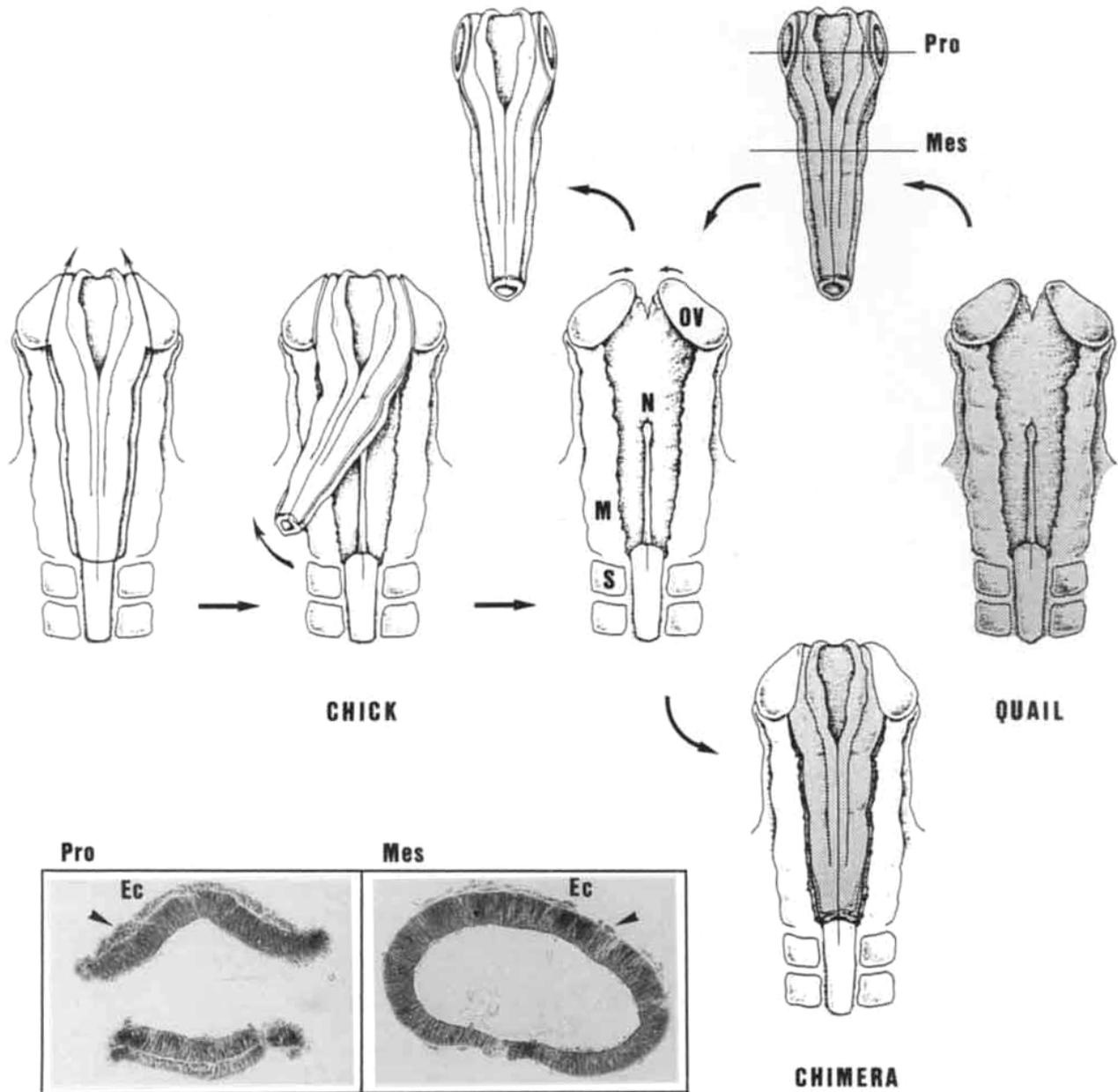


Fig. 1. Experimental procedure. Transsected at the level of the first somite pair (S), the encephalon of a chick embryo at H & H stage 9 or 10 is dissected from the surrounding mesenchyme and elevated from the underlying notochord (N). After complete excision between both optic vesicles (OV), it is replaced by a homologous fragment harvested

from a quail embryo at the same stage. **Insets:** Control sections at the level of the prosencephalon (Pro) and mesencephalon (Mes) show that the graft consists of the neural tube, with associated neural crest cells (arrowheads) and corresponding ectoderm (Ec), but does not include adjacent paraxial mesoderm (M).

paraffin. Serial 5 μm thick sections were then taken from frontal, transverse, and sagittal planes. Identification of quail cells in host mesenchymal tissues was allowed by the selective staining of their nuclear marker (Le Douarin, 1971, 1973) with Feulgen-Rossenbeck's method (1924). Picro-indigo carmine was used as light counterstain.

Adjacent sections were stained with methylene blue, Alcian blue-P.A.S., toluidine blue, or Goldner's trichrome stain, in order to differentiate the various skel-

etal tissues. Both latter methods have the singular property of simultaneously revealing large heterochromatic masses in the nucleus of quail cells. However, they specifically stain the two types of nucleic acids and are thus less efficient for the identification of the neural crest cells than the Feulgen-Rossenbeck's method which is a selective staining procedure for DNA.

In order to avoid "nuclear mass" staining, both toluidine blue and Goldner's stainings were thus preceded

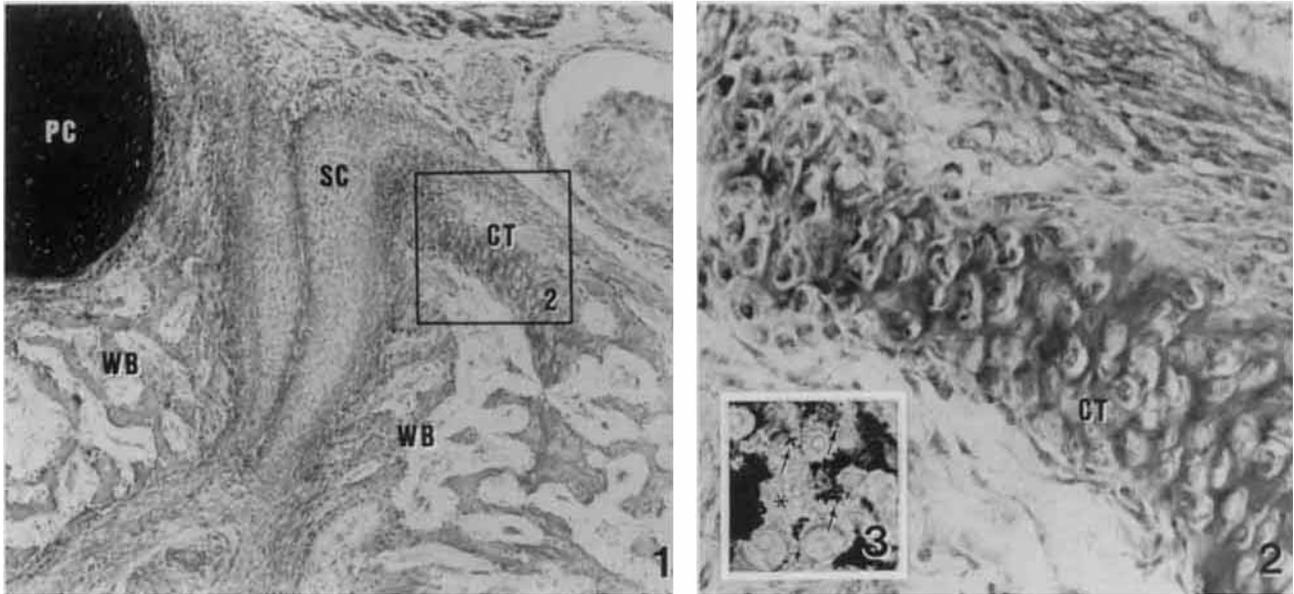


Fig. 2. General characteristics of the skeletal tissues involved in desmocranial morphogenesis. 1: Frontal section through the upper face of a 12-day incubated embryo (toluidine blue staining, $\times 100$), showing the differences between primary cartilage (PC), woven bone (WB), and chondroid tissue (CT). The latter is evidenced in 2: enlarge-

ment of the inset in 1 (toluidine blue, $\times 390$). Inset in 2 emphasizes the aspect of CT in a thick undecalcified section from a similar area (methylene blue staining, $\times 1,140$). Arrows: granular aggregations of mineral; asterisk: unmineralized matrix.

by a long time acid hydrolysis (20 min), and were simultaneously tested on control sections taken from six unoperated chick embryos at the same developmental stages.

RESULTS

Figure 2 illustrates the main characteristics of the various skeletal tissues involved in the development of the avian skull. Primary cartilage (PC) is intensively stained by toluidine blue and obviously differs from highly cellular secondary cartilages (SC) which are laid down on the woven bone (WB) trabeculae of the desmocranium (Fig. 2, inset). Otherwise, chondroid tissue (CT) appears to be constituted of cartilage-like rounded cells, closely packed in a bone-like matrix (Fig. 2, plate 2). This latter shows a metachromasia intermediate between that of woven bone and that of primary cartilage (Fig. 2, plate 2) while after Alcian blue-P.A.S. staining, it has the same appearance as bone matrix.

Moreover, the cellular lacunae of chondroid tissue, incompletely separated from each other, are partially confluent. This characteristic disposition is clearly evidenced on thick undecalcified sections after methylene blue staining (Fig. 2, insert in plate 2). The mineralized intercellular substance has indeed a heterogenous affinity for the stain and its dark and uneven edges circumscribe several neighbouring cells embedded in a variable layer of unmineralized extracellular matrix. Conspicuously, the boundaries between mineralized and unmineralized matrices are underlined by numerous granular aggregations (Fig. 2, arrows in inset). Very significant for chondroid tissue, these histological features indicate that its calcification starts at some

distance from the pericellular area, differing fundamentally from that of primary or secondary cartilages.

Lower Face

When the quail encephalon is transplanted in the chick embryo, grafted cells give rise to all skeletal derivatives of the mandibular arch (Fig. 3). Indeed, at the 9th day of incubation, the primordia of dentary, angular, surangular, and opercular bones consist of chondroid tissue, obviously deposited along the Meckel's cartilage by scleroblasts exhibiting the quail nuclear marker (Fig. 3, plates 1 and 2). At the 12th day of incubation, secondary cartilages are similarly formed by mesectodermal cells in the topographic areas where the bones are facing the Meckel's cartilage or overlapping each other (Fig. 3, plates 3 and 4). By the 14th day, these cartilaginous nodules undergo a characteristic cellular hypertrophy in their central portion (Fig. 3, plate 5) which is connected with the woven bony trabecular network by a transitional zone of chondroid tissue (Fig. 3, plates 3-5).

Notable is the fact that the adventitious cartilages are, at each stage of their maturation, bordered by a germinative layer of flattened mesenchymal cells which are of neural crest origin (Fig. 3, plate 4). This dense cambial sheet which separates primary and secondary cartilages is continuous with the meckelian perichondrium and with the periosteum of the mandibular bones (Fig. 3, plate 3).

At the opposite of all other articular aspects of the first branchial arch, the symphyseal edges of both hemimandibles are not covered with secondary cartilages. Like the sutural areas of the cranial vault, they are lined by chondroid tissue (Fig. 3, plate 6). The char-

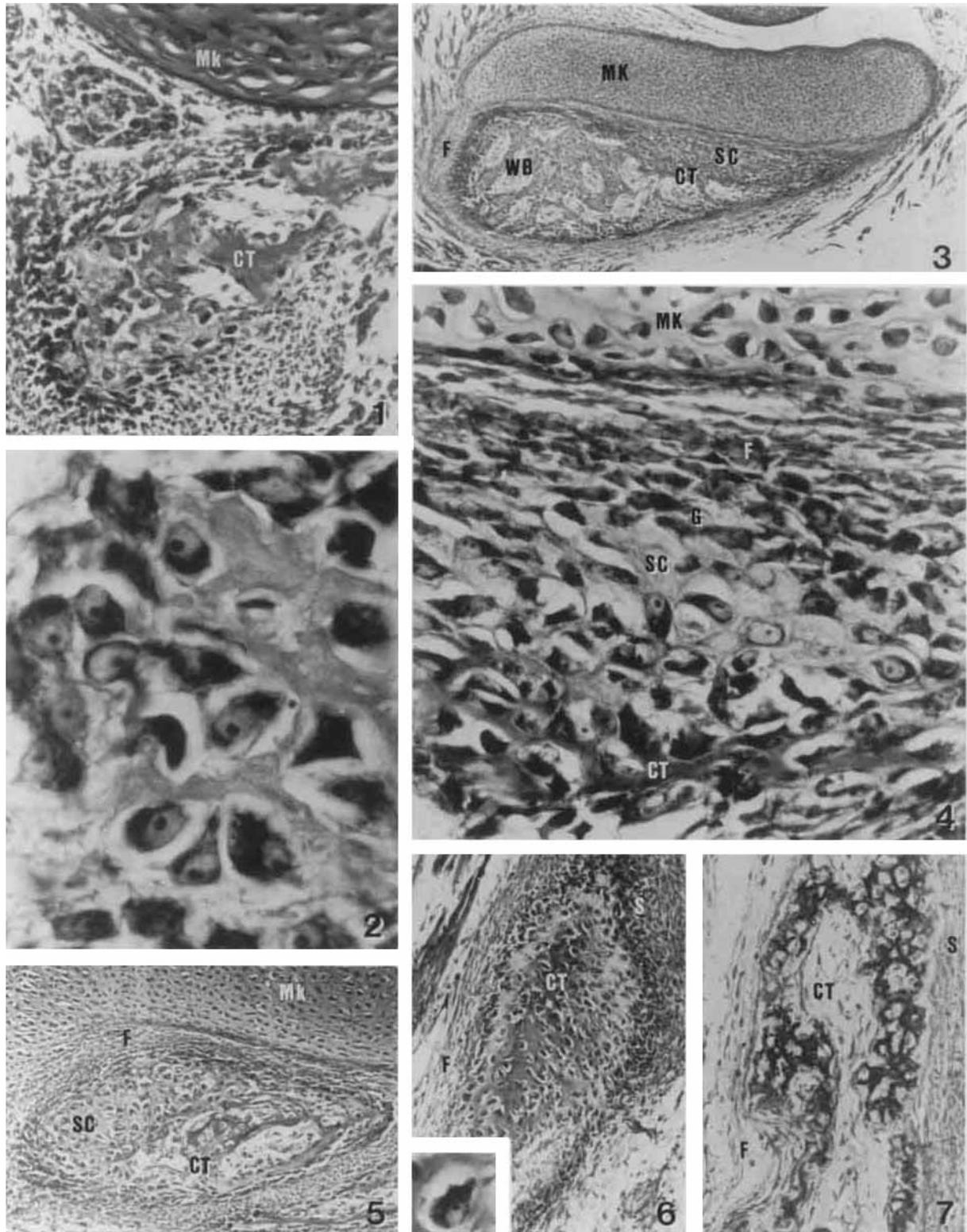


Fig. 3. Embryonic origin of CT and SC in the lower facial skeleton. The primordium of each mandibular bone at D9 consists of CT (1, toluidine blue, $\times 265$), deposited by NC cells (2, $\times 1,750$). At D12, secondary cartilages of the mandibular complex (3, toluidine blue, $\times 85$) share the same origin (4, $\times 800$), and show hypertrophy at D14 (5, toluidine blue, $\times 165$). The symphyseal edge of each hemimandible

is covered by CT (6, toluidine blue, $\times 165$), evidenced on a similar thick undecalcified section (7, methylene blue, $\times 180$). The latter tissue is deposited by quail cells (6: inset, Feulgen's staining, $\times 990$). CT: chondroid tissue; SC: secondary cartilage; WB: woven bone; F: fibrous sheath of the mandibular complex; G: germinative layer of the SC; S: mandibular symphysis; Mk: Meckel's cartilage.

acteristic features of this tissue are conspicuously emphasized after methylene blue staining of thick undecalcified sections (Fig. 3, plate 7) and the ubiquitous presence of the quail nuclear marker indicates that it is also deposited by neural crest cells (Fig. 3, plate 6).

Upper Face

The membranous bones of the upper facial skeleton are entirely of mesectodermal origin (Fig. 4). Indeed, in chimeric embryos, quail cells invade the membranous condensations of premaxillary, maxillary, palatal, and pterygoid bones and deposit in these fragments the chondroid anlage of the palate (Fig. 4, plates 1 and 2). Similar phenomena occur in the parasphenoid rostrum, in the vomer, and in both nasal and lacrymal bones. Moreover, woven bone formation in each of these ossification centers also results from graft-derived cell differentiation (Fig. 4, plate 5).

At the 12th day of incubation, secondary chondrogenesis is observed at both the ventral and dorsal extremities of the pterygoid bone. In the pterygo-quadrate and pterygo-palatal joints, the adventitious cartilages contain quail cells and show a close relationship with a band of chondroid tissue which is interposed between them and the newly formed bone (Fig. 4, plate 3). At the 14th day, chondrocytes adjacent to this transitional area become enlarged, surrounded by a fibrillar matrix, and exhibit similar modifications as those previously described in the mandibular bones (Fig. 4, plate 4).

On the contrary, the secondary chondrification of the pterygo-basicranial joint puts in place two distinct mesenchymal pads in which chondrocytes remain poorly differentiated and the matrix moderate in amount (Fig. 4, plate 6). Overlying the basicranial cartilaginous nodule, runs the perichondrium of the basisphenoid (Fig. 4, plate 6) and more ventrally, the periosteum of the parasphenoid rostrum (Fig. 2, plate 1). On the other aspect, the articular cap of the pterygoid is separated from its core by an intervening zone of dense tissue consisting of flattened cells which neither ossify nor chondrify (Fig. 4, plate 7). This picture, radically different from that encountered in any other secondary cartilage of the avian skull, is completed by the fact that the articular chondrifications deposited along the basal chondrocranium are of mesodermal origin. Indeed, their cells do not show the quail nuclear marker (Fig. 4, plate 7, inset) and this feature is strikingly correlated with the evidence that they fail to reach advanced stages of specific skeletal differentiation. Moreover, the pterygo-basicranial secondary cartilages have the capacity to develop in complete separation from the underlying bone which is covered by chondroid tissue. The latter obviously originates from the neural crest (Fig. 4, plate 7) and here the emphasized dual embryonic origin of contiguous chondroid tissue and secondary cartilages demonstrates that chondroid tissue formation in the sutural areas of the skull is the result of a specific pattern of skeletogenic differentiation rather than the intermediate stage of a metaplastic phenomenon necessarily linked with a preliminary secondary chondrogenesis.

Cranial Vault

The primitive cranial vault is deposited in the calvarial mesenchyme, along the connective tissue fibres of the meninges which simultaneously differentiate on the outer aspect of the growing brain (Fig. 5, plate 1). Careful examination of the calvarial tissues shows that the meningeal membranes are of neural crest origin while the more superficial loose mesenchyme mostly contain host-derived cells (Fig. 5, plate 2). Squamosal and frontal ossification centers appear at the 9th day of incubation. Located more rostrally, the parietal bone is detected on and after the 12th day and its primordium develops at this stage in the immediate vicinity of the densified ectomesenchymal layer of the calvaria (Fig. 5, plate 3). The further enlargement of the subarchnoid space definitively isolates the cranial vault anlage from the underlying nervous system (Fig. 5, plate 4).

Studied on thick undecalcified sections, the primer of frontal, parietal, and squamosal bones is obviously of chondroid nature. On a microradiograph, it is indeed constituted of a highly calcified tissue that contains numerous, large, irregular, and confluent cellular lacunae (Fig. 5, plate 5). Moreover, the methylene blue surface staining emphasizes the typical granular mineralization pattern of the newly deposited matrix (Fig. 5, plate 6). Finally, the Feulgen-Rossenbeck's nuclear reaction demonstrates that the scleroblasts arranged alongside each calvarial primordium originate from the quail transplanted neural folds (Fig. 5, plate 7). Thus, from the present data, it can be concluded that chondroid tissue deposited in the primitive cranial vault results from a direct and specific differentiation of the inner layer of the calvarial mesenchyme which is of neural crest origin.

The thickening of the cranial vault is initiated by the appearance in the cephalic mesenchyme of a new skeletogenic condensation located at a short distance from the external aspect of the primer (Fig. 6, plate 1). This phenomenon first occurs in the squamosal bone, then in the frontal and parietal anlagen. In the 12 day incubated chimeric embryos, each of these components consists of woven bony trabeculae scattered around residual islets of chondroid tissue (Fig. 6, plate 4). In the former tissue the cell lacunae are small, lenticular, and more regular than in the latter (Fig. 6, plate 4). At the periphery of the bone, mesenchymal cells condense and differentiate into a thin continuous periosteal layer which subsequently deposits lamellar bone (Fig. 6, plate 4).

Strikingly, the embryonic origin of both woven and lamellar bones involved in the thickening of the cranial vault depends on the area under consideration. In the dorsal part of the squamosal (SQ) and frontal (Fr) bones and on the whole surface of the parietal shell (P), bone tissue and its periosteum only contain cells from the chick host (Fig. 6, plates 2, 5, and 6). On the other hand, in the ventral portions of the squamosal and frontal bones, the fibrous, periosteal sheath conceals quail cells and gives rise to mesectodermal woven and lamellar bone formation (Fig. 6, plates 7 and 8). In the final analysis, the restricted portion of the cranial vault in which the outer cortex is of host origin corresponds to the rostral extension of the cephalic skeletogenic mesoderm which caudally generates the endo-

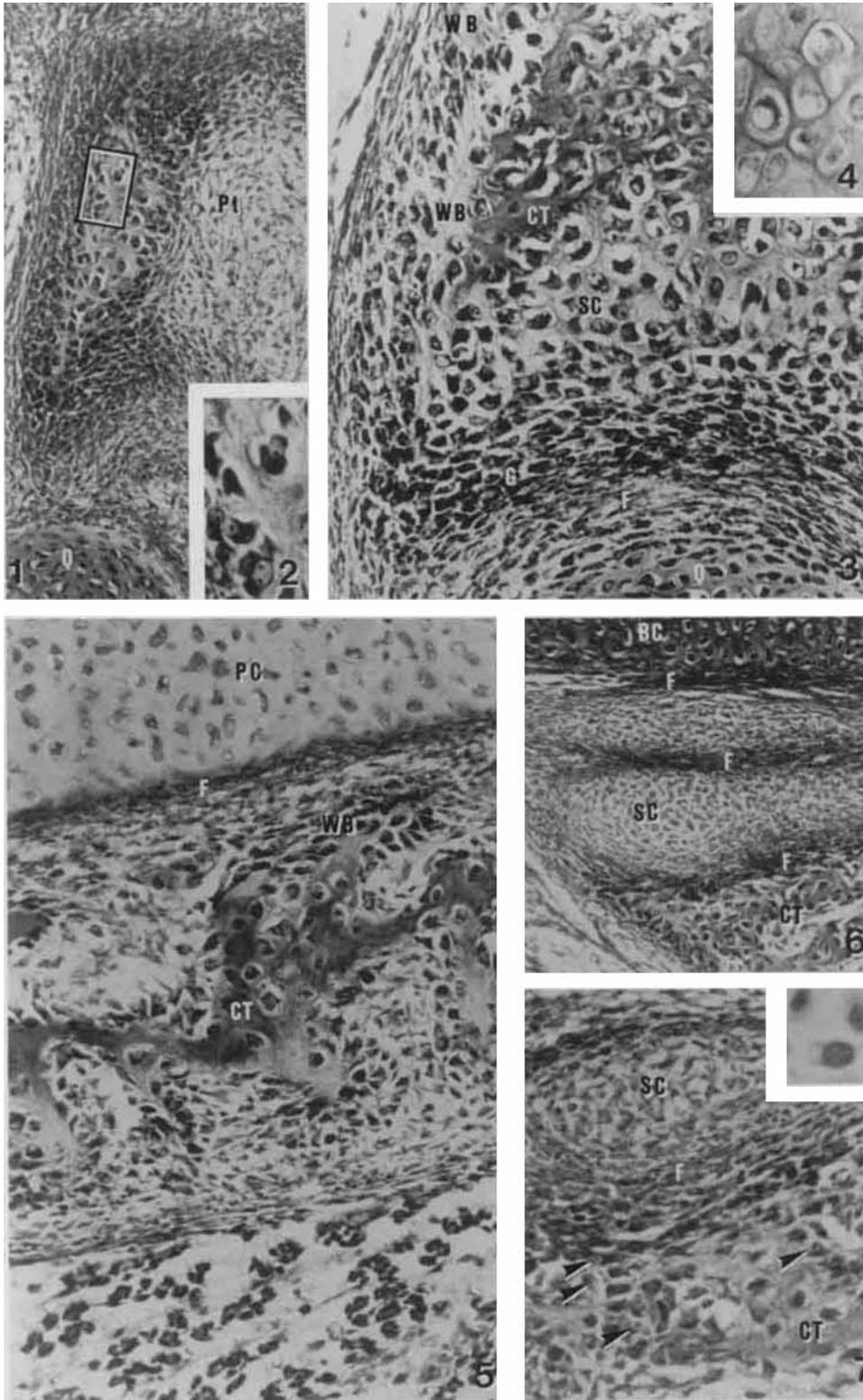


Fig. 4. Embryonic origin of CT and SC in the upper facial skeleton. At D9, the primordial analogue of the pterygoid bone (1, toluidine blue, $\times 200$), is of NC origin (2, toluidine blue, $\times 630$). At D12, the SC of the pterygo-quadrato articulation arises also from the NC (3, toluidine blue, $\times 410$) and subsequently hypertrophies at D14 (4, toluidine blue, $\times 490$). In the parasphenoid rostrum, both WB and CT are similarly deposited by quail cells (5, toluidine blue, $\times 330$). On the contrary, the

pterygo-basiscranial SC (6, toluidine blue, $\times 175$), which remains poorly differentiated at D14 (7, toluidine blue, $\times 250$), contains only host cells (7: inset, Feulgen's staining, $\times 660$). CT: chondroid tissue; PC: primary cartilage; SC: secondary cartilage; F: fibrous sheath of the upper facial skeleton; G: germinative layer of the SC; WB: woven bone; Pt: pterygoid; Q: quadrato; BC: basicranium; arrowheads: NC cells.

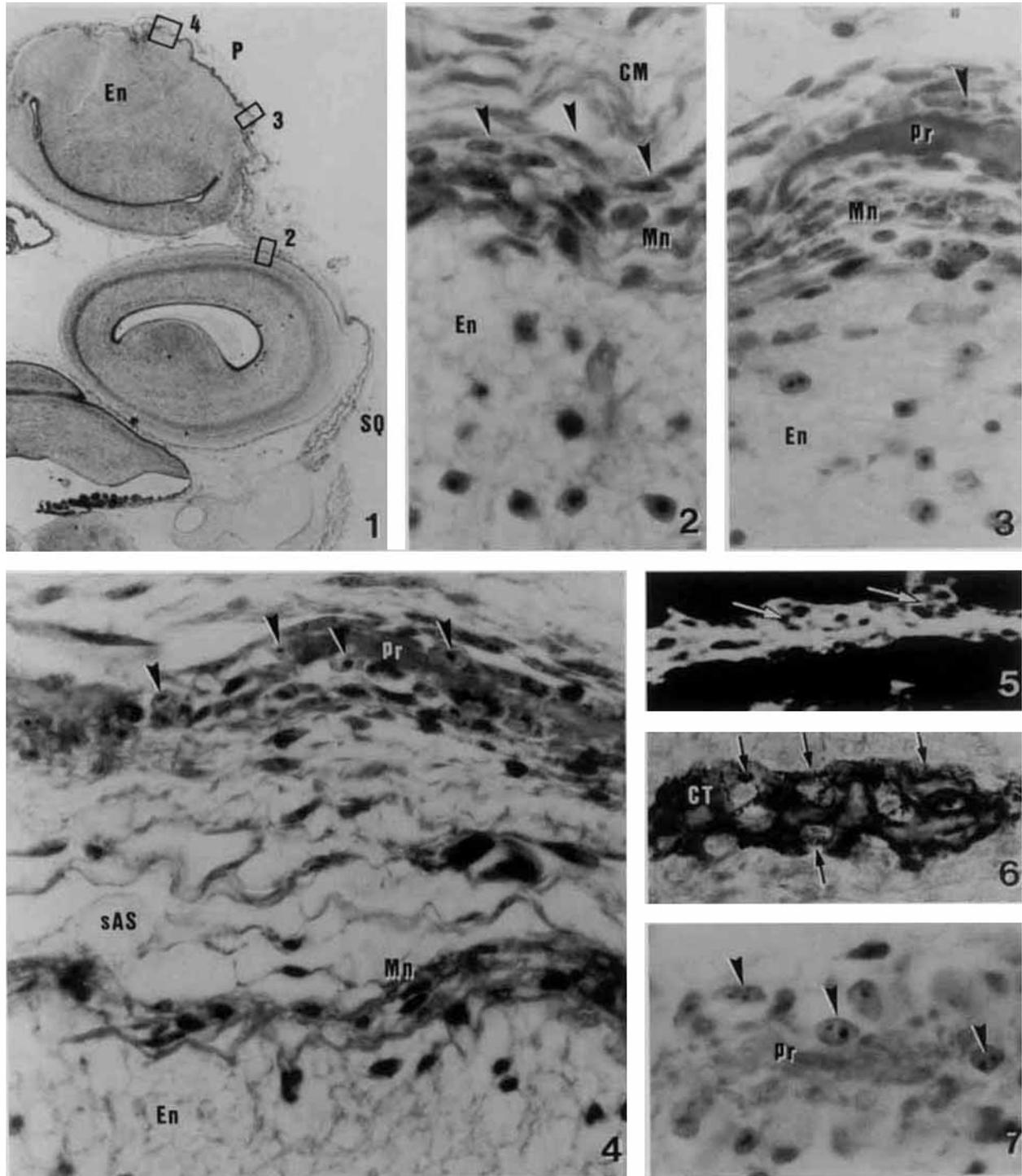


Fig. 5. Embryonic origin of the primitive cranial vault. Studied at D12 on a frontal oblique section (1, Goldner's staining, $\times 17$), the deep layer of the calvaria, including the meninx, is of mesotodermal origin (2, Goldner, $\times 1,130$). The parietal primer is deposited by NC cells alongside the meningeal sheath (3, Goldner, $\times 1,100$). The parietal anlage is subsequently separated from the encephalon by a wide subarachnoid space (4, Golner, $\times 650$). This skeletal primordium is of chondroid nature, as demonstrated by microradiographic analysis (5,

$\times 215$) and methylene blue staining (6, $\times 535$) of homologous thick undecalcified sections. Moreover, it is lined by NC cells (7, Feulgen's staining, $\times 1,420$). SQ: squamosal; P: parietal; CM: calvarial mesenchyme; En: encephalon; Mn: meninx; pr: primer; sAS: subarachnoid space; CT: chondroid tissue; arrowheads: NC cells; dark arrows: granular aggregations of mineral; white arrows: confluent cellular lacunae.

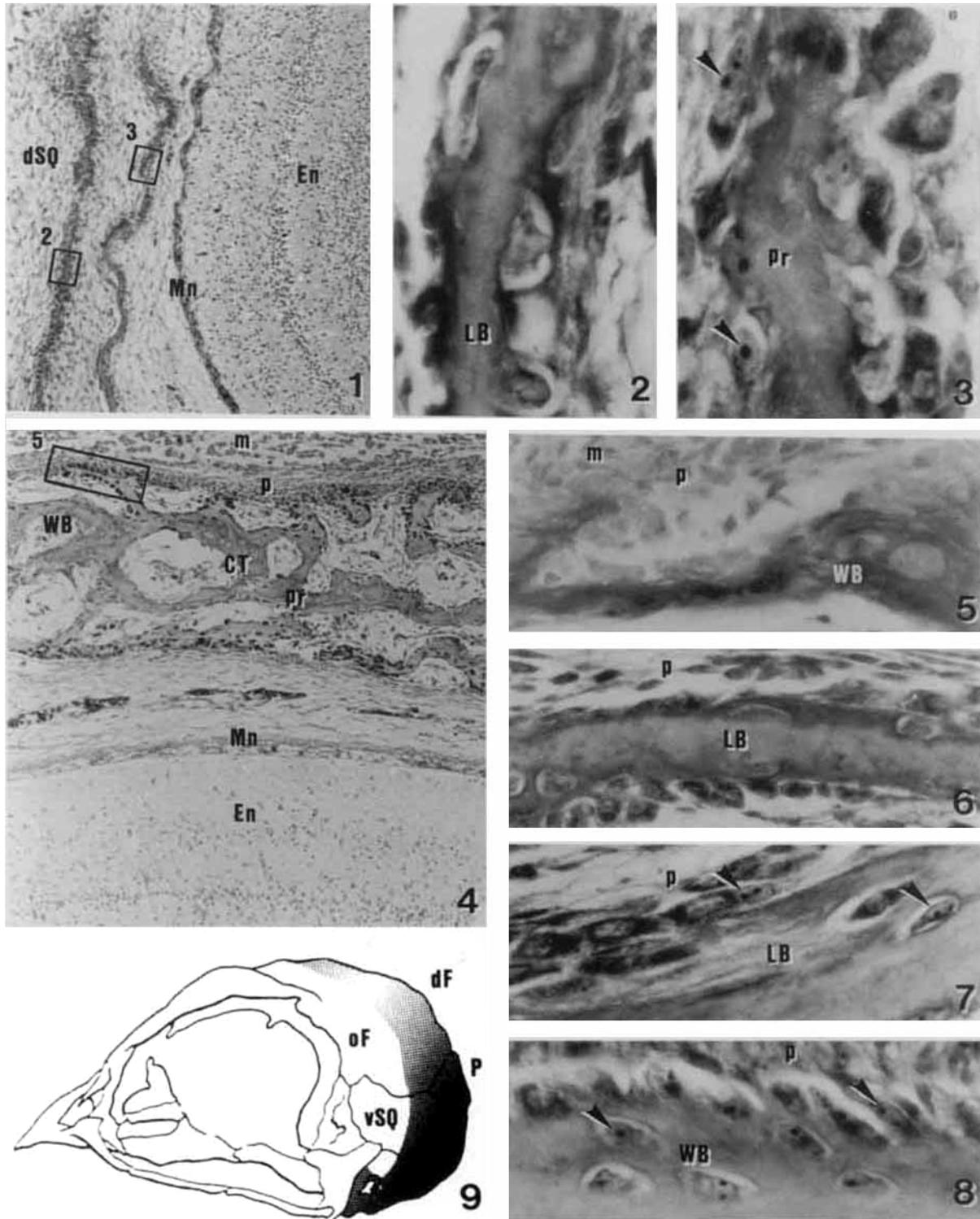


Fig. 6. Embryonic origin of the maturative cranial vault. At D12, the dorsal part of the squamosal bone (1, Goldner's staining, $\times 110$) shows an external lamina of bone deposited by host cells (2, Feulgen's staining, $\times 1,190$), at any distance of its mesectodermal primer (3, Feulgen, $\times 1,440$). At D14, the embryonic origin of woven or lamellar bone in the external layer of the calvaria depends on the area under consideration: arising from the host mesenchyme in the parietal bone (4, toluidine blue, $\times 160$; the framed area is enlarged in 5, Feulgen's staining of the neighbouring section, $\times 790$), and in the dorsal part of the frontal (6, Goldner, $\times 745$), they are derived from the grafted

neural folds in the anterior part of the squamosal (7, Goldner, $\times 1,030$), as in the orbital portion of the frontal bone (8, Feulgen, $\times 1,000$). The area of the cranial vault where bone of the outer cortex is of host origin is delineated in a schematic drawing in 9. CT: chondroid tissue; WB: woven bone; LB: lamellar bone; En: encephalon; Mn: meninx; pr: primer; p: periosteum; m: depressor mandibulae muscle; dSQ: dorsal part of the squamosal; p: parietal; dF: dorsal part of the frontal; vSQ: ventral part of the squamosal; oF: orbital part of the frontal; arrowheads: NC cells.

chondral skeleton of the skull base and that of the occipital complex (Fig. 6, plate 9).

Squamosal Bone

Among the bones of the avian skull, the squamosal is the one that contains the greatest amount of chondroid tissue and the largest secondary cartilages. Furthermore, as it has just been demonstrated, it is located at the level of the interface zone where the boundaries of graft- and host-derived skeletal tissues are overlapping each other. It therefore represents the most useful part of the desmocranium to study precisely the relationships between chondroid tissue and secondary cartilages during the histogenesis of membranous bones.

A first important feature is that in articular areas, chondroid tissue formation always precedes secondary chondrogenesis. Indeed, at the 9th day of incubation the expanded inferior border of the squamosal bone is covered by a cuff of chondroid tissue which is connected with the otic capsule by loose aerolar tissue (Fig. 7, plates 1, 2, and 3). More ventrally, a closer contact exists between the squamosal bone and the free edge of the chondrocranium (Fig. 7, plate 4). The joint consists of a layer of small flattened cells concentrically spread around the otic process of the quadrate and the dorsal border of the orbitosphenoid (Fig. 7, plate 4). Large rounded cells, with highly basophilic cytoplasm, are squeezed on the articular abutment of the squamosal bone (Fig. 7, plate 5). These germinal scleroblasts which deposit the chondroid tissue uniformly originate from the neural crest, as well as in the region where the squamosal bone faces the graft-derived quadrate, as in the areas where it is joined with the orbitosphenoid primary cartilage and the vestibular part of the otic capsule, which are both derived from the host mesoderm (Fig. 7, plates 1, 4, and 5).

In the 12 day incubated embryos, the basal part of the squamosal bone is still mostly constituted of a thick mass of chondroid tissue deposited by neural crest cells (Fig. 8, plates 1 and 3). On a microradiograph, the latter tissue obviously differs from the calcified primary cartilage of the ossifying otic capsule (Fig. 8, plate 1). Moreover, it continues with the highly mineralized chondroid primer of the squamosal bone, which is lined on its two faces by woven bony trabeculae (Fig. 8, plates 1 and 2). Rostrally, the sharp lengthening of this chondroid primordium ends within the densified sutural mesenchyme of the deep mesectodermal layer of the calvaria (Fig. 8, plate 2). On the major part of its circumference the squamosal bone is thus bordered by a crown of neural crest-derived chondroid tissue which ensures the centrifugal growth of its primordium.

Secondary chondrification of the articular mesenchyme is restricted to the basal part of the squamosal bone which faces the otic process of the quadrate and the ventral segment of the otic capsule. It starts after the 12th day of incubation and leads to the appearance of a large cartilaginous nodule which notably increases the diameter and the depth of the articular surface (Fig. 8, plate 4). The adventitious cartilage peripherally grows from germinal flattened cells of neural crest origin which are located below the fibrocellular membrane interposed between the squamosal bone and the quadrate (Fig. 8, plate 6). More central mature chondrocytes are scattered in a hyaline matrix, which remains un-

calcified and thus invisible on a microradiograph (Fig. 8, plate 5). Furthermore, a very conspicuous limit exists between the matrix of the newly formed secondary cartilage and that of the previously deposited chondroid tissue (Fig. 8, plates 6 and 7). Although the cells arranged on both sides of this boundary show the same mesectodermal origin, their fate is quite different. Indeed, between the 12th and 14th days, chondrocytes located in the deep portion of the secondary cartilage show a gradual hypertrophy with vacuolar degenerative changes of their cytoplasm and nucleolysis, while neighbouring chondroid tissue cells remain unaffected by these modifications (Fig. 8, plate 8). Remnants of the hypertrophied secondary cartilage are thereafter invaded by connective and vascular bundles arising from the marrow spaces of the underlying bone (Fig. 8, plate 9). The clear-cut segregation between chondroid tissue and secondary cartilage suggests that a switch occurred in the differentiation of the articular skeletal tissues rather than a progressive metaplasia of a well-differentiated mineralized tissue.

DISCUSSION

The present study focused its interest on the histogenic sequence of the different skeletal tissues involved in the morphogenesis of the avian desmocranium. Original data provided by the chick-quail model mainly concern the contribution of chondroid tissue and secondary cartilages to the early skeletogenesis of membranous bones, the hypothetical metaplastic relationship between the two tissues, and, finally, their respective embryonic origin.

Contribution of Chondroid Tissue and Secondary Cartilages to the Ontogenesis of Membranous Bones

The first morphological evidence of skeletogenesis in the desmocranium is a focal avascular condensation of mesenchymal stem cells. In all the dermal bones of the face (Figs. 3 and 4) and in those of the cranial vault (Fig. 5), these early aggregations are formed by neural crest derived cells which subsequently differentiate into chondroid tissue. As previously stated (Lengelé et al., 1990a, 1996), this tissue thus represents the initial pattern of skeletogenic differentiation in each cephalic ossification centre depositing in the absence of a cartilaginous anlage.

The deposit of woven bone in the mesenchymal spaces surrounding the chondroid primordium is the result of a second histogenic phenomenon which occurs more often than not at a short distance from the previously deposited anlage (Figs. 6 and 8). Strikingly, this beginning of osteogenesis, which leads to the peripheral bridging of the primary chondroid islets, is contemporary to the development of numerous blood vessels around the condensation, suggesting that the differentiation of stem cells into osteoblasts is, at least partially, initiated or influenced by an increased oxygen tension in their new environment (Hall, 1969; Thorogood, 1979; Bab et al., 1984).

Lamellar bone formation is a third sequential event which uses as support, previously deposited chondroid tissue or woven bone, and which mostly depends on the appositional activity of the periosteal layer that differentiates at the ultimate periphery of the condensation (Figs. 3, 6, and 8). This phenomenon essentially occurs

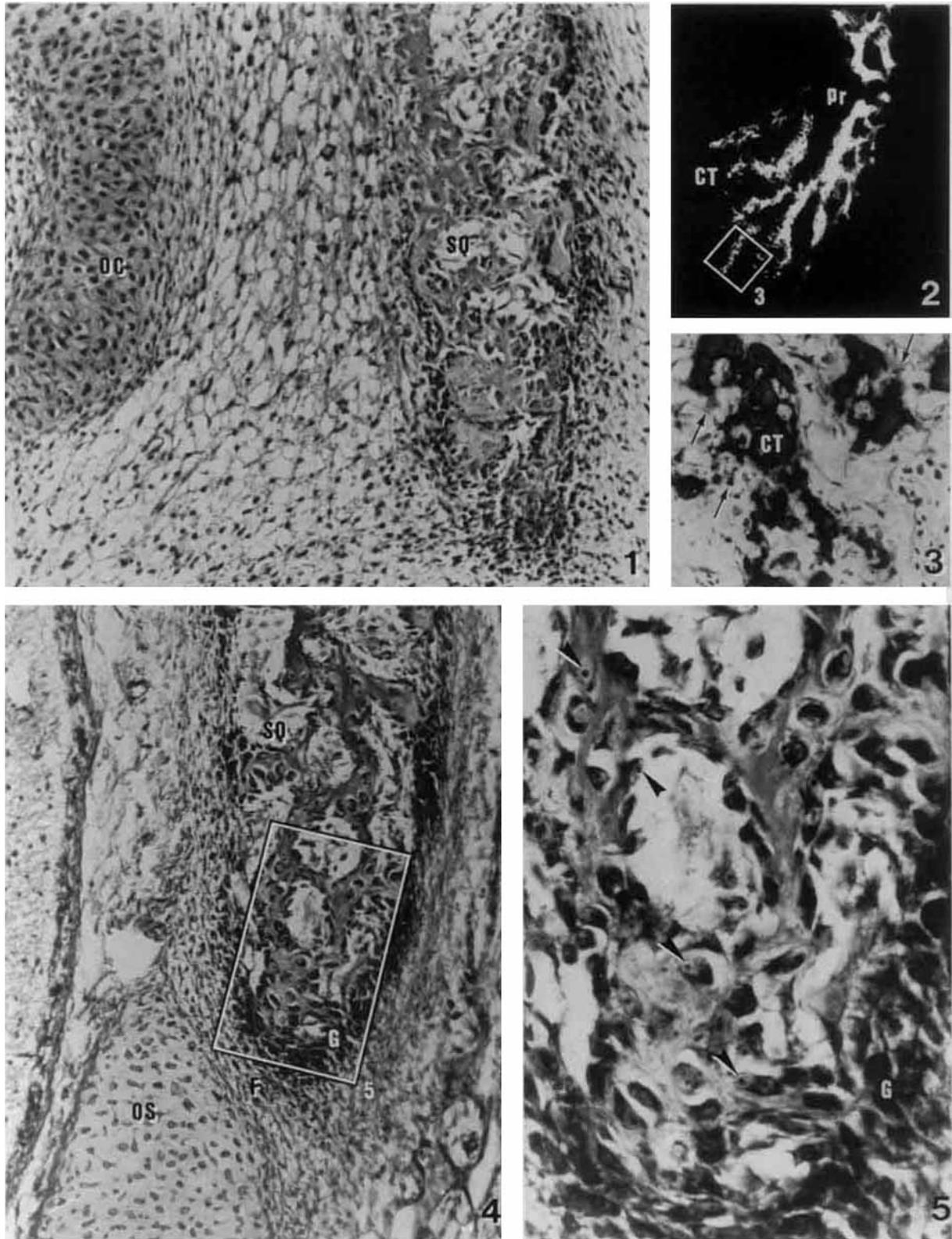


Fig. 7. Early embryogenesis of the articular surfaces of the squamosal bone. At D9, the squamoso-otic articulation (1, toluidine blue, $\times 275$) is covered by a cap of CT, evidenced by microadiographic analysis (2, $\times 150$) and methylene blue staining (3, $\times 325$) of homologous thick undecalcified sections. Ventrally, the closer abutment of the squamosal on the orbitosphenoid (4, toluidine blue, $\times 640$) is lined

by NC cells (5, enlargement of the framed area in 4, $\times 980$). CT: chondroid tissue; SQ: squamosal; OC: otic capsule; OS: orbitosphenoid; F: articular fibrous sheath; G: germinal scleroblastic layer; pr: primer; arrows: granular aggregations of mineral; arrowheads: NC cells.

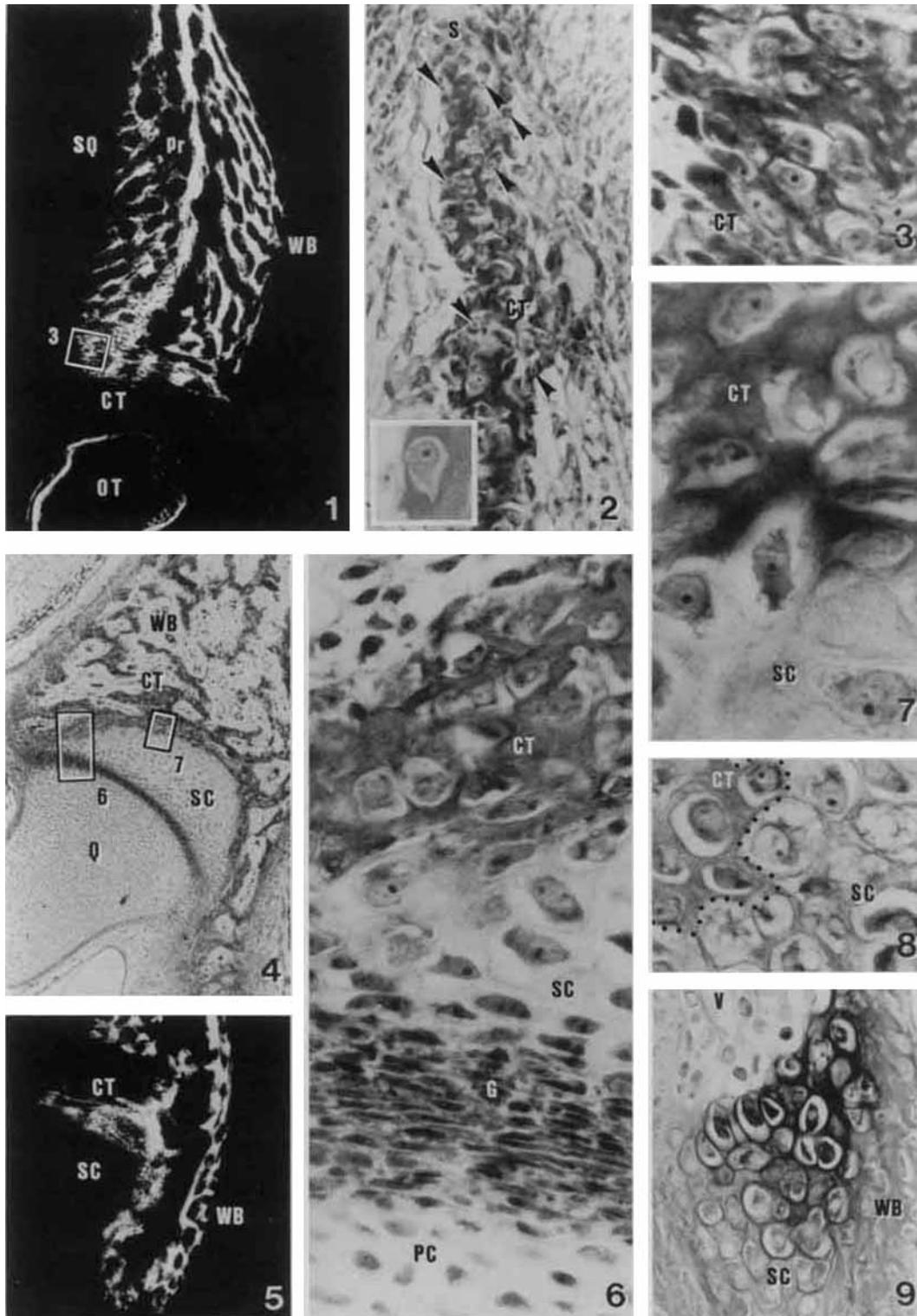


Fig. 8. Late embryogenesis of the articular surfaces of the squamosal bone. On a microradiograph at D12, the basal surface of the squamosal is still mostly covered by CT (1, $\times 80$) which is continuous with that of the primer; it extends rostrally to the sutural edge (2, toluidine blue, $\times 485$; inset: Feulgen's staining, $\times 1,150$) and arises from the NC (3, homologous thin section to the framed area in 1, Goldner's staining, $\times 1,010$). At D14, the squamoso-quadrate articulation is covered by a large SC (4, Goldner, $\times 75$). The latter is mainly uncalcified (5, microradiograph, $\times 50$) and shows a clear segregation

with CT (6, Goldner, enlargement of the framed rectangle in 4, $\times 905$). Cells located on both sides of this boundary are of NC origin (7, Feulgen's staining, $\times 1,450$). Hypertrophy occurs in SC only (8, toluidine blue, $\times 915$), and is followed by conjunctivo-vascular invasion (9, toluidine blue, $\times 660$). CT: chondroid tissue; PC: primary cartilage; SC: secondary cartilage; WB: woven bone; SQ: squamosal; OT: otic capsule; Q: quadrate; S: sutural edge; G: germinal articular layer; V: conjunctivo-vascular bundles; arrowheads: NC cells; dotted line in 8: boundary between CT cells and secondary chondrocytes.

on the external aspect of the bone, contributing to its thickening and growth. On the internal side, compensative resorptive mechanisms take place to adapt the shape of the bone to enlargements of the underlying organs.

This combined activity of calcified tissue apposition and resorption generates a spatial displacement of the skeletal piece moved by its expanding functional matrix (Moss and Salentijn, 1969; Goret-Nicaise et al., 1988). As an obvious consequence of this cortical drift (Enlow, 1975), the chondroid primordium located at the internal aspect of each dermal bone anlage is destined to be resorbed. Nevertheless, chondroid tissue persists in membranous bones at later stages of their development, as residual blocks scattered in bone trabeculae (Figs. 4 and 6) as well as in articular and sutural areas (Fig. 8).

In these peculiar growth centres, chondroid tissue is often closely associated with secondary cartilages. However, its formation occurs in the prolongation of the primer and always precedes the beginning of secondary chondrogenesis (Fig. 8). The deposit of adventitious cartilage thus originates from a fourth histogenic program which seems to be specifically initiated when close articular contact appears between the skeletal pieces. As a matter of fact, it has to be pointed out that secondary cartilages are never formed on the sutural edges of the bones of the calvaria, nor in the mandibular symphysis, which are only covered with chondroid tissue (Figs. 3 and 8). On the contrary, they are ubiquitous in topographic conflicting regions where membranous bones are squeezed upon the primary cartilages of the chondrocranium (Figs. 3, 4, and 8) or overlapping each other (Fig. 2). According to these features, chondroid tissue formation should be characteristic for sutural growth sites where facing bones are disjoined by the expansion of their related functional matrix (Goret-Nicaise et al., 1988). Otherwise, secondary cartilages should be preferentially produced in articular zones where skeletal elements are stressed against each other, either as a consequence of their intrinsic growth, or as a result of relative movements generated by the simultaneous development of the myogenic mesenchyme (Hall, 1967, 1968, 1979, 1986). Mechanical evocation of the secondary cartilages remains, however, a matter of controversy. Indeed, recent investigations seem to indicate that muscle function is not a prerequisite for secondary chondrification as has been previously claimed (Vinkka-Puhakka and Thesleff, 1993).

About the So-Called Chondroid Metaplasia of Secondary Cartilages

Due to its frequent proximity with the secondary cartilages in the avian growing skull, chondroid tissue has often been considered as an intermediate stage of a progressive metaplasia of hyaline cartilage into bone (Murray and Smiles, 1965; Hall, 1972). Moreover, the co-distribution of collagen types I and II in the deep layer of mammalian secondary cartilages was similarly interpreted (Silbermann et al., 1983, 1987; Tsao and Chuah, 1988; Mizoguchi et al., 1990, 1993; Ben-Ami et al., 1993), then more recently described as the result of a "modulative" process by which osteochondroprogenitor cells, initially differentiated into chon-

drocytes, slowly transform into osteocytes without any proliferation (Richman and Diewert, 1988). Although such a phenomenon corresponds to the description of a true metaplasia and has been favored by many authors (reviewed in Beresford, 1981), its outcome necessitates several sophisticated modifications which not only involve sequential changes of the cellular phenotype but also complex biochemical remodelling of the surrounding mineralized matrix.

The present study of the chick-quail chimeras raises arguments against the current hypothesis of the metaplastic origin of the avian "chondroid bone" (Hall, 1972).

- The first consists in the skeletogenic cascade of intramembranous ossification, since differentiation of mesenchymal stem cells in chondroid tissue always occurs before the beginning of secondary chondrogenesis (Figs. 3, 4, and 8). The present evidenced histogenic sequence is quite obviously the reverse of that of the postulated metaplasia.

- The second conflicting feature concerns the development of the adventitious cartilage of the pterygo-basiscranial joint (Fig. 4). The latter is double, contains poorly differentiated chondrocytes, and remains separated from both adjacent chondroid tissue and bone by a continuous fibrous sheath. In the chimeric embryo, this cartilage is derived from the host mesenchyme while the underlying chondroid tissue is of graft origin. Such a dual embryonic origin of the neighbouring tissues, probably related to their relative proximity with the basal chondrocranium, obviously demonstrates that no metaplastic relationship exists between them.

- A last relevant fact lies in the well-defined limit always observed between the respective matrices of closely associated chondroid tissue and secondary cartilage: that histochemical boundary indeed corresponds to two different fates for the cell populations arranged on both its sides (Figs. 4 and 8). The calcification of the deep layer of secondary cartilage involves several nuclear and subcellular changes leading to chondrocyte death (Hall, 1971b) while contiguous chondroid cells never show such irreversible ultrastructural injuries (Goret-Nicaise and Dhem, 1987). The clear-cut segregation between the two tissues in articular areas thus suggests more likely that a "shift" happened in the differentiation pattern of the scleroblasts produced beneath a common germinal layer.

- So, the progenitor cell layer of membranous bones appears to contain cells that are capable of three development pathways, producing chondroid tissue, secondary cartilage, or bone. Although these cells are initially phenotypically indistinguishable, they are segregated early according to their embryonic origin, differentiative potential, and fate. Local influences and functional stimuli probably induce the sequential activation of each specific pattern of skeletogenesis (Ben-Ami et al., 1993). No metaplasia occurs. The selective production of mineralized tissue changes from one type to another to adapt bone growth and shape to the new morphogenetic factors acting in its microenvironment. In such a manner, the here described "switch phenomenon" should be homologous to that observed at the level of the ossification groove of endochondral bones (Ranvier, 1873) where osteochondroprogenitor cells

change from cartilage to bone formation cells (Shapiro et al., 1977). Interestingly, the perichondrial ring (Lacroix, 1951) deposited in this transitional zone is precisely of chondroid nature (Lengelé et al., unpublished data).

Contribution of Neural Crest Cells to Histogenesis of Chondroid Tissue, Bone, and Secondary Cartilage

The major fact enlightened by the present experimental study concerns the embryonic origin of chondroid tissue. Indeed, the chick-quail model obviously demonstrates that the latter arises from a specific differentiation of neural crest cells in the whole cephalic skeleton. If this feature doesn't appear to be surprising for the chondroid tissue deposited in the facial desmocranium (Figs. 3 and 4) which is known for a long time to be entirely of mesectodermal origin (Lelièvre, 1978; Noden, 1978, 1982), it is much more pertinent for the chondroid primordium of the cranial vault (Figs. 5–8), classically described as a mesodermal derivative (Lelièvre, 1978; Le Douarin, 1982; Noden, 1982, 1983a, 1991). Thus our results emphasize an early contribution of the neural crest to the histogenesis of the calvaria and so partially corroborate the conclusions of Couly et al. (1992, 1993) who suggested, in contradiction to the previously prevailing hypothesis, that the whole cranial vault, including its sutures and corresponding dermis, originated from the mesectoderm (reviewed in Le Douarin et al., 1993).

The here detailed observations more precisely indicate that several populations of cells from various origins are involved in the morphogenesis of the calvarial bones. Each dermal anlage is indeed first invaded by neural crest cells which deposit chondroid tissue (Fig. 5). However, the embryonic origin of bone tissue laid down at the periphery of the mesenchymal condensation depends on the area under consideration (Fig. 6). In the most rostral part of the skull, bone and its periosteum only contain cells of the chick host; more ventrally, they arise from the quail graft (Fig. 6). Strikingly, the corresponding calvarial mesenchyme was found to be uniformly of host origin (Fig. 5). Nevertheless, the migration of neural crest cells in the rostral part of the head occurred since the meninx deeply, and melanocytes superficially, exhibited the quail nuclear marker (Fig. 5).

The discrepancy between our data and those reported by Couly et al. (1992, 1993) may be explained by the earlier embryonic stage at which these authors carried out their microsurgical experiments. According to Crochard and Coltey (1983), neural crest cells start to leave the neural fold at the mesencephalic level at the 6- to 7-somite stage (H & H stage 9). At the 8- to 9-somite stage (H & H stage 9⁺), they dorsally cover a portion of the medial paraxial mesoderm (Meier and Packard, 1984; Couly et al., 1992). Since our operations were performed at H & H stages 9 or 10 (between 6 and 10 pairs of somites), the medial paraxial mesoderm of the host embryo which yields a part of the cephalic skeleton and overlying dermis, might have been contaminated by the early migratory group of neural crest cells. By the way, some mesenchymal or skeletal constitutives of the vault may appear, at least partially, of mesodermal origin, whereas they are in fact entirely derived from the mesectoderm. However, it has to be

mentioned here that in our material, no significant differences were noted between embryos grafted at the various developmental stages (H & H 9 vs. H & H 9⁺ or 10). Moreover, if the chick hosts should have been contaminated earlier by some of their own neural crest cells, this should indicate that the mesectodermal cell migration into the rostral part of the head includes two waves of morphogenic movements: the first one, putting in place the external sheath of the calvarial mesenchyme and dermis, and the second one generating the internal layer of the cranial vault, including meninges and the chondroid primordium of membranous bones. A secondary recruitment of the external layer from the internal one should then occur in order to deposit bone and consequently to complete the ontogenesis of the calvaria.

Nevertheless, this scenario can only serve to explain a part of the apparently contradictory results. Indeed, in our experiments, the connective tissue of the depressor mandibulae muscle, which is inserted on the external aspect of both parietal and squamosal bones, was found to be of mesectodermal origin as previously recorded by Noden (1983b). Combined with the already mentioned presence of quail melanocytes in the calvarial skin, this feature suggests that at least a fraction of the grafted neural crest cells migrated in both layers of the scalp. Furthermore, another troublesome fact is that, in the illustrations provided by Couly et al. (1993), chick flattened cells are conspicuously seen in the "periosteal sheath" or in the immediate vicinity of the presented fragments of frontal and parietal bones (see Couly et al., 1993, p. 413, fig. 2C, 2D, and p. 417, fig. 5C). Corroborating recent investigations which have shown a wide co-distribution of cranial paraxial mesoderm and neural crest cells in the rostral cephalic mesenchyme of the mouse embryo (Trainor et al., 1994; Trainor and Tam, 1995), our observations indicate thus that previous conclusions about the so-claimed "exclusively mesectodermal" origin of the cranial vault and associated "dermis," should be nuanced.

In our material, the composite constitution of parietal, frontal, and squamosal bones is interpreted as additional evidence that cephalic skeletoblasts are segregated early in function of their embryonic origin and developmental capacities. Chondroid tissue is specifically deposited by neural crest cells because the latter belong to a highly adaptative cell lineage (Hall, 1975), and are able to differentiate quickly in response to inductive influences encountered during their migration or in the target site to which they home (Le Douarin et al., 1993). On the contrary, both mesodermal and mesectodermal cells are equally competent for bone formation or secondary chondrogenesis. Migrating skeletogenic cells should thus present the same heterogenous differentiative potentialities as those evidenced *in vitro* for trunk melanoblasts (Sieber-Blum and Cohen, 1980) and for various cell populations in the peripheral nervous system (Ziller et al., 1983).

Finally, the data detailed here about the distribution and embryogenesis of chondroid tissue in the avian desmocranium validate, at the tissular level, the evolutionary hypothesis of the dual embryonic and phyletic origin of the skull in higher vertebrates. According to Gans and Northcutt (1983), the transition from protochordates to vertebrates is indeed characterized by the

development of a prechordal cranial skeleton having as precursor the diffuse epidermal nerve plexus of Deuterostomes. The acquisition of this desmocranial "new head" occurred as a result of the switch from passive feeding to active predation, and coincided with the appearance of the neural crest derivatives in intimate relationship with the simultaneous development of the brain, sensory placodes, and branchial apparatus. Relevantly, intermediate calcified tissues listed as "chondroid bone" have been evidenced in the cephalic skeleton of Agnatha, placoderm fossils (Orvig, 1951, 1967), then in the pharyngeal jaws and neurocranium of Teleost fishes (Huyssseune and Verraes, 1986; Benjamin and Ralphs, 1991). Seemingly to respect the phylogenesis of calcified tissues in the lower branches of the vertebrate tree (Moss, 1964; Hall, 1975), chondroid tissue is the first pattern of skeletogenic differentiation in the desmocranium of higher vertebrates. Moreover, it is uniformly derived from the neural crest and its formation is initiated by tenuous microenvironmental influences and specific cellular interactions with the pharyngeal endoderm (Tyler and Hall, 1977; Tyler, 1978) or embryonic encephalon (Schowing, 1968a,b). Primary cartilages of the cranial base lack these highly adaptive capacities. Originating from the para-axial and somitic mesoderm, they are strongly dependent upon a notochordal induction (Schowing, 1968c; Pourquie et al., 1993).

In the neoskull, chondroid tissue seems to have overtaken and supplanted the ancestral functions devoted, in the arche- and paleoskulls, to the primary cartilage. First acting as the primordial support for bone formation, it thereafter becomes the privileged vector for longitudinal bone growth in the sutural areas. Along the cranial base, the boundary between neo- and paleoskulls precisely corresponds to the anterior tip of the notochord (Couly et al., 1993). In the calvaria, this limit appears to be more confusing. However, the observed overlapping between meso- and mesectodermal territories should simply imply a mixing of cellular populations, which follows the intense morphogenetic movements generated during the cranial vault ontogenesis.

CONCLUSIONS

The main facts enlightened by the present study may be summarized as follows:

- Chondroid tissue arises from a specific differentiation of neural crest-derived cells and its formation results from an original histogenic program that differs from that of secondary chondrogenesis. However, both phenomena contribute to the harmonious shaping of membranous bones.

- An early segregation exists between the various populations of mesenchymal cells which migrate according to their definitive pattern of skeletogenic differentiation. The recruitment of several of those populations is necessary for the normal development of some parts of the skull.

- The uniform participation of the neural crest and chondroid tissue to the early morphogenesis of the desmocranium is in agreement with the evolutionary concept of the dual embryonic and phyletic origin of the skull in higher vertebrates. Indeed, in the ectomesen-

chyme of the neoskull everything happens just as chondroid tissue had replaced and supplanted the ancestral functions devoted to the mesodermal primary cartilages of the arche- and paleoskulls: initial support of the early bone apposition, it acts thereafter in the sutural growth centers as the driving force for bone lengthening.

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