Secondary chondrocyte-derived Ihh stimulates proliferation of periosteal cells during chick development

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Summary

The development of the skull is characterised by its dependence upon epigenetic influences. One of the most important of these is secondary chondrogenesis, which occurs following ossification within certain membrane bone periostea, as a result of biomechanical articulation. We have studied the genesis, character and function of the secondary chondrocytes of the quadratojugal of the chick between embryonic days 11 and 14. Analysis of gene expression revealed that secondary chondrocytes formed coincident with *Sox9* upregulation from a precursor population expressing *Cbfa1/Runx2*: a reversal of the normal sequence. Such secondary chondrocytes rapidly acquired a phenotype that is a compound of pre-hypertrophic and hypertrophic chondrocytes, exited from the cell cycle and upregulated *Ihh*. Pulse and pulse/chase experiments with BrdU confirmed the germinal region as the highly proliferative source of the secondary chondrocytes, which formed by division of chondrocyte-committed precursors. By blocking Hh signalling in explant cultures we show that the enhanced proliferation of the germinal region surrounding the secondary chondrocytes derives from this *Ihh* source. Additionally, in vitro studies on membrane bone periosteal cells (non-germinal region) demonstrated that these cells can also respond to Ihh, and do so both by enhanced proliferation and precocious osteogenesis. Despite the pro-osteogenic effects of Ihh on periosteal cell differentiation, mechanical articulation of the quadratojugal/quadrate joint in explant culture revealed a negative role for articulation in the regulation of *osteocalcin* by germinal region descendants. Thus, the mechanical stimulus that is the spur to secondary chondrocyte formation appears able to override the osteogenic influence of Ihh on the periosteum, but does not interfere with the cell cycle-promoting component of Hh signalling.

Key words: *Cbfa-1*, *Ihh*, *Sox9*, Hypertrophy, Chondrocyte, Membrane bone, Proliferation, Chick

Introduction

Intramembranous, or direct, ossification is distinguished from endochondral ossification by the absence of a cartilage scaffold in the former. The significance of this difference is highlighted as we learn more about the key processes of endochondral ossification, although knowledge regarding direct ossification remains scarce. A linear pathway can be discerned for the endochondral maturation of chondrocytes, during which molecular and histological markers of differentiation accrue along the axis of the element from epiphysis to diaphysis. The endpoint for this series is the hypertrophic chondrocyte, a post-proliferative, enlarged cell that possesses characteristics of both chondrocyte and osteoblast (Gerstenfeld and Shapiro, 1996). It is reached via an intermediate, the pre-hypertrophic chondrocyte, which is characterised by exit from the cell cycle, but not expressing the full hypertrophic phenotype. Coincident with the formation of pre-hypertrophic and hypertrophic chondrocytes, changes in the cells surrounding the cartilage element occur. The perichondrium overlying the pre-hypertrophic and hypertrophic zone begins to secrete mineralised matrix; by loosely followed convention this is designated the periosteum (Lacroix, 1951). The pathway of chondrocyte maturation, and its effects on the attendant endochondral ossification, can therefore be distilled into two processes: cell cycle regulation and differentiation. The periosteum comprises pre-osteoblasts and osteoblasts, which undergo a parallel but less overt differentiation pathway than do chondrocytes (Aubin and Liu, 1996).

Skeletogenic tissues are highly responsive to applied forces, and hence are uniquely shaped by epigenetic influences (which we define as cellular responses to macroscopic influences); an example in development is the evocation of secondary cartilage. Secondary cartilage arises after membrane bone formation at a variety of sites in birds and mammals (reviewed by Fang and Hall, 1997; Beresford, 1981). In the chick, secondary cartilage has been shown to occur at articulations between membrane bones and cartilage elements of the head (Murray, 1963). Muscle action across these joints leads to the induction of cartilage within the bounds of the periosteum (Murray, 1963), and this tissue is believed to be the source of the chondrocytes. This evocation process can be mimicked in explant culture by mechanical articulation (Hall, 1967), or

prevented through in ovo paralysis (Murray and Smiles, 1965). In the mouse temporo-mandibular joint (TMJ), immunohistochemical data indicate a rapid hypertrophy of secondary chondrocytes (Silbermann et al., 1990; Shibata et al., 1997). Despite the unusual epigenesis, the lifetime of a secondary chondrocyte is short, and follows the normal course for a chondrocyte: resorption of matrix by chondroclasts and replacement with endosteal bone (Hall, 1972).

The identification and study of two transcription factors, Sox9 and Cbfa1/Runx2, has greatly enhanced our understanding of the skeletal tissues. Sox9 is recognised as the key regulator of chondrocyte differentiation: it is obligate for chondrocyte specification (Bi et al., 1999), regulates cartilage specific genes (Bell et al., 1997; Lefebvre et al., 1997; Sekiya et al., 2000; Xie et al., 1999) and can induce ectopic chondrogenesis when misexpressed (Bell et al., 1997; Healy et al., 1999). Its transcriptional regulation is consistent with these roles (Wright et al., 1995; Zhao et al., 1997), and excludes any putative role in osteoblast development.

The *runt*-box-containing transcription factor *Cbfa1/Runx2* is similarly essential for osteoblast maturation (Ducy et al., 1997; Komori et al., 1997); haploinsufficiency leads to the human skeletal disorder cleidocranial dysplasia (Mundlos et al., 1997). Hypertrophy of chondrocytes in the *Cbfa1*-knockout mouse is also defective in some long 'bone' elements (Inada et al., 1999; Kim et al., 1999), which is consistent with upregulation of *Cbfa1* expression in chondrocytes preceding hypertrophy (Kim et al., 1999). The requirement for *Cbfa1* in chondrocyte maturation has been further investigated using the *Col2* promoter to drive *Cbfa1* expression in immature chondrocytes and in those that would not normally hypertrophy (Takeda et al., 2001). *Cbfa1* is sufficient in these cells to promote hypertrophy and to rescue the chondrocyte phenotype of *Cbfa1*-deficient mice. Moreover, these studies identify Cbfa1 as the first transcription factor to regulate both chondrocyte and osteoblast differentiation (Takeda et al., 2001).

As mentioned above, *Cbfa1* plays an important role in the attainment of hypertrophy by some chondrocytes; an example of the regulatory integration between bone and cartilage. The co-dependence of these tissues has also been illustrated by elucidation of the role of Indian hedgehog (Ihh) in skeletogenesis. Overexpression of *Ihh* in the chick limb leads to suppressed hypertrophy and an increase in bone collar formation (Vortkamp et al., 1996). The former effect results from a feedback loop involving *Pthrp* upregulation. *Ihh* expression in the pre-hypertrophic chondrocytes coincides with osteogenesis in the adjacent perichondrium; a link shown to be causal by the failure of osteogenesis in the long bones of $Ihh^{-/-}$ mice (St-Jacques et al., 1999). Thus, in conjunction with its role in chondrocyte proliferation, achieved directly (Karp et al., 2000) and indirectly (Vortkamp et al., 1996), Ihh couples cartilage differentiation with differentiation of adjacent osteoblasts (Chung et al., 2001). It can be seen, therefore, that a crucial step in osteoblast induction is chondrocyte exit from the cell cycle leading to pre-hypertrophy and *Ihh* upregulation. The tight choreography of chondrocyte differentiation and osteogenesis in the perichondrium/periosteum cedes the primary role to cartilage. It is appropriate therefore that in the membrane bones of $Ihh^{-/-}$ mice, where cartilage forms secondarily, osteogenesis is normal (St-Jacques et al., 1999).

Owing to the likely periosteal origin of secondary chondrocytes, one may expect their genesis to challenge the established bifurcation between *Cbfa1*-specified osteoblasts and *Sox9*-specified chondrocytes. The regulation of these pivotal genes has not previously been examined in secondary chondrogenesis, but the importance of epigenetic influences on skull development and evolution is widely acknowledged (Thorogood, 1993). We have studied the evocation and function of secondary cartilage in the chick, and found that the integration of epigenetic, and more familiar genetic, pathways underlies its role as a signalling centre. Our study also indicates that *Sox9*, like *Cbfa1* and chondrocyte differentiation, can divert pre-osteoblasts to chondrogenesis: the hypertrophic chondrocyte being the common endpoint, reached via the functionally crucial *Ihh*-expressing pre-hypertrophic chondrocyte.

Materials and methods

In situ hybridisation and probe details

Radioactive in situ hybridisation was performed as described (Francis et al., 1994). In brief, embryos were fixed in 4% paraformaldehyde and processed through to wax, sectioned at 8 μm and hybridised with [^{35}S]-labelled riboprobes at 55°C. Chick probes used were as follows: *Cbfa1/Runx2 – Xho*I (T3; gift from Dr J. Helms), *Sox9* (Kent et al., 1996), *Col2* (Devlin et al., 1988), *Col10* (Kwan et al., 1989), *Ihh* (Vortkamp et al., 1996), *Ptc2* (Pearse et al., 2001), *Bmp7* (Houston et al., 1994), *Frzb* (Ladher et al., 2000), and *Osteocalcin* and *Osteopontin* PCR fragments (gift from Dr V. Church). Sections were viewed on a Zeiss Axioskop and photographed using a Sony DSC75 digital camera. Montages were arranged using Adobe Photoshop. In some cases bright field images were overlayed with inverted dark field images.

Manipulation of the quadratojugal/quadrate joint

Fertilised wild-type chicken eggs (Ross White) were obtained from Henry Stewart and Co., Lincolnshire, UK. Eggs were incubated at ~38°C for the stated number of days. Embryos at stage e14 were used for the ex vivo experiments, as the joint was less liable to dislocation when manipulated. The quadratojugal/quadrate (QJ/Q) was dissected from either side of the head and stripped of most attendant connective tissue, except that holding the two elements together. The Q and QJ were trimmed and then placed on a filter (Millipore; 0.2 μM) upon a metal grid at the air/medium interface. Explants were cultured in DMEM (Gibco) plus 15% chick serum (Sigma), at 37°C/5% CO_2. Articulation regimes involved operating the joint through its normal movement (10 times on the hour) in order to mimic the sporadic movements in ovo, for the specified time (following Hall, 1967).

Treatment with blocking antibody

Anti-hedgehog antibody, 5E1 (Ericson et al., 1996), was obtained from the DSHB, University of Iowa, IA. Partially purified antibody was obtained and used at a dilution of 1:10.

Cell proliferation studies

Explants were labelled in 10×BrdU diluted in culture medium (as above), according to the manufacturer's protocol (Roche), immediately following dissection (for 0 hour timepoint) or at the specified time post-dissection for 90 minutes. Explants were then fixed in 4% paraformaldehyde, processed through to wax and sectioned. BrdU incorporation was revealed using a biotin-conjugated anti-BrdU antibody (MD-5215; Caltag, USA). ABC kit from Vectastain (Vector Labs, Peterborough, UK) was then used, followed by DAB (Vector Labs) for the colour reaction. Sections were counterstained with Toluidine Blue (0.02%) for cell counting, or Alcian Blue (prior to immunochemistry) and Chlorantine Fast Red

(post-immunochemistry) to assess differentiation. For pulse-chase experiments the QJ/Q joint was rinsed in medium, placed on a fresh filter in fresh medium and re-incubated. Parathyroid hormone (PTH) was used at a concentration of 10 nM.

High-density micromass culture

High-density micromass culture was carried out essentially as described by Fang and Hall (Fang and Hall, 1996), based on the sequential digestion method for calvaria (Wong and Cohn, 1974). 2-3 dozen eggs were harvested to provide e13 QJs and Qs that were then dissected free from muscle and connective tissue, and pooled in PBS. The hook, containing the secondary chondrocytes, was removed and the shafts were digested in 0.5% trypsin/collagenase type 1A (200 units/ml; Sigma) in PBS, sequentially for 30 minutes, 30 minutes and 45 minutes. Between each digestion the solution was removed and replaced. The solution containing the cells was then spun down and rinsed in serum-containing medium. For establishing the micromass cultures we used fractions 2 and 3. These were pooled, passed through a cell strainer (Falcon), spun down and resuspended in 200 μl of medium. A 10 μl drop was added to the centre of a well of a multi-well plate, allowed to settle for 2 hours before flooding with medium (3/4 Ham's F12+1/4 BGJb, containing 10% FBS, L-glutamine, ascorbate 50 μg/ml, penicillin/streptomycin and β-glycerophosphate). Proliferation was assessed by adding BrdU for 30 minutes on the fourth day, before fixing and processing as outlined above.

Infection with retroviruses was achieved by adding filtered conditioned medium at the flooding stage. Alkaline phosphatase activity was detected using Napthol AS MX-PO_4 (Sigma) and Red Violet (Sigma), and mineralised matrix was detected using 2.5% silver nitrate enhanced by carbonate formaldehyde (von Kossa). The RCAS retroviral construct used in this study, *RCAS-Ihh*, has been previously described (Vortkamp et al., 1996); *RCAS-Gfp* was constructed as described by Church et al. (Church et al., 2002).

Results

Genetic basis of secondary chondrocyte initiation

Previous studies in the chick by Murray, and by Fang and Hall, have demonstrated that secondary chondrocytes arise in the quadratojugal (QJ) between embryonic days (e) 10 and 11 (Murray, 1963; Fang and Hall, 1997). In order to characterise the formation of secondary chondrocytes in this location, we studied initially the expression of *Cbfa1* and *Sox9*. Using radioactive in situ hybridisation of adjacent sections at e10 (Fig. 1A), we found *Cbfa1* (Fig. 1B) and *Sox9* (Fig. 1C) to be expressed in exclusive tissues: *Cbfa1* was expressed in membrane bone periostea and osteoblasts, and *Sox9* was expressed in the primary cartilaginous elements (e.g. the quadrate; Q). *Cbfa1* was expressed throughout the periosteum (Fig. 1E), but at the tip of the QJ at e10, where the secondary chondrocytes will arise, there was neither Alcian Blue staining (Fig. 1D), nor expression of *Sox9* (Fig. 1C), *type II collagen* (*Col2*; Fig. 1G) or *type X collagen* (*Col10*; data not shown). Analysis of *Gdf5* expression, a potent pro-chondrogenic growth factor of the TGFβ superfamily (Francis-West et al., 1999), revealed it to be expressed only in the perichondrium of the Q (Fig. 1F).

Alcian Blue staining can first be detected within the boundary of the periosteum of the membrane bone at e11, on the inner face of the QJ-hook (Fig. 1H), the earliest histological evidence of secondary chondrocytes (SCs). Observed simultaneously with this change, (though necessarily slightly in advance of it; data not shown) *Sox9* transcripts could be detected within the same region of the QJ (Fig. 1J). The *Sox9* expression domain was confined to the inner region of the QJ tip (the secondary chondrocytes) and did not extend to the adjacent periosteum, which, from hereon, we shall call the germinal region (Hall, 1968). *Cbfa1* continued to be expressed unaltered in the periosteum and germinal region, and was also expressed in the forming chondrocytes (Fig. 1I). Detection of the same genes at e13 (Fig. 1K,L,M) show clearly that *Cbfa1* was expressed in secondary chondrocytes (Fig. 1L), the periosteum and the germinal region. *Sox9* was also expressed throughout the SCs at this time point, but was excluded from the germinal region (Fig. 1M).

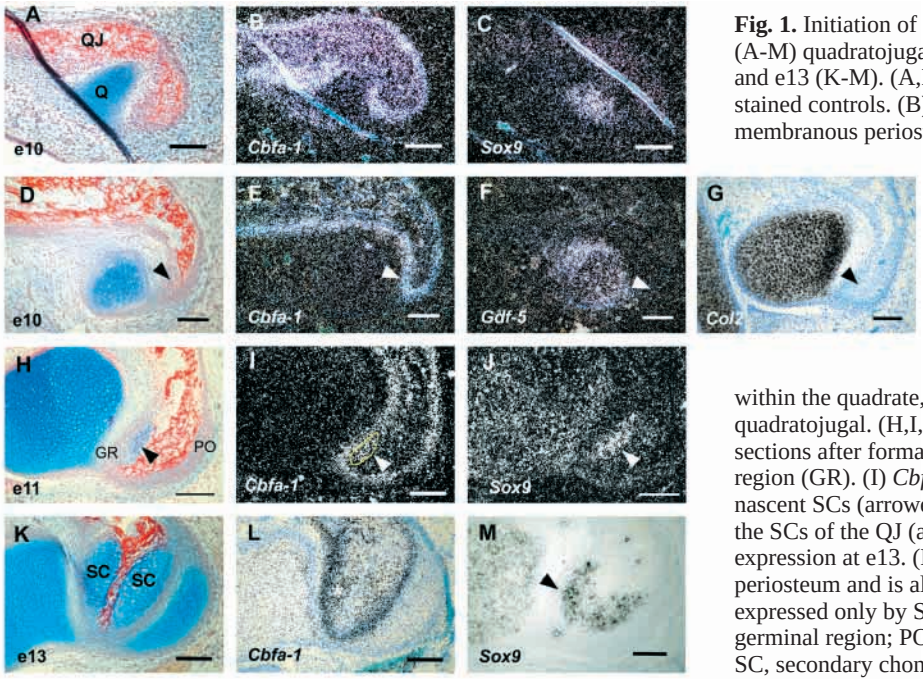

Fig. 1. Initiation of secondary chondrocyte formation. (A-M) quadratojugal/quadrate joints at stage: e10 (A-G), e11 (H-J) and e13 (K-M). (A,D,H,K) Alcian Blue/Chlorantine Fast Red stained controls. (B) *Cbfa1* expression is found only in the membranous periosteum of the quadratojugal (QJ). (C) *Sox9* expression is only found in the cartilaginous quadrate (Q). (D-G) Comparison of *Cbfa1*, *Gdf5* and *Col2* expression. Arrowheads (D-J) indicate future sites of secondary chondrocytes (SCs). (E) *Cbfa1* expression is maintained in the periosteum prior to SC formation. *Gdf5* (F) is expressed around the quadrate and *Col2* (G) is expressed within the quadrate, but neither is expressed within the quadratojugal. (H,I,J) *Cbfa1* and *Sox9* expression on adjacent sections after formation of the first SCs, adjacent to the germinal region (GR). (I) *Cbfa1* is expressed by the periosteum, and by the nascent SCs (arrowed and circled). (J) *Sox9* is expressed only by the SCs of the QJ (arrowhead). (K,L,M) *Cbfa1* and *Sox9* expression at e13. (L) *Cbfa1* continues to be expressed in the periosteum and is also expressed by SCs (asterisk). (M) *Sox9* is expressed only by SCs (arrowed), not in the periosteum. GR, germinal region; PO, periosteum; Q, quadrate; QJ, quadratojugal; SC, secondary chondrocytes. Scale bars: 100 μm.

We further characterised the secondary chondrocytes by examining the expression of *Col2*, an early chondrocyte matrix constituent; *Ihh*, expressed by pre-hypertrophic chondrocytes (Vortkamp et al., 1996); *Col10*, a marker for terminally differentiated chondrocytes (Devlin et al., 1988); and *Frzb*, expressed by condensing mesenchyme and early chondrocytes (Ladher et al., 2000).

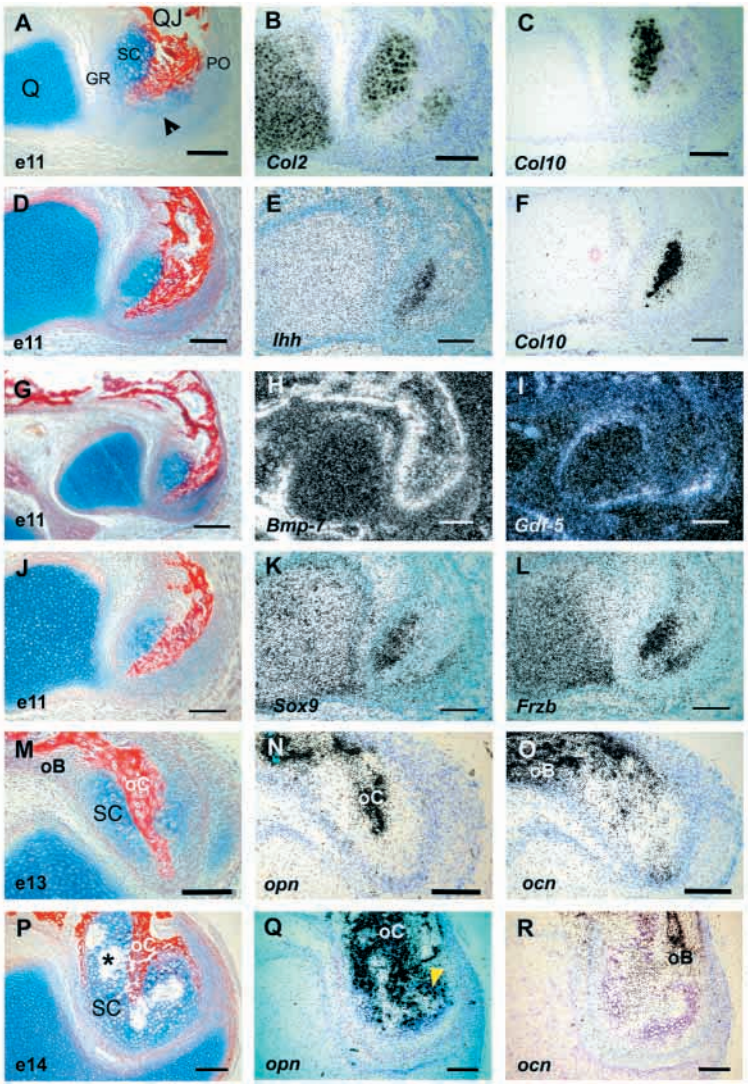

Fig. 2. Differentiation of secondary chondrocytes. (A-L) e11, (M-O) e13, (P-R) e14. (A,D,G,J,M,P) Alcian Blue/Chlorantine Fast Red stained controls. (A) SCs forming on the anterior and posterior (arrowhead) aspect of the QJ. (B,C) Adjacent sections to A, showing that *Col2* (B) is upregulated as SCs form, and that *Col10* (C) upregulation is delayed. (E,F) Adjacent sections to D, showing *Ihh* expression (E) in the same domain as *Col10* (F). (H,I) Adjacent sections to G. (H) *Bmp7* expression is observed throughout the periosteum, and (I) *Gdf5* expression is restricted to the perichondrium of the Q; neither reveal a change in the germinal region. (K,L) Adjacent sections to J, which show that *Sox9* (K) expression is confined to the SCs of the QJ, and that *Frzb* (L) expression is upregulated by SCs. (N,O) Adjacent sections to M, showing *opn* expression (N) in osteocytes; *ocn* expression (O) in osteoblasts adjacent to the PO precedes *opn* expression. (P,Q,R) At e14, chondroclast activity is evident (asterisk in P), and *opn* expression (Q) is additionally upregulated by late SCs (yellow arrowhead); *ocn* expression (R) remains specific for osteoblasts. oC, osteocytes; oB, osteoblasts. Scale bars: 100 μm.

Col2 was expressed in a domain coincident with that of Alcian Blue staining (Fig. 2A,B); in this example, both in anterior and posterior SC domains of the tip of the QJ, but not in the germinal region. *Col10* expression on an adjacent section (Fig. 2C) revealed that although the domain of *Col10* was contained within that of *Col2*, it had been rapidly upregulated by the secondary chondrocytes. This apparent rapid hypertrophy was borne out by the co-expression of *Ihh* (Fig. 2E) and *Col10* (Fig. 2F), and was consistent with the enlarged appearance of the secondary chondrocytes. Additionally, a negative regulator of chondrocyte differentiation, *Pthrp*, was undetectable at e11 and at e14 (data not shown). As secondary chondrocytes are believed to arise from the germinal region, we examined the expression of known perichondrial/periosteal markers to see whether changes in the expression of these genes presaged or reflected the formation of SCs. We found that *Bmp7* was expressed in the periosteum of the membrane bones (Fig. 2H), including around the SCs. *Gdf5* expression was not detected in the germinal region (Fig. 2I), even after SC formation, nor was it expressed in the remaining periosteum, but it could be detected in the quadrate perichondrium. We also looked at further markers of chondrocytes, for example, the pre-chondrogenic marker *Frzb* (Fig. 2L), which was expressed coincident with *Sox9* upregulation (Fig. 2K) and in advance of Alcian Blue staining. For markers of definitive osteoblasts and late stage hypertrophic chondrocytes we also compared the expression of *osteocalcin* (*ocn*) and *osteopontin* (*opn*) in the quadratojugal. Neither gene was expressed in SCs up to e13 (Fig. 2M,N,O; data not shown). In the membrane bone, *opn* was expressed by the osteocytes of the trabecular bone (Fig. 2N), whereas *ocn* was expressed earlier, in the osteoblasts adjacent to the periosteum (Fig. 2O). *Opn* was upregulated in SCs at e14 (Fig. 2P,Q), coinciding with the erosion of secondary cartilage by chondroclasts. By contrast, *ocn* expression remained osteoblast specific (Fig. 2R).

Secondary chondrocytes rapidly exit the cell cycle

Exit from the cell cycle is a key event in the maturation of chondrocytes, occurring at the transition to pre-hypertrophic chondrocyte. To determine how, or whether, this transition occurred in secondary chondrocytes, we explanted the quadratojugal/quadrate joints (QJ/Q), from e14 embryos, onto filters and labelled them with bromo-2′-deoxyuridine (BrdU) for 90 minutes whilst culturing at the air/medium interface. Explants were then fixed and processed for detection of BrdU. Labelling revealed striking differences in proliferation between the SCs, the germinal region and the periosteum – very few SCs were found to be in S-phase. Comparison of the percentage of BrdU labelled cells in the three regions confirmed the clear variation in proliferation rates (Fig. 3): periosteum (5.2%; 114/2175), germinal region (15.9%; 563/3531) and secondary chondrocytes (0.51%; 10/1978) (nine sections from three QJs were assessed for each result). Histologically, these results represented a marked cut-

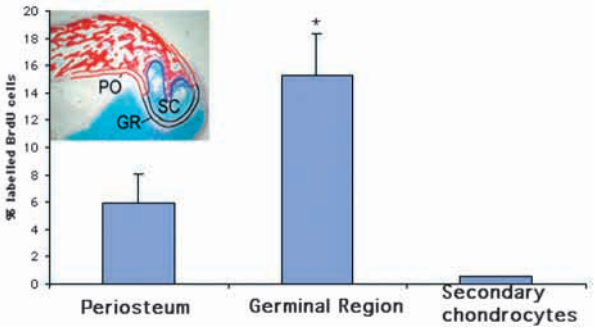

Fig. 3. Proliferation in the QJ at e14, as shown by the percentage of S-phase cells after BrdU labelling for 90 minutes at 0 hours. Figure (inset) indicates regions of the QJ counted for each value. Values shown are mean±s.d. from three QJs, a total of 6 sections. Asterisk indicates a significant difference between the periosteum and the germinal region (P<0.001, Student's t-test).

off in proliferation at the boundary between the SCs and germinal region (Fig. 4A), with only occasional cells at the periphery of the SC region being labelled (Fig. 4C; n=6, 36 sections).

Pulse and chase confirms origin of secondary chondrocytes

Given the distinction between proliferation and non-proliferation, and the parallel contrast between undifferentiated germinal region and differentiated secondary chondrocytes (as assayed by genetic markers), we realised that it would be possible to use BrdU incorporation to label the germinal cells and then follow their fate. The immediate labelling by BrdU of S-phase cells would therefore assess commitment to the chondrocytic pathway under the in vivo conditions of articulation. Subsequently, however, in the absence of mechanical stimulation ex vivo, the germinal region should start to give rise to osteoblasts, not chondrocytes (Hall, 1968) (see also Fig. 8). All the following experiments were carried out in the absence of mechanical stimulation, unless otherwise stated. In order to follow the fate of proliferating cells, paired QJ/Q joints were incubated in BrdU, as described above, and one was immediately fixed (time 0). The contralateral side was rinsed of BrdU, moved to a new well with a fresh filter/medium and re-incubated for 24 hours (the chase) prior to fixation.

By following the pulse/chase protocol, we found that pairs of BrdU-labelled nuclei could be detected within the chondrocyte zone (Fig. 4B,D). The daughter cells shared a lacuna within the hypertrophic-like domain of the secondary chondrocytes, where at time 0 BrdU data showed proliferation to be very low or undetectable. To confirm that self-renewing pre-chondrocytes were not present, we incubated the explanted QJ/Q joint for 16 hours prior to labelling with BrdU, and followed this by a 24-hour chase in the absence of mechanical stimulation. If there was an amplification zone of pre-chondrocytes, these would be labelled at the second division and labelled chondrocytes would be observed. We found that pulsing at 16 hours, followed by immediate fixation, labelled the germinal region (Fig. 4E), but the chase of the contralateral side failed to label chondrocytes (Fig. 4F; labelling osteoblasts instead), even though conditions were clearly permissive for

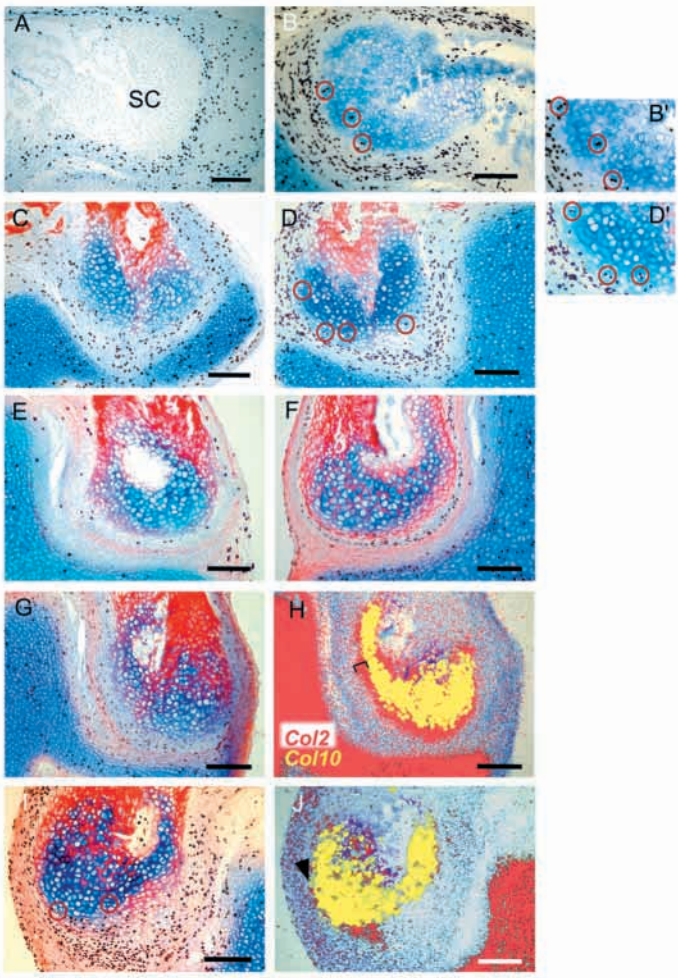

Fig. 4. BrdU labelling and the origin of secondary chondrocytes at e14. Counterstains: Toluidine Blue (A); (B-G,I) Alcian Blue/Chlorantine Fast Red. BrdU labelled cells are black. (A) Pulse and immediate fix shows cycling cells in the germinal region, and a relative absence of BrdU label within the SCs. (B) Contralateral side to A, with a 24-hour chase following pulse. Doublets found within the SC domain are highlighted with red circles. (B′) Higher magnification of image in B. (C) BrdU labelled cells at the boundary between the germinal region and the SCs. (D) Contralateral side to C shows labelled doublets after a 24-hour chase (circled). (D′) Higher magnification of image in D. (E) BrdU labelling at 16 hours after explant and immediate fixation labels cells in the germinal region. (F) Contralateral side to E after a 24-hour chase shows no labelled chondrocytes in the SC domain. (G) BrdU label at 0 hour. (H) Adjacent section to G, which shows *Col10* expression (yellow) superimposed on, and contained within, *Col2* expression (red). Bracket indicates delay in *Col10* upregulation. (I) 24-hour chase on contralateral side to G,H results in labelled doublets. (J) Adjacent section to I, showing that the *Col10* domain has expanded to coincide with *Col2* domain, black arrowhead. Scale bars: 100 μm.

chondrocyte proliferation as judged by that detected in the quadrate.

To explore the role of cell division in differentiation, we assessed the expression of *Col2* and *Col10* in explanted QJ/Q joints labelled with BrdU, on adjacent sections. As at e11 and e13 (see Fig. 2B,C; data not shown), *Col2* expression (Fig. 4H) slightly preceded that of *Col10* (Fig. 4H), although BrdU

labelling within the *Col2*-expressing area was rare (Fig. 4G). When we compared this to the situation after a 24 hour chase (Fig. 4I, BrdU labelling at 0 hours), we found that the *Col2* and *Col10* expression domains at the germinal region/SC boundary were now coincident (Fig. 4J). Also, cell doublets could now be observed within the *Col10* expression domain (Fig. 4I). This observation was consistent with a cell committing to the chondrocyte lineage, dividing once, and then upregulating *Col10* (n=3). The rapid hypertrophy indicated by this result, led us to assess whether this was an intrinsic property of SCs, or was related to the absence of *Pthrp* expression. We attempted to delay hypertrophy by adding PTH peptide to cultured QJ/Q explants. We found no significant difference between PTH-treated and untreated explants when we compared *Col2* with *Col10* expression on adjacent sections after 24 hours culture (n=4; data not shown).

Ihh signalling during secondary chondrogenesis

The proliferation rate in the germinal region was almost three times that of the periosteum in other regions, indicating that the germinal region was subject to influences unique to this location. In order to assess whether hedgehog (Hh) signalling from SCs was responsible, we analysed the expression of *Ptc2* in relation to *Ihh*. As an inducible transcriptional target, *Ptc2* is a sensitive marker for receipt of Hh signal (Pearse et al., 2001; Hartmann and Tabin, 2001). At e11, although *Ihh* was readily detectable (Fig. 2E, Fig. 5B), *Ptc2* expression was at the limit of detection (Fig. 5C). However, at e12 and e14 *Ptc2* expression was evident within the germinal layer (Fig. 5F,I), adjacent to the *Ihh* domain in the SCs (Fig. 5E,H). *Ptc2* was not expressed in the periosteum surrounding the membrane bone, and we were unable to detect *Ptc1* expression at either e11 or e14 (data not shown).

Ihh stimulates proliferation of the germinal region

We addressed the functional significance of the *Ihh* domain by using the Hh blocking antibody 5E1 to ablate Ihh function in our explant system (Ericson et al., 1996; Wu et al., 2001). We then assessed proliferation after 16 hours of incubation by BrdU labelling as before. We found that proliferation in the contralateral joint in 5E1-treated sections, as measured by the number of cells in S-phase, was dramatically reduced when compared with non-Hh antibody treated, or no antibody treated controls (Fig. 6A,D). *t*-test analyses on scores for four pairs of QJ/Qs revealed significant differences in each case (P<0.05): on average the total number of BrdU-labelled cells in the germinal region of 5E1-treated samples was reduced to a third of that in the controls. To ensure the effect was specific, we analysed adjacent sections for *Ihh* and *Ptc2* expression: *Ihh* was unaffected (Fig. 6B,E), whereas *Ptc2* was downregulated in the treated side relative to the control (Fig. 6C,F).

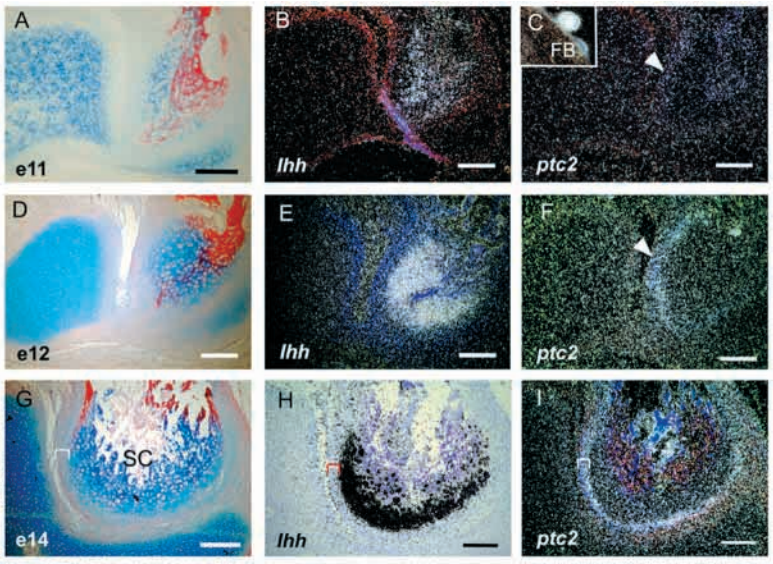

Fig. 5. Ihh signalling from secondary chondrocytes. (A,D,G,) Alcian Blue/Chlorantine Fast Red stained controls. (A-C) At e11, *Ihh* is expressed by SCs (B), but, as shown on an adjacent section (C), *ptc2* expression is at the limit of detection in the germinal region (white arrowhead); however, it is expressed in the feather buds (FB; inset in C). (D-F) At e12, *Ihh* is expressed by SCs (E), and *ptc2* (F) is expressed in the germinal region (arrowhead). (G-I) At e14, the relationship between *Ihh* (H) and *ptc2* (I) expression is maintained, brackets indicate extent of the germinal region. Scale bars: 100 μm.

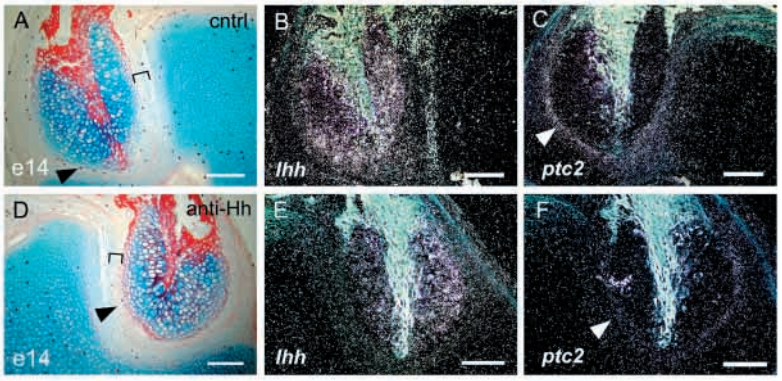

Fig. 6. Inhibition of Ihh signalling reduces proliferation in the germinal region at e14. (A) control Q/QJ cultures cultured for 16 hours and labelled with BrdU. Bracket demarcates the germinal region, black arrowhead indicates proliferating cells. (B) *Ihh* expression in an adjacent section. (C) *ptc2* is expressed in the germinal region (white arrowhead). (D-F) Hh blocking-antibody treated contralateral side. (D) The reduction in number of BrdU labelled cells is indicated by the black arrowhead. (E) An adjacent section showing unaffected *Ihh* expression. (F) An adjacent section showing downregulation of *ptc2* expression in the germinal region (arrowhead). Scale bars: 100 μm.

Our data indicated that Ihh signalling to the periosteal cells was having a proliferative effect. We assessed whether this was a general property of membrane bone periosteal cells by using high-density micromass cultures with QJ-derived periosteal cells that excluded the germinal region (Fang and Hall, 1996). Such cultures are alkaline phosphatase (AP) positive, express *osteocalcin* and *osteopontin*, do not produce Alcian Blue staining matrix (data not shown) or type II collagen (Fang and

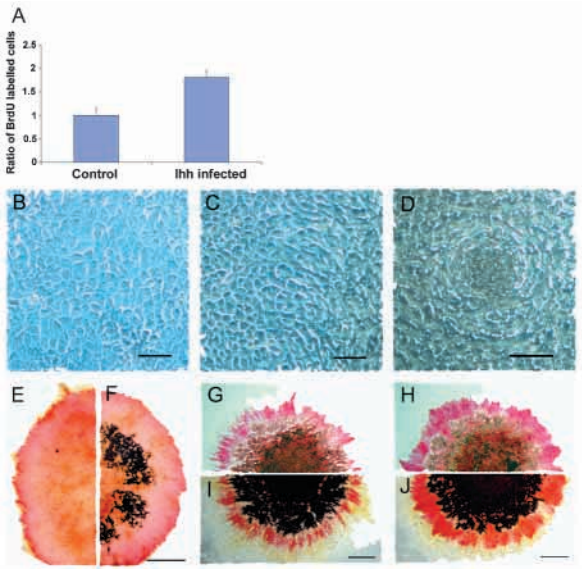

Fig. 7. The response of periosteal cells to exogenous Ihh. (A) Comparison of proliferation rates in control (*RCAS-GFP* infected) and experimental (*RCAS-Ihh* infected) micromass cultures after 4 days. Values shown are means±s.d. of BrdU labelled cells per unit area after a 30-minute pulse. (B) Phase contrast image of control micromass after 11 days in culture, showing polygonal osteoblast-like cells, but no nodules. (C) *Ihh*-infected micromass from the same series as B, again showing polygonal cells at day 11. (D) Same micromass as in C, shown as an example of the precocious bone nodules found in *Ihh* infected micromasses. (E) Day 11 control micromass displaying alkaline phosphatase (AP) activity (red). (F) *Ihh*-infected micromass additionally demonstrates mineralised bone matrix (black stain). (G,H,I,J) Cultures at day 14. (G) AP activity is evident at the periphery of the control micormass. (H) AP activity is increased in *Ihh*-infected cultures. (I,J) Sections stained additionally for von Kossa, demonstrating mineralised bone matrix at the centre of both micromasses; the matrix is increased in *Ihh*-treated cultures (J). Scale bars: B-D, 50 μm; E-J, 1 mm.

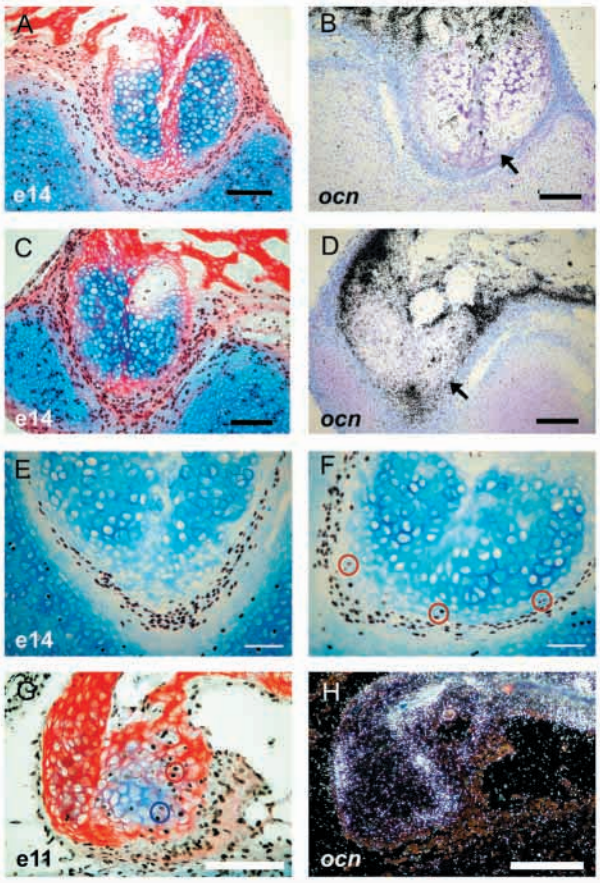

Fig. 8. Mechanical stimulation in secondary chondrogenesis. (A-F) e14, (G-H) e11. (A) BrdU labelled at 0 hours, with a 24-hour chase with mechanical stimulation; labelled doublets are observed. (B) *osteocalcin* (*ocn*) expression in an adjacent section: *ocn* is not seen around the SCs (black arrow). (C) BrdU labelled at 0 hours, with a 24-hour chase without mechanical stimulation; again labelled doublets are found. (D) Upregulation of *ocn* expression around the SCs on an adjacent section (black arrow). (E) BrdU labelled at 16 hours followed by 24-hour chase without mechanical stimulation; labelled cells are found in the germinal region. (F) As E, but with mechanical stimulation; BrdU labelled cells are found in the chondrocyte zone (circled). (G) e11 BrdU pulse at 0 hours followed by a chase for 48 hours without mechanical stimulation, both chondrocytes (blue circle) and osteoblasts (red circle) are labelled. (H) Adjacent section to G, showing *ocn* expression in BrdU-labelled cells. Scale bars: E, 100 μm; F, 50 μm.

Hall, 1996), and go on to form bone after 14 days (Fang and Hall, 1996). We assayed proliferation by BrdU incorporation 4 days after seeding and infection with *RCAS-Ihh* or *RCAS-GFP*. We found that Ihh almost doubled the proliferation rate (1.8×, s.d. ±0.15, three experiments in triplicate; Fig. 7A). In longer-term cultures (day 11), we observed polygonal cells in both control (Fig. 7B) and *RCAS-Ihh* infected cultures (Fig. 7C), but observed widespread bone nodule formation only in the latter (Fig. 7D). Both control and *RCAS-Ihh*-infected cultures were AP positive (Fig. 7E,F), but the pronounced nodules present in *Ihh*-infected cultures also reacted strongly for von Kossa stain, demonstrating precocious formation of mineralised matrix (Fig. 7E,F; n=6). After 14 days in culture, we found greatly enhanced AP activity around the periphery of the *Ihh*-infected micromasses compared with the control (Fig. 7G,H). The core of the micromass did not express AP activity in either control or *Ihh*-infected cultures (osteocytes are AP negative) (Aubin and Liu, 1996), but the AP-negative core of *Ihh*-infected culture was larger. Visualisation of mineralised bone matrix in the same samples confirmed that osteocytes had formed in the centre of each micromass (Fig. 7I,J; n=8). Thus, *Ihh*-infected cultures exhibited increased proliferation of pre-osteoblasts at 4 days, and at the later timepoints showed enhanced progression to osteocytes. Together these findings indicated an osteogenic effect of Ihh on periosteal cells.

Mechanical regulation of secondary chondrogenesis

To explore the role of mechanical cues in the genesis of SCs, we used the transcriptional regulation of *osteocalcin* as an assay; this would also provide evidence on whether the *Cbfa1*-expressing nature of the germinal cells was truly indicative of pre-osteoblastic commitment. We utilised an hourly articulation regime, based on the methods of Hall (Hall, 1968), in which the joint is manually flexed whilst in explant culture

to recapitulate the effects of jaw opening and closing as occurs in ovo. We coupled analysis of *ocn* expression with BrdU labelling in order to determine potential effects on the cell cycle simultaneously. Pulsing at 0 hours, followed by hourly articulation for 9 hours and analysis at 24 hours, revealed labelling of chondrocyte pairs (Fig. 8A) and an absence of *ocn* expression (Fig. 8B). The control joint possessed similar labelling of chondrocyte pairs (Fig. 8C) after 24 hours, but in contrast to the articulated joint, *ocn* expression was readily detected at the periphery of the SC domain (Fig. 8D; 0/4 in articulated joints; 3/4 in non-articulated joints). Thus, in the absence of mechanical stress, the cells of the germinal region rapidly give rise to definitive osteoblasts. Having established that mechanical stress resulting from the articulation of the QJ/Q joint was sufficient to repress osteoblast differentiation we wanted to assess whether it was also promoting chondrogenic differentiation. Using the same regime but with a delayed labelling strategy incorporating a chase (i.e. pulse at 24 hours, chase for 24hours), we could follow the fate of cycling cells. By doing so we found that, as before, in the absence of articulation chondrocytes were not labelled (Fig. 8E), but after the articulation regime labelled chondrocytes could be detected (Fig. 8F; n=3). This indicated that germinal region cells had been committed to the chondrocyte pathway.

The rapid shift in cell-type differentiation, dependent upon the presence or absence of mechanical stimulation, indicated that the germinal region was extremely plastic, with the fate decision being a late event in germinal region expansion. To determine whether this was the case, we labelled e11 explanted QJ/Qs at 0 hours followed by a 48 hour chase (n=3). The resultant BrdU-labelling of both chondrocytes and osteoblasts (Fig. 8G) indicated that precursor cells capable of giving rise to osteoblasts, as well as cells committed to giving rise to chondrocytes, had both been proliferating under the influence of mechanical stimulation (Fig. 8H).

Discussion

Unique ontogeny of secondary chondrocytes

Our study of the events underlying secondary chondrogenesis has revealed the details and potential function of a unique differentiation pathway: the formation of hypertrophic-like chondrocytes from osteoblast precursors. In essence, this transition reflects the transcriptional upregulation of the *Sox9* gene within a precursor population that already expresses *Cbfa1*. Subsequently, a raft of genetic markers, normally associated with the hypertrophic chondrocyte phenotype, become rapidly expressed within these cells: *Ihh*, *Col10*, *Wnt11* (data not shown) and, later, *osteopontin*. Some stratification within the secondary chondrocytes is apparent; *Col2* expression precedes *Ihh* and *Col10* expression, and *osteopontin* expression reflects the last phase of hypertrophy. Thus the hypertrophic character that Murray observed (Murray, 1963) and used to distinguish secondary chondrocytes from their primary counterparts is found to be bona fide, in common with mammalian secondary chondrocytes (Shibata et al., 1997). In itself, the evidence that *Sox9* expression is upregulated is unsurprising given its pivotal role in chondrocyte specification and gene regulation (reviewed by de Crombrugghe et al., 2000; Bi et al., 1999); what is unusual is the succession of gene upregulation.

It is well known that immature chondrocytes of the growth plate gradually acquire the range of markers outlined above, but start from a population of precursors expressing *Sox9*. Misexpression of *Cbfa1* in immature, *Sox9*-expressing chondrocytes has shown that it is sufficient to promote hypertrophy and exit from the cell cycle (Enomoto et al., 2000; Takeda et al., 2001). What our study of SCs shows is that the reverse is also true: mechanical evocation of *Sox9* in ovo, in a population expressing *Cbfa1*, leads to the same array of gene targets becoming upregulated. Consequently, we have shown that the key motivator of chondrogenesis on the one hand (*Sox9*) and osteogenesis on the other (*Cbfa1*) appear to affect the same outcome (pre-hypertrophic chondrocytes) when co-expressed, irrespective of their primacy. Consequently, a bifurcating pathway of endochondral ossification and secondary chondrogenesis can be described in which early lineage determination from a mesenchymal precursor, arguably governed by Sox9 and Cbfa1 activity, much later rejoins with the differentiation of the hypertrophic chondrocyte (Fig. 9).

This reversibility of succession is evidence for a simple mechanism underlying hypertrophy and the control of chondrocyte maturation. It is also noteworthy that although the endpoint for pre-osteoblasts and chondrocytes can be the same, the genes expressed by (pre-) hypertrophic chondrocytes are not merely the sum of Sox9 targets (de Crombrugghe et al., 2000) plus Cbfa1 targets (Ducy, 2000). We characterise the germinal cells as pre-osteoblastic because they express *Cbfa1*, and, if not diverted into chondrogenesis, they will upregulate *osteocalcin* and produce matrix that binds Chlorantine Fast Red when they differentiate. Furthermore, prior to the

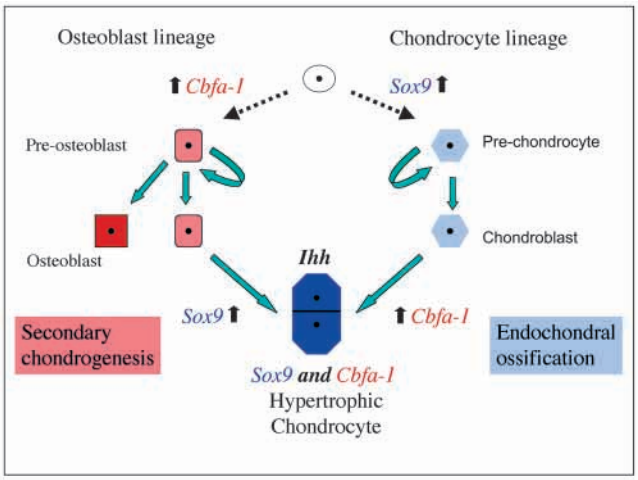

Fig. 9. Two routes to chondrocyte hypertrophy. A common skeletogenic precursor gives rise to both chondrocytes and osteoblasts, a division arguably dictated by *Sox9* and *Cbfa1/Runx2*. Chondrocytes are self-renewing, but differentiate and exit the cell cycle to form pre-hypertrophic chondrocytes, an event that can be mediated by the upregulation of *Cbfa1*/*Runx2*. Pre-osteoblasts are also self-renewing, and exit the cell cycle to form osteoblasts; however, when subjected to compression, they undergo chondrogenesis correlated with the upregulation of *Sox9*, which results in symmetric division followed by exit from the cell cycle and the acquisition of a pre-hypertrophic phenotype. *Ihh* from both sources of pre-hypertrophic chondrocytes signals to the periosteum: in long bones to induce osteogenesis and in SCs to stimulate proliferation and, in vitro, to promote osteogenesis.

evocation of secondary chondrocytes, the periosteum at the tip of the QJ gives rise to osteoblasts and no changes in the expression of peripheral markers occurred coincident with the genesis of secondary chondrocytes. However, we must note that SCs do derive from these *Cbfa1*-expressing cells, and this can be interpreted as evidence that Cbfa1 does not dictate commitment to the osteoblast lineage, but rather confers bi-potentiality.

Exit from the cell cycle follows commitment to the chondrogenic lineage

The transition from perichondrium to periosteum during endochondral ossification follows exit from the cell cycle and pre-hypertrophy in the underlying chondrocytes. This process of chondrocyte maturation is meticulously regulated and deficiency of any of a number of genes that affect proliferation or cell cycle control causes its perturbation, for example, *Pthrp* (Karaplis et al., 1994), *Pthrp-r* (Lanske et al., 1996), *Ihh* (St-Jacques et al., 1999), *p57* (Zhang et al., 1997) and *Cyclin D1* (Beier et al., 2001). When we consider secondary chondrogenesis, the periosteum (germinal region) is the source of the chondrocytes as well as the responding tissue. However, the chondrocytes formed rapidly exit the cell cycle and undergo hypertrophy. Thus, commitment to the chondrocyte lineage appears not to be accompanied by the formation of a self-renewing proliferative layer; this is in contrast to the growth plate of the long bone, where chondrocyte precursors are maintained independent of movement of the joint. This conclusion is also supported by in ovo experiments that show that, following paralysis of the embryo, proliferating secondary chondrocyte-committed cells are not found (Hall, 1979).

This achieves one end – rapid hypertrophy – but necessitates continued mechanical articulation of the joint to re-commit precursors to the chondrocyte lineage (Murray and Smiles, 1965; Hall, 1967; Hall, 1968; Hall, 1979) (this paper). Our finding that *Col10* expression expanded to coincide with the *Col2* domain after 24 hours in culture indicated that commitment, differentiation and exit from the cell cycle are intimately correlated in SCs. Thus the initial spur to chondrogenesis – compression and upregulation of *Sox9* (Takahashi et al., 1998) – appears to be followed by a single round of division and hypertrophy.

Ihh drives proliferation of the germinal region

The mouse chondrodysplasias mentioned above result in either delayed or accelerated hypertrophy. These changes then feed into ossification via *Ihh* signalling, resulting in reduced or increased bone formation, respectively. As in endochondral ossification, accelerated exit from the cell cycle in SCs is accompanied by *Ihh* upregulation, which then signals to the germinal region but not to the chondrocytes. As a result, cycling cells in the germinal region express *Ptc2*, but, as they become chondrocytes, the cells exit the cell cycle, downregulate *Ptc2* and upregulate *Ihh*. Hence, the cells progressed from Hh-signal receiving to Hh-signal producing at the germinal region/SC boundary, superficially resembling an extreme form of the growth plate of the *Pthrp-r* knockout mouse (Lanske et al., 1996).

The threefold enhanced proliferation evident in the germinal region around the SCs was quenched by blocking Ihh function with the 5E1 antibody (Ericson et al., 1996; Wu et al., 2001). This finding was consistent with the demonstrated role of Ihh signalling to immature chondrocytes to drive proliferation (Long et al., 2001), but was transposed to the germinal region. Given the pivotal roles of Ihh in chondrogenesis and osteogenesis of the long bones, it might also be expected to affect differentiation of the germinal cells. However, we did not find precocious or delayed expression of the osteogenic marker *osteocalcin* in 5E1 treated samples (data not shown). It was also apparent from the *osteocalcin* expression data at 24 and 48 hours that Ihh alone, in the absence of mechanical stimulation, was not sufficient to cause chondrogenic differentiation in germinal region descendants. This is consistent with the fact that *Ihh* is expressed only after SCs have formed, excluding it from any role in the initiation of SCs themselves. Thus the principal, and perhaps sole, function of Ihh signalling in secondary chondrogenesis is to drive proliferation of the germinal region and thereby increase indirectly recruitment to the chondrogenic fate. In turn, exit from the cell cycle appears to be a prerequisite for the synthesis of Ihh.

The mitogenic effect of Ihh in micromass culture was not directly comparable with the role of Ihh in SC genesis owing to the different fates of the responding cells. However, it was consistent with the conclusion above. Our in vitro micromass data additionally demonstrate that Ihh promotes osteogenesis, as measured by nodule formation and deposition of mineralised matrix. This conclusion is comparable to that from the phenotype of the $Ihh^{-/-}$ mice, that *Ihh* is necessary for osteogenesis in cartilage-replacement bones (St-Jacques et al., 1999). Our findings are significant not only for understanding secondary cartilage but also because they extend the similarities between periostea of membrane bones and long bones. This implies that it is ontogenetic differences that separate these periostea, not genetic ones (St-Jacques et al., 1999).

Mechano-transduction regulates gene expression and cell fate in the germinal region

One of the most striking features of the SCs described is that their formation is dependent upon articulation of the QJ/Q joint. In ovo this is achieved by muscular contraction that opens and closes the beak, which can be mimicked in explant by the manual operation of the joint (Hall, 1968) (this paper). Whereas evocation of chondrogenesis has certainly been described, we found that the discreet nature of the germinal region as proliferative centre allowed us to explore the short-term response to mechanical articulation. The ability of mechanical articulation to prevent the default osteogenic pathway, as determined by *ocn* upregulation, indicates a direct role for mechanical stimulation in the regulation of gene expression. *Ocn* is upregulated by osteoblasts as they exit the cell cycle (Aubin and Liu, 1996), and in the absence of mechanical stimulation the germinal cells follow this route. Thus, the cells of the germinal region that give rise to secondary chondrocytes would normally exit the cell cycle at this juncture, but the acquisition of chondrogenic fate diverts them – a limited form of bi-potentiality (Fang and Hall, 1996). The fact that no self-renewing chondrocyte-committed precursors appear to be established, as evidenced by gene expression and BrdU labelling (see also Hall, 1979), accentuates this as a defining feature of secondary chondrogenesis in the chick.

Conclusion

The unusual differentiation undergone by SCs, and their transient nature, alludes to a role for SCs that is not structural, but is rather as a growth and signalling centre; our study demonstrates that a molecular basis for this function is Ihh production. The clear inference is that the pre-hypertrophic chondrocyte is of such utility as a source of extracellular signals for bone morphogenesis that, even when no cartilage scaffold exists, pre-hypertrophic chondrocytes are evoked. The finding that Ihh stimulates proliferation of the germinal region highlights the reciprocity between epigenetic and genetic pathways that characterise the development of the cranial tissues, and that underpin their evolution. Such a scenario resonates with other phenomena, such as fracture repair, where a short-lived, *Ihh*-synthesising soft callus forms from the periosteum under the influence of movement (Vortkamp et al., 1998; Ferguson et al., 1999). Conversely, the common characteristics that we have found between endochondral and secondary chondrogenesis indicate that the subordinate role of bone to cartilage [and other epigenetic influences (Herring, 1993)] is a skeleton-wide phenomenon. Moreover, although requiring confirmation in mammals, our findings provide additional mechanistic evidence in support of Scott's view of cartilage as a pacemaker of cranial bone growth (Scott, 1954).

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