High frequency of cephalic neural crest cells shows coexistence of neurogenic, melanogenic, and osteogenic differentiation capacities

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The neural crest (NC) is a vertebrate innovation that distinguishes vertebrates from other chordates and was critical for the development and evolution of a ''New Head and Brain.'' In early vertebrates, the NC was the source of dermal armor of fossil jawless fish. In extant vertebrates, including mammals, the NC forms the peripheral nervous system, melanocytes, and the cartilage and bone of the face. Here, we show that in avian embryos, a large majority of cephalic NC cells (CNCCs) have the ability to differentiate into cell types as diverse as neurons, melanocytes, osteocytes, and chondrocytes. Moreover, we find that the morphogen Sonic hedgehog (Shh) acts on CNCCs to increase endochondral osteogenesis while having no effect on osteoblasts prone to membranous ossification. We have developed culture conditions that demonstrate that ''neural–mesenchymal'' differentiation abilities are present in more than 90% of CNCCs. A highly multipotent progenitor (able to yield neurons, glia, melanocytes, myofibroblasts, chondrocytes, and osteocytes) comprises 7–13% of the clonogenic cells in the absence and presence of Shh, respectively. This progenitor is a good candidate for a cephalic NC stem cell.

clonal culture | dermal bone | endochondral bone | multipotency | quail embryo

Most of the skull of vertebrates is derived from the cephalic neural crest cells (CNCCs), a population of embryonic cells that appears at the border of the neural plate. CNCCs undergo extensive migration and give rise to a wide array of adult cell types that include, in addition to chondrocytes and osteocytes, diverse nonskeletal mesenchymal cells as well as neurons and glial cells of the peripheral nervous system and pigment cells (1). CNCC-derived osteoblasts yield either endochondral bones (e.g., hyoid bone), which replace cartilaginous templates, or membranous (dermal) bones (e.g., in skull vault), which differentiate directly from mesenchymal condensations (2). How skeletogenic cells become specified in the CNCC population and whether they arise from multipotent progenitors need to be further documented. Through previous in vitro clonal analysis of avian CNCCs, a rare subset of common progenitors for chondrocytes and pigment, glial, and/or neuronal cells was identified (3–6). Recently, we found that chondrocytes differentiate in vitro from highly multipotent progenitors of NC origin (7), able to yield both ''neural'' (i.e., neurons, glia, pigment cells) and ''mesenchymal'' (i.e., smooth muscle and connective cells, chondrocytes) cell types. The morphogen Sonic hedgehog (Shh), a crucial factor for brain and face development (8–13), increased the number of such progenitors in vitro (7). It has not yet been established whether multipotent ''neural–mesenchymal'' cephalic NC (CNC) progenitors can also give rise to osteocytes, the alternative being that osteogenic cells constitute a separate lineage within the CNCC population. It is also unknown whether the skeletogenic cells that form endochondral and dermal bones in the head derive from common CNC progenitors.

Based on the expression of *Runx2*, a master transcription factor for osteoblast differentiation and bone formation (14–16), we have analyzed the osteogenic properties of quail CNCCs in mass and clonal cultures and investigated the effect of Shh on the development of skeletal progenitors. We describe that osteoblasts differentiate in CNCC cultures either at a distance from, or closely associated with, the perichondrium surrounding cartilage nodules. Only the perichondrial endochondral-like osteoblasts, but not those unconnected to chondrogenic islets, showed enhanced differentiation and proliferation in presence of Shh. In single-cell culture, the great majority of clone-forming CNCCs were capable of generating osteoblasts together with cells of the neural and melanocytic lineages, and all of the osteogenic progenitors were multipotent (or at least bipotent). Moreover, we identify an NC progenitor yielding neurons, glia, pigment cells, myofibroblasts, chondrocytes, and osteoblasts, which has never been described so far and could be assimilated to a highly multipotent stem cell in the early CNC.

Results

Differentiation of *Runx2*$^+$ Osteoblastic Cells in CNC Cultures and Influence of Shh on Perichondrial Osteoblasts. With the aim of analyzing the osteogenic capacity of CNCCs in vitro, we used culture conditions that had been proven in our previous investigations to be suitable for differentiation of CNCCs into various mesenchymal phenotypes (7). Briefly, mes-rhombencephalic NCCs from 6–7 somite-stage quail embryos were harvested after 15 h of primary culture of the neural primordium, and after 6 days of subculture they generated mesenchymal (i.e., myofibroblasts/smooth muscle cells and chondrocytes) and nonmesenchymal, neural cell types (i.e., neurons, glia, and melanocytes). Because in these experiments addition of Shh during the first 48 h of culture vigorously promoted chondrogenesis, we have investigated the osteogenic properties of CNCCs grown in the absence and presence of 100 ng/mL recombinant Shh.

To recognize the cells engaged in the osteogenic differentiation pathway, we examined the expression of *Runx2*, a transcription factor required for endochondral and membrane bone formation in vivo (14–16) that is expressed in avian craniofacial skeleton from embryonic day 6.5 (E6.5) onward (17, 18). In day 10 (d10) cultures, we identified 3 types of osteoblastic CNCCs: (*i*) *Runx2*$^+$ cells closely associated with and surrounding cartilage nodules, designated as perichondrial cells (Fig. 1*C*), (*ii*) *Runx2*$^+$ cell islands not associated with chondrocytic aggregates (Fig. 1*I*), and (*iii*) isolated *Runx2*$^+$ cells located randomly (Fig. 1*G*). Continuous treatment with Shh from d0 to d10 increased by 50% the percentage of perichondrial areas that expressed *Runx2* compared with control cultures (Fig. 1 *A–C*). To investigate whether Shh acted during a particular window of time, we

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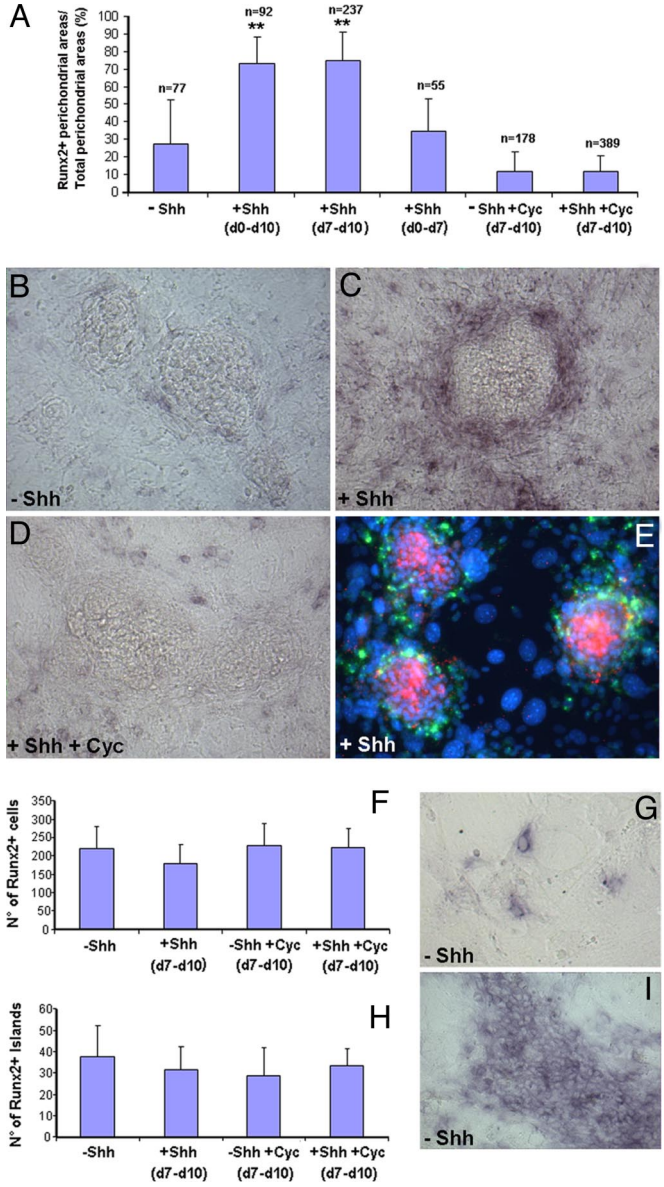

Fig. 1. *Runx2*-expressing osteoblasts differentiate in CNCC cultures; Shh influences perichondrial osteoblasts. *Runx2* expression was studied by in situ hybridization in d10 cultures of CNCCs grown in the absence and presence of 100 ng/mL Shh. Cultures were treated with Shh, cyclopamine, or both Shh and cyclopamine during 3 different time periods: d0–d10, d0–d7, and d7–d10. The total number of perichondrial areas and the number of those expressing *Runx2* were evaluated in the cultures. (*A*) Continuous treatment (d0–d10) and d7–d10 treatment increase the percentage of perichondrium containing *Runx2*$^+$ cells; this effect of Shh is blocked by cyclopamine (n indicates the number of cultures for each condition). **, $P < 0.01$. (*B–D*) CNCC cultures show *Runx2*$^+$ cells in perichondrium in control medium (*B*), in the presence (d7–d10) of Shh (*C*), or in the presence of both Shh and cyclopamine (*D*). (*E*) Double in situ hybridization showing *Sox9* (red) and *Runx2* (green) expression in chondrocytes and osteoblasts, respectively (blue shows Hoechst nuclear staining). (*F–I*) Nonperichondrial *Runx2*$^+$cells are isolated (*G*) or grouped in cell islands (*I*); the number of *Runx2*$^+$ isolated cells (*F*) and the number of cell islands expressing *Runx2* (*H*) do not change in the different media (*F* and *H*; $n = 12$). Data are shown as mean (±SEM) of 3 independent experiments. (Magnifications: *B–E* and *I*, 55×; *G*, 220×.)

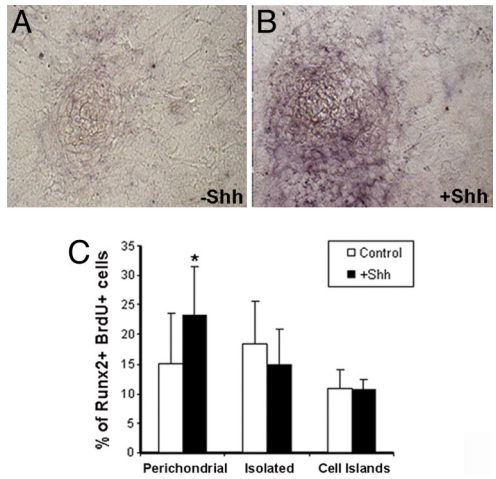

Fig. 2. Shh promotes *Gli2* expression and proliferation of *Runx2*$^+$ cells. *Gli2* expression increased in CNCC cultures treated with Shh from d7 to d10 (*B*) compared with d0–d7 treatment (*A*), similarly to *Runx2* (Fig. 1*A*). (Magnification: 80×.) (*C*) BrdU incorporation and *Runx2* expression in Shh-treated (d7–d10) and untreated d10 cultures; the proportion of BrdU-incorporating *Runx2*$^+$ cells is indicated for *Runx2*$^+$ perichondrial cells, *Runx2*$^+$ isolated cells, and *Runx2*$^+$ cell islands. Data are given as mean (±SEM) of 3 independent experiments ($n = 12$ cultures). *, $P < 0.05$.

submitted CNCC cultures to Shh treatment from d0 to d7, from d7 to d10, or from d0 to d10 as described above (Fig. 1*A*). Similar to continuous exposure to Shh, treatment with Shh between d7 and d10 significantly increased the number of perichondrial areas that expressed *Runx2* at d10 compared with controls (Fig. 1 *A–C*). The positive effect of Shh on perichondrial osteoblast differentiation took place between d7 and d10. Accordingly, addition of both Shh and the alkaloid cyclopamine, an inhibitor of Shh signaling (19), from d7 to d10 blocked Shh-mediated increase of *Runx2* perichondrial expression (Fig. 1*D*).

In addition to its requirement for osteogenesis, *Runx2* plays a role in chondrocyte maturation and hypertrophy (20). Therefore, we looked for expression of both *Runx2* and *Sox9* transcription factors, because the latter is activated in vivo specifically in chondrocytes (21, 22). The chondrocyte nodules only expressed *Sox9*, whereas *Runx2* expression was detected mainly in the perichondrium in d10 cultures (Fig. 1*E*). *Runx2*$^+$ osteoblastic cells and *Sox9*$^+$ chondrocytic cells thus formed nonoverlapping, adjacent cell populations.

We also found that *Runx2* was expressed in d10 cultures independently of the presence of cartilage nodules, in either isolated cells (Fig. 1*G*) or cell islands (Fig. 1*I*). Quantification of *Runx2*$^+$ isolated cells and of islands expressing *Runx2* showed no difference between Shh-treated and untreated cultures (Fig. 1 *F* and *H*). Moreover, addition of both cyclopamine and Shh did not change the amount of *Runx2*$^+$ isolated cells and cell islands recorded in the cultures (Fig. 1 *F* and *H*).

Effect of Shh Treatment on *Gli* Expression and Cell Proliferation in *Runx2*-Expressing CNCCs. To gain further insight about the role of Shh in osteogenesis by cultured CNCCs, we first examined the expression of the *Gli* genes encoding Shh signaling effector proteins Gli1, Gli2, and Gli3 (23). In d10 cultures exposed to Shh from d7 to d10, we observed an increase in *Gli2* expression in perichondrial regions compared with control cultures and with those treated from d0 to d7 (Fig. 2 *A* and *B*). We did not detect *Gli1* and *Gli3* transcripts in either culture condition. Therefore, in the perichondrium, enhancement of *Gli2* by Shh coincides with the increase in *Runx2*, suggesting that Gli2 mediates the effect of Shh on the development of perichondrial osteoblasts.

To examine osteoblast proliferation, we performed a pulse labeling with BrdU at d10. CNCC cultures exposed to Shh exhibited a higher proportion of *Runx2*$^+$ perichondrial cells incorporating BrdU, whereas the proportion of *Runx2*$^+$ isolated

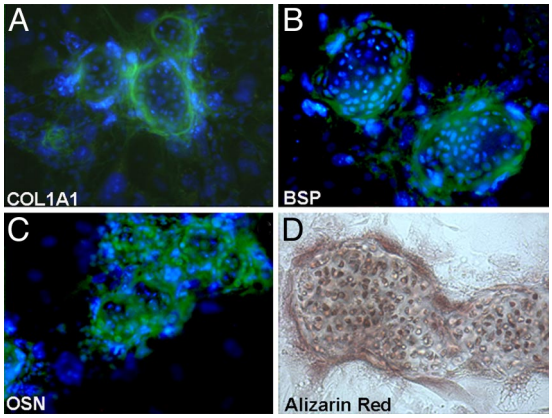

Fig. 3. Cultured CNCCs express bone matrix proteins. Shh-treated CNCC cultures maintained from d7 to d15 in osteogenic medium (see *Materials and Methods*) comprise perichondrial cells immunoreactive to collagen-1α1 (COL1A1) (*A*), bone sialoprotein 1 (BSP) (*B*), and osteonectin (OSN) (*C*) and which stain with Alizarin red (*D*). (Magnification: 50×.)

cells and *Runx2*$^+$ cells in islands, which were labeled with BrdU, was unchanged compared with control cultures (Fig. 2*C*).

Expression of Bone Matrix Proteins by Differentiated Osteogenic CNCCs. To assess whether the osteogenic CNCCs that develop in the perichondrium in the presence of Shh are able to acquire mature ossification markers, Shh-treated cultures were further grown from d7 to d15 in the presence of an osteogenic medium (24). These conditions triggered synthesis of the bone matrix proteins collagen-1α1, bone sialoprotein, and osteonectin in the perichondrial areas (Fig. 3 *A–C*) and promoted mineral deposition, as detected by Alizarin red staining (Fig. 3*D*).

Characterization of Osteoblastic Progenitors in CNCC Clonal Cultures. To identify osteogenic progenitors, we performed clonal cultures of CNCCs in the absence and presence of Shh from d7 to d10 (as described above as promoting perichondrial osteogenesis). Quantification of d10 colonies showed that the clonal efficiency of CNCCs slightly increased in Shh-treated cultures compared with untreated cultures [65% (n = 146) and 52% (n = 109), respectively; P = 0.04]. We analyzed the CNCC clonal progeny from the main NC-derived cell types by using phenotypic markers (Fig. 4*A*). *Runx2*$^+$ osteoblasts differentiated in nearly all colonies in both control (94%) and Shh-supplemented (96%) medium. Addition of Shh increased the frequency of the clones containing chondrocytes (from 20% to 50%) and myofibroblasts (from 54% to 78%) compared with control medium (Fig. 4*A*). According to the combinations of cell types they contained, 30 distinct clone types were recorded (Fig. 5). Noticeably, we identified a highly multipotent GNMFCO progenitor able to give rise to all of the expected phenotypes (glial, neuronal, melanoblastic, myofibroblastic, chondrocytic, and osteoblastic; Fig. 4 *B–E*), which exhibited a higher frequency in the presence of Shh (13%) than in controls (7%; P = 0.009; Fig. 5). The majority of the other progenitors in both media were pentapotent, quadripotent, and tripotent CNCCs endowed with osteoblastic potential. The most frequent in Shh-treated cultures corresponded to a GMFCO pentapotent progenitor (23%), a progenitor very rare in control conditions (0.5%). In contrast, Shh decreased the frequency of GNMO and GMO progenitors 2.5-fold and 4-fold, respectively (Fig. 5).

Three kinds of skeletal progenitors were identified in these experiments: (*i*) O^+C^+ osteochondrogenic progenitors, (*ii*) O^+C^- progenitors yielding osteoblasts but no chondrocytes, and (*iii*) O^-C^+ progenitors generating chondrocytes but no osteoblasts. The last group of chondrogenic CNCCs was recorded at a low frequency in both media. By contrast, the osteochondrogenic progenitors increased and the osteogenic progenitors decreased significantly in the presence of Shh (Fig. 5). Therefore, although it did not modify the overall frequency of osteogenic CNCCs (Fig. 4*A*), Shh favored the development of osteochondrogenic progenitors at the expense of those able to yield osteoblasts but no chondrocytes. Taken together, because of the widespread osteogenic capacity of CNCCs, neural–mesenchymal progenitors accounted for more than 92% of clonogenic cells in both control and Shh-supplemented media (Fig. 5, gray progenitors). Hence, the proportion of CNCCs with exclusively neural or mesenchymal potentials (Fig. 5, yellow and blue, respectively) was very low in both conditions.

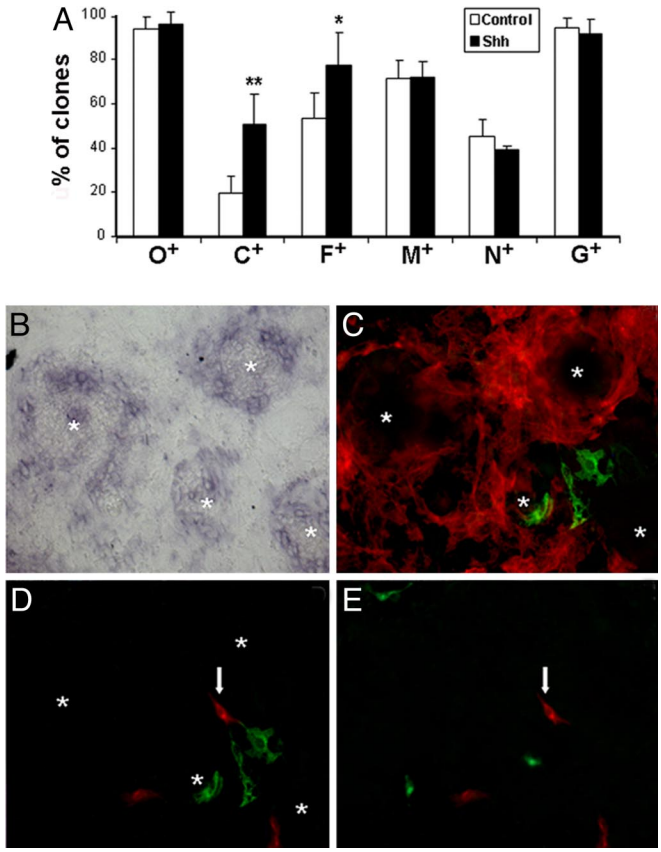

Fig. 4. Evidence for highly multipotent osteogenic CNC progenitors in clonal cultures. Clonal cultures of CNCCs grown with and without Shh (d7–d10) were analyzed by in situ hybridization and immunocytochemistry to detect osteoblasts, chondrocytes, myofibroblasts, melanocytes, neurons, and glial cells (see *Materials and Methods*). (*A*) Quantification of the clones containing each phenotype. Nearly all colonies include osteoblasts; Shh increases the total frequency of the clones containing chondrocytes and myofibroblasts. Data are expressed as mean percent ± SEM from 4 independent experiments (n = 109 and n = 146 colonies in control and Shh-supplemented media, respectively; **, $P < 0.01$; *, $P < 0.05$). (*B–E*) GNMFCO multiphenotypic colony including: (*B*) *Runx2*$^+$osteoblasts, (*C*) α-SMA$^+$ myofibroblasts (red) and HNK1$^+$ glial cells (green), (*D*) neurons (tyrosine hydroxylase-tubulinβIII$^+$ in red, indicated by the arrow next to the glial cells), and (*E*) melanocytic cells (MelEM$^+$ in green, next to the neurons). Asterisks in *B–D* indicate cartilage nodules. (Magnification: 140×.)

Discussion

In contrast to the trunk skeleton, which derives from mesodermal cells, most of the head skeleton arises from the CNC in higher vertebrates. CNCCs give rise to intramembranous bones in the skull vault, the otic capsule and jaw, and to endochondral

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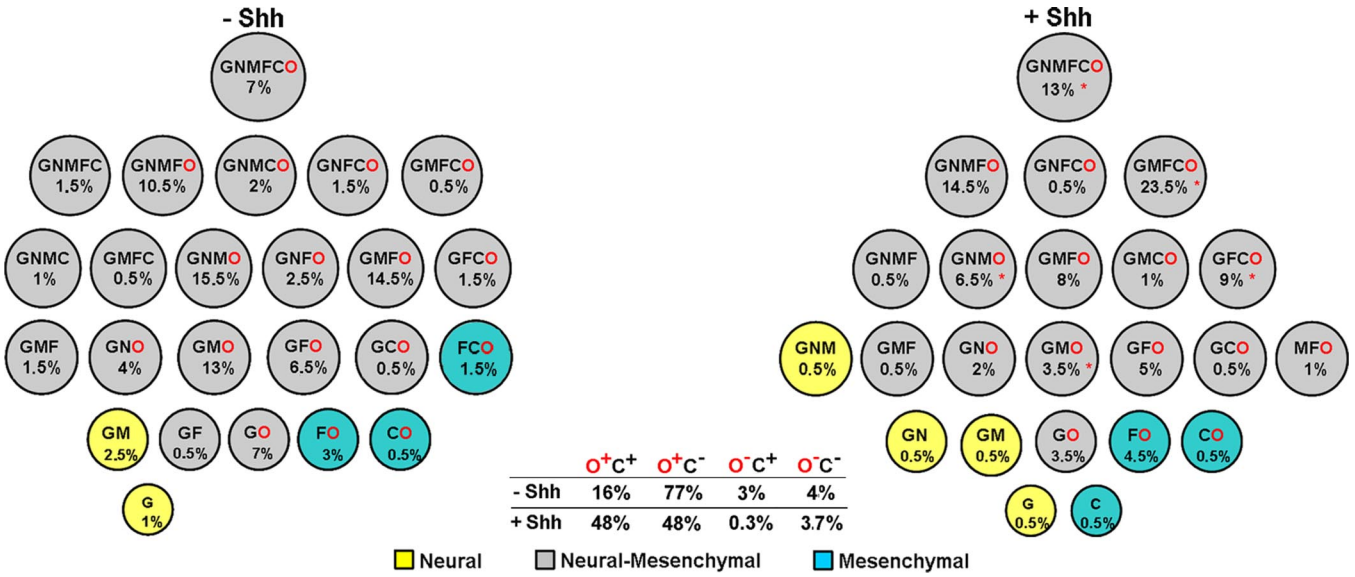

Fig. 5. Osteogenic CNCCs are multipotent and the targets of Shh. The analysis of control ($n = 109$) and Shh-treated ($n = 146$) colonies (Fig. 4) is summarized in a schematic lineage tree in which progenitor types are classified according to the number of cell types in their progeny (G, glial cells; N, neurons; M, melanocytes; F, myofibroblasts; C, chondrocytes; and O in red, osteoblasts). The frequency of each progenitor type (percent of clones) is shown in both medium conditions, and red asterisks indicate those modified upon Shh treatment (*, $P < 0.05$). In both medium conditions, progenitors endowed with both mesenchymal (i.e., F, C, O) and neural (i.e., G, N, M) potentials (in gray) are widespread compared with those yielding only neural (in yellow) or only mesenchymal (in blue) cells. The table summarizes progenitor frequency with respect to chondrogenic (C^+) and osteogenic (O^+) potentials, which define nonskeletogenic CNCCs (O^-C^-) and 3 types of skeletogenic CNCCs—i.e., osteochondrogenic (O^+C^+), osteogenic nonchondrogenic (O^+C^-), and chondrogenic nonosteogenic (O^-C^+) progenitors.

bones, such as the nasal capsule and the quadrate and hyoid bones, which originate from a cartilage rudiment (1, 25, 26). Despite the importance of the CNC in building craniofacial structures, little is known about the emergence and specification of skeletogenic NC progenitors. Here, we have characterized the developmental potentials and response to Shh of osteogenic CNCCs in vitro.

CNCCs Differentiate in Vitro into Perichondrial and Dermal-like Osteoblasts. By exploiting a culture system that previously allowed quail CNCCs to develop along the main NC-derived lineages, including the chondrocytic lineage (7), we have characterized in vitro osteoblastic differentiation of CNCCs isolated at early migratory stages from the mes-rhombencephalon of 6–7 somite-stage quail embryos. Osteoblastic cells, as defined by expression of the early marker gene of osteogenesis *Runx2* (14–16), were detected from d7, increased in number at d10, and were still present in d15 cultures. This is consistent with the temporal pattern of *Runx2* described in vivo (17, 18).

Runx2$^+$ cells were found in association with chondrocyte nodules or in chondrocyte-free areas of the cultures as isolated cells or grouped in cell islands. The first type of osteoblasts differentiated in the perichondrium of cartilage nodules that had formed at earlier stages of CNCC culture (7). When CNCCs were further maintained in medium conditions promoting osteogenesis (24), the perichondrial osteoblasts underwent synthesis of bone matrix proteins and showed mineral deposits. In contrast, the *Runx2*$^+$ isolated cells and cell islands in these cultures did not reach the matrix-secreting mature state. Based on their differential bone marker expression and distinct locations relative to chondrocyte nodules, the CNCC-derived perichondrial osteoblasts can be considered as undergoing endochondral-like ossification (27, 28), whereas the *Runx2*$^+$ cell islands can be assigned to a dermal-like type of osteoblast, which does not require a cartilage template to develop (17, 18, 29).

Shh Regulates in Vitro Proliferation and Differentiation of the CNC-Derived Perichondrial Osteoblasts. Differences between the types of *Runx2*$^+$ osteoblasts recorded in CNCC cultures are further exemplified by their differential response to Shh. When added to CNCC cultures from d7 to d10 (once cartilage nodules have been formed), Shh has a critical role in promoting differentiation and proliferation of *Runx2*$^+$ perichondrial cells. This result is consistent with the ability of Shh to trigger osteogenic differentiation in mesenchymal and preosteoblastic cell lines (30–32). The Shh-induced increase in perichondrial osteoblasts was blocked by cyclopamine and correlated with up-regulation of perichondrial *Gli2* expression, suggesting that Shh acts on perichondrial osteoblasts in CNCC cultures through Smoothened and Gli2 effector proteins. This possibility is supported by the osteopenic phenotype of *Gli2* knockout mice (33) and by in vitro studies showing that *Gli2* mediates Shh action on *Runx2* expression and osteoblast differentiation by mesenchymal cells (34, 35). In contrast to perichondrial osteoblasts, we found no detectable effect of Shh on dermal-like osteoblasts, which differentiated and proliferated independently of the presence of Shh (whenever added continuously or from d7 to d10). Shh treatment did not up-regulate *Gli2* expression in *Runx2*$^+$ cell islands.

Taken together, these findings are consistent with in vivo data showing that Hedgehog signaling mediated by Indian hedgehog (Ihh) is indispensable for endochondral skeleton development (36–39). Although reduced, membranous ossification occurs in the skull of mice deleted for *Ihh* (39). Therefore, compared with endochondral ossification, a distinct, hedgehog-independent mechanism likely regulates osteoblast commitment in membranous bones in vivo and in CNCC cultures.

CNCCs Comprise 3 Types of Multipotent Skeletogenic Progenitors with Differential Responsiveness to Shh Signaling. In single-cell cultures, more than 90% of clonogenic CNCCs turned out to be able to yield osteoblasts. Nearly all of these osteogenic CNCCs were multipotent progenitors with diverse combinations of neural and mesenchymal differentiation potentials. According to their ability to generate chondrocytes and/or osteoblasts, skeletogenic multipotent CNCCs were identified as osteochondrogenic, osteogenic only and, less frequently, chondrogenic only. These

types of skeletogenic progenitors strikingly recapitulate the 3 distinct skeletal structures arising from the CNC—i.e., endochondral bones, dermal bones, and persistent cartilages (18).

These results have several implications for skeletogenic cell lineage segregation in the CNC. First, the CNCCs do comprise common progenitors for bone and cartilage, designated as osteochondrogenic progenitors, the existence of which was inferred by genetic fate mapping (40). Second, our finding that most CNCCs can generate osteoblasts but no chondrocytes strongly argues that dermal bone cells can arise from specific progenitors distinct from the osteochondrogenic, endochondral-like ones. Hence, the endochondral and dermal bone cell lineages may be independently regulated in different multipotent CNCCs that can be distinguished by their response to Shh; this factor specifically favors osteochondrogenic CNCCs (O^+C^+) at the expense of the progenitors yielding dermal-like osteoblasts (O^+C^-) (Fig. 5). This action of Shh on clonogenic CNCCs is consistent with the effects of Shh observed in mass cultures, in which Shh stimulated the development of perichondrial, endochondral-like osteoblasts but did not influence significantly the dermal-like osteoblasts located in cell islands. The increase in osteochondrogenic progenitors also results from stimulation of chondrogenesis exerted by Shh during the first 48 h of culture (7, 41).

An Shh-Responsive, Multipotent Osteochondrogenic Progenitor Lies Upstream of CNCC Hierarchy. We identified a highly multipotent CNCC that generated a progeny containing glial cells, neurons, melanocytes, myofibroblasts, chondrocytes, and osteoblasts (GNMFCO). This hexapotent progenitor lies upstream of all of the other NC progenitors described thus far (42). It is a good candidate for being the NC stem cell comparable to the hematopoietic stem cell able to yield all of the blood cell types. For deserving this status, however, it lacks the proven self-renewal capacity fully demonstrated for the hematopoietic stem cell (43). The GNMFCO progenitor responds to Shh treatment by a 2-fold increase in frequency, arguing that Shh regulates the survival of multipotent CNCCs, as shown in our previous work for the GNMFC progenitor (7). Taken together, the pentapotent and hexapotent progenitors identified in the present experiments accounted for 51% of total clones recorded in the presence of Shh, compared with 23% in the absence of Shh (Fig. 5). These results suggest that Shh regulates the survival and proliferation of multipotent stem cells in the CNC, as observed in the enteric nervous system (44, 45) and CNS (46–48). Finally, in both control and Shh-treated cultures, more than 90% of CNCC progenitors were endowed with neural–mesenchymal potentialities, whereas progenitors of exclusively neural or mesenchymal phenotypes were very rare. This reflects the high proportion of progenitors for glia and osteoblasts in CNCCs, which are thus the most frequent representatives of the neural and mesenchymal NC lineages, respectively.

The present results thus uncover a previously unsuspected prevalence of the osteogenic potential in the early CNC, which may be related to the notion drawn from fossil data that, in all likelihood, the NC was at the origin of the bony armor of primitive vertebrates, such as ostracoderms (49). Subsequently, an internal skeleton of mesodermal origin replaced the exoskeleton, permitting a significant increase in body mobility. The skeletogenic capacities of the NCCs were retained in the dorsal fin of teleosts and in the cranial and facial skeleton, even in the most evolved vertebrates. The capacity to yield osteocytes and chondrocytes together with glia, neurons, and melanocytes, which is present in single CNCCs, thus might be a striking remnant of the initial role played by the NC during vertebrate evolution.

Materials and Methods

Cell Cultures. CNCCs were isolated from the neural primordium (mesrhombencephalon) of 6–7 somite-stage quail embryos. After 15 h of primary culture, CNCCs that had migrated from explanted neural tubes were harvested for secondary plating on a feeder layer of growth-inhibited 3T3 fibroblasts (7) in either mass cultures (400 cells per well) or clonal cultures in 96-well plates (TPP). Control culture medium was DMEM containing 10% FCS. In Shh-treated cultures, the medium was supplemented with 100 ng/mL mouse N-Shh (R & D Systems) during the whole culture period (d0–d10), during only the first 7 days (d0–d7), or from d7 to d10 (d7–d10). In the last of these conditions, an initial treatment during the first 48 h of culture (d0–d2) was also performed, which enhanced differentiation into cartilage (7, 41) but did not affect osteogenesis by CNCCs (Fig. 1*A*). In clonal cultures, 2% chicken embryo extract was added to the medium. Inhibition of Shh signaling was performed by treatment with 5 μM cyclopamine (19) (Toronto Research Chemicals). When indicated, Shh-treated cultures were maintained from d7 to d15 in osteogenic medium (24)—i.e., control medium supplemented with 10 nM dexamethasone, 1 mM β-glycerol phosphate, and 50 μg/mL ascorbic acid (all from Sigma). Cultures were maintained at 37 °C in a humidified 5% CO_2/95% air atmosphere.

Phenotype Analysis. Cultures were fixed with 4% paraformaldehyde at d10 or d15. Quail CNCCs were distinguished from mouse 3T3 fibroblasts by Hoechst nuclear staining (3). Osteoblastic *Runx2*-expressing cells were identified by in situ hybridization with a digoxygenin-labeled probe for chicken *Runx2* [gift from T. Jaffredo, Centre National de la Recherche Scientifique Unité Mixte de Recherche 7622, Paris, France] according to an already described procedure (7). Differentiation of chondrocytes in 3-dimensional nodules was assessed by phase-contrast microscopy and after in situ hybridization for chick *Sox9* (50) as described previously (7), except that a fluorescein-labeled RNA probe was revealed by using a Tyramide System Amplification Kit (Invitrogen). Bone matrix proteins were detected by using antisera against bone sialoprotein-1 (BSP; LF-84, LF-119; 1:100), osteonectin (LF-8, LF-45; 1:200), and collagen-1α1 (LF-67; 1:100), a generous gift from L. W. Fisher (51) (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda). Immunocytochemical detection of non-skeletal phenotypes was performed essentially as described previously (7) by using the following antibodies: Melanoblast/melanocyte Early Marker (MelEM) for melanocytic cells (52), HNK1 for glial cells, α-smooth muscle actin (αSMA; 1A4; Sigma) for myofibroblasts/smooth muscle cells, and Tubulin βIII (5G8; Promega) and tyrosine hydroxylase for neurons and adrenergic cells, respectively. Secondary antibodies were purchased from Southern Biotechnology Associates. Cell proliferation activity was analyzed by BrdU incorporation (1-h pulse before fixation) detected by immunofluorescence using a Cell Proliferation Kit (Roche). Fluorescence was observed with an X70 Olympus microscope. Statistical analysis was performed by using a 2-tailed Student's *t* test (for cell numbers) or χ^2 analysis (for clone frequency). Differences between control and Shh-treated cultures were considered significant when $P < 0.05$.

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