RESEARCH ARTICLE

Precise spatial restriction of BMP signaling is essential for articular cartilage differentiation

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ABSTRACT

The articular cartilage, which lines the joints of the limb skeleton, is distinct from the adjoining transient cartilage, and yet, it differentiates as a unique population within a contiguous cartilage element. Current literature suggests that articular cartilage and transient cartilage originate from different cell populations. Using a combination of lineage tracing and pulse-chase of actively proliferating chondrocytes, we here demonstrate that, similar to transient cartilage, embryonic articular cartilage cells also originate from the proliferating chondrocytes situated near the distal ends of skeletal anlagen. We show that nascent cartilage cells are capable of differentiating as articular or transient cartilage, depending on exposure to Wnt or BMP signaling, respectively. The spatial organization of the articular cartilage results from a band of noggin-expressing cells, which insulates these proliferating chondrocytes from BMP signaling and allows them to differentiate as articular cartilage under the influence of Wnt signaling emanating from the interzone. Through experiments conducted in both chick and mouse embryos we have developed a model explaining simultaneous growth and differentiation of transient and articular cartilage in juxtaposed domains.

KEY WORDS: Articular cartilage, BMP, Nog, Wnt, Proliferating chondrocytes

INTRODUCTION

Long bones of vertebrate limbs are formed by endochondral ossification. In this process, a cartilage scaffold pre-figures the developing skeleton (Akiyama et al., 2005). Chondrocytes at the center of this cartilage undergo hypertrophic differentiation and are referred to as transient cartilage. Eventually, bone forms within the domain of hypertrophic differentiation. However, a small population of cartilage cells on either side, at the site of segmentation, resists this change. This remnant cartilage, which lines the ends of skeletal elements, is referred to as articular cartilage or permanent cartilage (Karsenty and Wagner, 2002; Archer et al., 2003; Kronenberg, 2003; Pacifici et al., 2005).

Current literature suggests that a Sox9-expressing, contiguous cartilage condensation forms most of the skeletal elements of the developing limb by branching and segmentation (Shubin and Alberch, 1986; Bi et al., 1999). The first sign of segmentation becomes apparent with the formation of the interzone, characterized by a region of densely packed flattened cells. The cells of the interzone turn off the expression of Col2a1, a marker of cartilage cells, while turning on expression of growth differentiation factor 5 (Gdf5), Wnt9a and autotaxin (Atx; Enpp2 – Mouse Genome Informatics Database) (Hartmann and Tabin, 2001; Karsenty and Wagner, 2002; Pacifici et al., 2005).

It is currently not well understood how two spatially and molecularly distinct cell populations are formed within a uniform cell population at the time of interzone induction. However, the interzone and the Wnt/BMP signaling pathways play important roles in this process. BMP signaling has been particularly well studied in the context of joint development. It has been reported that any molecular manipulation leading to ectopic activation of BMP signaling through (1) overexpression of BMP ligands or Gdf5 in developing chick or mouse cartilage (Duprez et al., 1996; Tsumaki et al., 1999); (2) misexpression of constitutively active BMP receptor in developing chick cartilage (Zou et al., 1997); or (3) knockout of Nog, a BMP signaling inhibitor (Brunet et al., 1998), results in transient cartilage differentiation throughout the cartilage anlagen, including in the cells of the presumptive interzone.

Nevertheless, Wnt ligands secreted from the interzone are crucially required for embryonic articular cartilage development (Hartmann and Tabin, 2001; Guo et al., 2004; Später et al., 2006a, b). Gain-of-function experiments with BMP and Wnt signaling indicate that the cells of the nascent cartilage anlagen are competent to differentiate into transient or articular cartilage in response to one signal or the other. Therefore, the central issue that remains to be solved in the context of cartilage development is how two distinct populations, namely transient and articular cartilage cells, are formed adjacent to each other from one nascent population of cartilage cells upon interzone induction.

Proliferating chondrocytes near the ends of developing skeletal anlagen contribute to longitudinal growth of transient cartilage (Colnot, 2005); here, we show that these cells also contribute to the appositional growth of articular cartilage. Further, we demonstrate that a band of noggin-expressing cells insulates a region of these proliferative cells from the influence of BMP signaling, and allows them to differentiate as articular cartilage upon exposure to Wnt signaling emanating from the interzone. Thus, we propose a model that explains the concomitant longitudinal growth of transient cartilage and the appositional growth of articular cartilage.

RESULTS

Increase in cell number in the interzone and articular cartilage during early embryonic development

Growth of chicken articular cartilage during early embryonic development has not been systematically characterized before. We first investigated whether the growth in volume is associated with a
concomitant increase in cell number in this tissue. Throughout this article we use the term ‘articular cartilage’ only when cavitation of the synovial joint is histologically obvious. For developmental stages prior to cavitation we use the term ‘interzone’. We measured the increase in cell number between successive stages in the interzone/articular cartilage, as well as transient cartilage of the metatarsophalangeal (MTP) joint of hindlimb digit 3, starting from Hamburger–Hamilton (HH) stage 28, when the interzone first becomes apparent, up to HH38, the stage by which cavitation has already taken place at this joint. We observed a nearly 12-fold increase in cell number in the developing articular cartilage during this period (Table 1; supplementary material Fig. S1A,E). It should also be noted that there was a steady increase in the number of cells per unit area in the interzone/articular cartilage region (supplementary material Fig. S1D).

Our data (Table 1) suggest a significant increase in the number of cells in the interzone/articular cartilage during early chick development. However, the developing interzone has been reported to be mitotically deficient (Mankin, 1962). Therefore, we investigated whether this increase in cell number of articular cartilage cells results from proliferation of interzone cells.

Interzone is proliferation deficient

We used three independent criteria to identify proliferating cells in developing chicken embryonic cartilage: (1) expression of PCNA mRNA (a marker for S-phase cells) (Köhler et al., 2005); (2) detection of phosphorylated histone 3 (pH3) immunoreactivity; and (3) incorporation of 5-ethynyl-2'-deoxyuridine (EdU), a thymidine analogue. Using any of these methods, we could rarely detect proliferating cells in the interzone of developing interphalangeal joints and MTP joints in any of the stages examined (Fig. 1A-I; supplementary material Fig. S2C). This suggests that the interzone cells proliferate at a very slow rate, if at all, which cannot explain the observed rate of increase in cell number. However, we observed many proliferating cells on either side of the interzone (Fig. 1A-I; supplementary material Fig. S2C). This zone of proliferating cells is situated at the distal ends of the developing skeletal anlagen and will be referred to as ‘distal proliferative zone’ or DPZ throughout the manuscript. Likewise, in mouse embryos the interzone cells also did not incorporate EdU, whereas the DPZ cells did (Fig. 1J-L). Further molecular characterization of the DPZ is described below. In contrast to our observations, two previous reports (Kahn et al., 2009; Gunnell et al., 2010) showed BrdU-labeled cells in the interzone of mouse forelimb humeroulnar or humeroradial joints at 14.5 days post coitum (dpc). This discrepancy might be due to the difference in joints analyzed.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Fold increase in volume of interzone/articular cartilage</th>
<th>Fold increase in cell number in interzone/articular cartilage</th>
<th>Fold increase in cell number in cartilage between successive stages</th>
<th>Compared with HH28</th>
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<td>HH38</td>
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Proliferative chondrocytes of the DPZ contribute to the growth of developing interzone and articular cartilage

As the cells of the interzone are non-proliferative, we hypothesized that cells from outside the interzone must be contributing to the growth of this tissue.

A previous study found that articular cartilage cells are descendants of Gdf5-expressing cells (Koyama et al., 2008). To investigate whether there are Gdf5 mRNA-expressing cells in the interzone that are proliferative, we co-labeled BrdU+ proliferating and Gdf5 mRNA-expressing cells in developing digits at HH31 in chick and 14.5 dpc in mice (Fig. 2). We observed that, in a distal phalangeal joint, some of the Gdf5-expressing cells flanking the interzone are also BrdU immunoreactive (Fig. 2B,E). However, within proximal joints of the same digit, cells expressing Gdf5 are confined within the interzone and are not BrdU immunoreactive (Fig. 2C,F). These observations suggest that flanking cells of the newly formed interzone are proliferative and lie within the domain of Gdf5 expression, whereas, in more mature interzone/articular cartilage, Gdf5-expressing cells are primarily non-proliferative. Therefore, the further interstitial growth of matured interzone cannot be accounted for by proliferation of cells within the interzone.

To investigate whether flanking cells are incorporated into the interzone, we conducted a pulse-chase DNA-labeling experiment (Fu et al., 2012). Such pulse-chase experiments are not possible with developing chick embryos because the label is neither diluted (as is possible in cell culture experiments) nor can it be removed by the circulatory system (Hämmerle and Tejedor, 2002). Therefore, we used developing mouse embryos and pulse-labeled with EdU and/or BrdU. Interzones of mouse 13.5 and 15.5 dpc harvested 2 h post EdU injection were largely devoid of EdU+ cells, whereas proliferating cells were detected within the regions immediately flanking the interzone (Fig. 3B,D). By contrast, embryos harvested at 14.5 and 16.5 dpc, 24 h post EdU injection, showed many EdU+ cells in the interzone and very few EdU+ cells in the flanking regions (Fig. 3C,E). Col2a1 and Gdf5 mRNA expression domains on sections adjacent to the ones used in Fig. 3D,E are presented in supplementary material Fig. S3A-D. Intermediate chases conducted for up to 5 h post BrdU injection failed to detect any BrdU+ cells in the interzone (supplementary material Fig. S3G-J).

Finally, to rule out the possibility that the EdU-labeled cells which appear in the interzone after a 24-h chase might be a result of slow proliferation, we conducted a competition experiment. We established that, if a fivefold molar excess of unlabeled dTTP is injected along with EdU, no EdU+ cells could be detected in the hindlimb of these animals, demonstrating that the unlabeled dTTP can successfully outcompete EdU for incorporation (Fig. 3G). On the other hand, when unlabeled dTTP is injected 2 h post EdU injection, many EdU+ cells could still be detected in the interzone 24 h post EdU injection (Fig. 3H), thus demonstrating that cells which incorporated EdU within 2 h of its injection, and before dTTP administration, are eventually incorporated in the interzone.

Furthermore, we injected pregnant females with EdU at 15.5 dpc, followed by BrdU injection after 22 h, and harvested 2 h post BrdU injection, i.e. 24 h post EdU injection (Fig. 3I-M). Most of the interzone cells were labeled with EdU (Fig. 3J,L). On the other hand, many chondrocytes in the DPZ, flanking the interzone, were labeled with BrdU (Fig. 3J,K). This domain is marked by Col2a1 mRNA expression (supplementary material Fig. S3F,F’). Taken together, these data suggest that proliferative
chondrocytes in the DPZ are labeled with EdU at 15.5 dpc but become incorporated in the interzone 24 h later. However, cells in the DPZ remain proliferative at 16.5 dpc (as they are also labeled with BrdU), whereas interzone cells remain non-proliferative at both 15.5 and 16.5 dpc.

Our data suggest that the cells that contribute to the growth of the interzone during embryonic development are the proliferative DPZ cells located within the Col2a1 mRNA expression domain (supplementary material Fig. S3F'). However, pulse-chase labeling with thymidine analogs cannot completely rule out the possibility that the labeled cells that are incorporated in the interzone are not coming from some other nearby pool of proliferative cells. To test whether the proliferative cells that are incorporated into the interzone have a history of Col2a1 expression, we traced the lineage of Col2a1-expressing cells in the developing cartilage (Fig. 3N). Col2a1 mRNA-expressing cells were marked at 15.5 dpc in Col2a1-CreERT2;Rosa26mT/mG embryos (Nakamura et al., 2006); at this stage, there is no expression of Col2a1 mRNA in the interzone (Fig. 3O). Nevertheless, marked cells were found in the newly cavitated articular cartilage at 18.5 dpc (Fig. 3P). Some of these GFP-expressing cells also have incorporated EdU (Fig. 3Q,R), demonstrating that at least some of the cells that contribute to the growth of interzone/embryonic articular cartilage come from the Col2a1-expressing, proliferating cells of the DPZ.

Fig. 1. Chick and mouse interzone cells are proliferation deficient. (A-D) mRNA in situ hybridization (ISH) of PCNA in chick hindlimb digit from HH31 (A) and HH36 (C). (B,D) Higher magnification views of the boxed regions in A and C, respectively. (E-I) EdU incorporation in chick digits after 2 h of EdU injection. (E) Digit 3 of HH36 chick hindlimb. (F-I) MTP joints of digit 3 at HH28 (F), HH31 (G), HH34 (H) and HH38 (I). (J) EdU incorporation in 16.5-dpc mouse hindlimb digits. (KL) Higher magnification views of the boxed regions in J. Dashed lines represent the margins of the skeletal elements in the developing digits. Red asterisks denote interzone, green arrowheads indicate distal proliferative zone (DPZ) and yellow arrowheads highlight hypertrophic region. MTP, metatarsophalangeal joint. Scale bars: 100 μm.

To test the possibility that the proliferative DPZ cells contribute to postnatal articular cartilage after incorporation into the interzone, we conducted a long-term chase of EdU- or BrdU-labeled cells. We monitored the presence of EdU+ (P0, P3 and P7) or BrdU+ (P21 or P42) cells in the articular cartilage of the MTP joint in postnatal stages of mouse development after three consecutive pulses of EdU or BrdU on 13.5, 14.5 and 15.5 dpc. We observed a number of EdU+ or BrdU+ cells in the articular cartilage of P0, P3, P7, P21 and P42 MTP joints of mouse hindlimbs (Fig. 3S-W; supplementary material Fig. S3P,Q). It should be noted that the number of BrdU+ cells detected in the articular cartilage is lower in P21 or P42 compared with the EdU+ cells at earlier time points. Whereas most of the BrdU-labeled cells detected in P21 or P42 articular cartilage are located in the deeper layers, some of the EdU-labeled cells detected at P0, P3 and P7 are also located in the superficial layers of articular cartilage and have a flattened morphology (Fig. 3T,U; supplementary material Fig. S3Q).

Based on our observations (Figs 1-3), we define DPZ as the zone of proliferative cells, which is mostly confined to the distal zone of the Col2a1 mRNA expression domain (supplementary material Fig. S3F'). Our observations suggest that cells of the DPZ contribute to the growth of both transient as well as articular cartilage.
Dynamic changes in gene expression during cartilage differentiation

In order to understand how both transient and articular cartilage can differentiate from the same cell population within the DPZ, we examined expression of markers for both transient cartilage (Col2a1) and interzone/articular cartilage (Atx and Gdf5) (Hartmann and Tabin, 2001; Ikeda et al., 2004; Hyde et al., 2008) during the course of early cartilage development.

Col2a1 and Atx mRNAs are co-expressed in a broad domain, spanning the interzone and the adjoining proliferative chondrocytes in the MTP joints of the chick hindlimb at early stages (HH28 and HH31) (Fig. 4A,B). There is a graded expression of Atx extending from the interzone towards the center of the element, and an opposite graded expression of Col2a1, extending from the center towards the interzone. At later stages (HH34 and HH38), the interzone cells continue to express high levels of Atx but turn off Col2a1, whereas the opposite happens outside the interzone. Eventually, a few layers of cells emerge between these two domains, in which both Col2a1 and Atx expression are downregulated (Fig. 4A-D). Thus, it seems that there is an overlap of Atx and Col2a1 mRNA expression domains in the nascent cartilage cells, but this is resolved over time to two separate domains in more mature proximal joints.

It is possible that early chondrocytes express both Col2a1 and Atx and, as development progresses, the cells of the transient cartilage maintain expression of Col2a1, whereas the cells of the interzone maintain expression of Atx or Gdf5. To examine such a possibility, we took advantage of the temporal gradient of differentiation from the proximal to the distal tip of the digit, with proximal being at a more advanced stage of differentiation than the distal phalanx. We carried out fluorescent double mRNA in situ hybridization for Atx and Col2a1 on a HH36 chick hindlimb digit 3 (Fig. 4E-I). Many cells in the two distal phalanges co-express Atx and Col2a1 (Fig. 4E-G), whereas, in the proximal phalanges, cells expressing Atx are distinct from those expressing Col2a1 (Fig. 4E,H,I). Furthermore, the cells flanking the proximal interzone have downregulated both Atx and Col2a1 expression (compare the regions marked by white asterisks in Fig. 4F and G with those in Fig. 4H and I, respectively).

We have also examined the relative expression patterns of Col2a1 and Gdf5 in the digit 3 of chick hind limb. Our results with Gdf5 are similar to those obtained with Atx, i.e. nascent cartilage cells express Col2a1 and Gdf5 in an overlapping manner, whereas, in more developed cartilage, transient cartilage cells express Col2a1 mRNA and interzone/articular cartilage cells express Gdf5 mRNA (Fig. 4J-L; supplementary material Fig. S4A). We observed a similar dynamic resolution of gene expression patterns in mice as in chick (Fig. 4M-O).

Dynamic expression of noggin and pSMAD1/5/8

To understand the molecular basis of such dynamic changes in gene expression, we surveyed mRNA expression of many signaling molecules that are expressed in the articular cartilage between HH28 and HH38 (supplementary material Table S1). We found the mRNA expression of Nog, a secreted inhibitor of BMP signaling, to be particularly informative. In agreement with earlier reports (Brunet et al., 1998; Francis-West et al., 1999), we found that, at earlier stages, Nog mRNA is expressed in a broad domain throughout the cartilage, other than in the interzone (HH28, supplementary material Fig. S5A; and HH31, Fig. 5A,B). In more developed cartilage, Nog mRNA is expressed in a tight band of cells with a distinguishable boundary (Fig. 5C-G; supplementary material Fig. S5B) towards the interzone of an MTP joint. This results in a few layers of cells on either side of the interzone that are devoid of Nog mRNA expression (Fig. 5C-G; supplementary material Fig. S5B). The distinct domain of Nog mRNA expression is clearly visible around a more mature joint, e.g. the tibiotarsal joint at HH36 (Fig. 5E).

These observations prompted us to investigate the spatial domains of active BMP signaling in the time window when Nog mRNA expression undergoes these dynamic changes. Thus, we assayed BMP signaling using pSMAD1/5/8 indirect immunofluorescence. In early stages (HH28), we find that pSMAD1/5/8 activity is primarily restricted to the perichondrium and absent from the interzone (supplementary material Fig. S5C,D). Later (HH31, HH34, HH36), pSMAD1/5/8 activity is present in a precisely restricted manner in a subpopulation of cells in the epiphysis but is undetectable in the interzone or the cells flanking it (Fig. 5H-J; supplementary material Fig. S5E-G). To directly compare noggin protein expression and the domain of active BMP signaling we performed double immunohistochemistry in the MTP joint of HH34 (supplementary material Fig. S5H-K). We observed that, in some cells of the
Fig. 3. Proliferating cells from the DPZ contribute to the growth of embryonic articular cartilage. (A-E) EdU pulse-chase experiments were carried out in pregnant mice 13.5 and 15.5 dpc. (A) Schematic of the experimental design. (B,D) Mouse hindlimbs 2 h post EdU injection. (C,E) Mouse hindlimbs 24 h post EdU injection. Red asterisks show EdU-incorporated cells of DPZ and white asterisks indicate DPZ cells devoid of EdU. White arrowheads denote interzone cells devoid of EdU and red arrowheads highlight EdU-incorporated cells in the interzone. For D,E, corresponding Col2a1 and Gdf5 expression domains are presented in supplementary material Fig. S3A-D. (F) Schematic showing the experimental design for EdU-dTTP competition in mouse embryos. (G) Simultaneous injection of EdU and excess dTTP abolished any selective uptake of EdU. (H) Many EdU+ cells are detected in the interzone when excess dTTP is administered 2 h post EdU injection. White arrowhead indicates interzone. MT, metatarsus; PH, phalangeal element. (I) Schematic of EdU/BrdU double pulse-chase experiment. (J-M) J is merged image of K-M. Many cells in the interzone of MTP incorporated EdU (J,L, white arrowheads), but did not incorporate BrdU. However, cells flanking the interzone displayed BrdU immunoreactivity (J,K, asterisks). (M) DAPI-stained section. For J-M, corresponding Col2a1 and Gdf5 expression domains are presented in supplementary material Fig. S3E,F. (N-R) Genetic lineage tracing using Col2a1-CreERT; RosamT/mG. (N) Schematic for the experimental design. (O) At 15.5 dpc, Col2a1 is not expressed at the interzone of the MTP joint (black asterisk). (P) Adjacent sections from 15.5 dpc Col2a1-CreER<sup>2</sup>;RosamT/mG MTP joint. (Q) Col2a1 mRNA ISH (pseudocolored in red) and anti-GFP antibody (green). (R) EdU (red) and anti-GFP antibody (green). Individual frames are provided in supplementary material Fig. S3L-O. (R) Higher magnification view of the region marked in Q shows multiple EdU<sup>+</sup> and GFP<sup>+</sup> double-positive cells (arrowheads). (S) Schematic showing long term pulse-chase of thymidine analogs EdU or BrdU in mice. (T-W) EdU and BrdU incorporation (arrowheads) were detected in the MTP joint articular cartilage at P0, P7, P21 and P42. The corresponding data for P3, P21 (higher magnification view) and P42 (higher magnification view) are presented in supplementary material Fig. S3P-S. Dashed lines represent the margins of the skeletal elements in the developing digits.
epiphysis, both Nog protein and pSMAD1/5/8 are detectable, whereas cells flanking the interzone on either side express only Nog (supplementary material Fig. S5H-K).

It is important to note that the region which shows downregulation of BMP activity, flanking the interzone, is the same region that upregulates Wnt signaling, as can be seen from β-catenin immunostaining (Fig. 5K).

**Restricted domains of BMP and Wnt signaling are crucial for proper transient and articular cartilage differentiation**

Our expression analysis reveals that the cells of the DPZ are flanked by separate BMP and Wnt signaling domains (Fig. 6A). This led us to predict that ectopic activation of BMP or Wnt signaling in the DPZ would result in ectopic transient or articular cartilage differentiation of these cells, respectively.

To activate ectopic BMP signaling, HH18 hindlimbs were infected with viral particles expressing BMP4 (RCAS-BMP4) and constitutively active BMPRIIB (RCAS-ca-BMPRIIB).

We observed that, upon overexpression of the BMP ligand using RCAS-BMP4 (Duprez et al., 1996), Col2a1 mRNA is expressed in a broad domain encompassing the domain of presumptive interzone (Fig. 6B). We further observed that the cells that ectopically expressed Col2a1 mRNA retained neither β-catenin immunoreactivity (compare Fig. 6B and D, white asterisks) nor endogenous Gdf5 mRNA expression (Fig. 6C, white asterisk). By contrast, the interzone cells that lacked ectopic Col2a1 mRNA expression retained β-catenin immunoreactivity (Fig. 6B,D) as well as expression of Gdf5 mRNA (Fig. 6C). Thus, our data suggest that active BMP signaling suppresses Wnt signaling in developing cartilage cells.

As reported earlier (Zou et al., 1997), we observed loss of segmentation of skeletal elements upon ectopic expression of caBMPRIIB. Furthermore, virally infected patches (as determined by 3C2 immunoreactivity, Fig. 6E) of HH36 digit cartilage ectopically turned on Col2a1 mRNA expression in the cells of both the presumptive interzone and the adjoining region (Fig. 6F). By contrast, the cells of the interzone that were not infected did not express Col2a1, and instead continued to express Gdf5 (supplementary material Fig. S5L). It is also apparent that the infected cells of the interzone not only ectopically turned on Col2a1 mRNA expression but also have altered morphology to resemble the rounded cells of the epiphysis, as opposed to the flattened cells of the interzone (compare Fig. 6H and I).

In the viral misexpression experiments, it is possible that the limb mesenchymal cells experienced ectopic activation of BMP signaling even before the formation of interzone or establishment of the cell populations flanking the interzone. We therefore used an organ culture system (see Materials and Methods) to test the effect of later administration of BMP or Wnt, after the interzone has been established. Following administration of recombinant BMP4 protein, ectopic pSMAD1/5/8 immunoreactivity (Fig. 6J) and ectopic Col2a1 mRNA expression (Fig. 6L) were detected in the cells of both the interzone and the adjoining region, in contrast to the sham-injected joint (Fig. 6K, M). Similarly, as opposed to a sham-injected
control, ectopic application of exogenous mWnt3a induced Wnt signaling in a broader population of cells around the interzone, as demonstrated by β-catenin immunostaining (compare Fig. 6N and O). Notably, Atx mRNA expression could also be detected in a broader domain in the Wnt protein-injected interzone compared with a sham-injected control interzone (compare Fig. 6P and Q). It is important to note that, upon ectopic BMP activation at the joint site, the interzone cells start to express markers of transient cartilage but do not lose their characteristic flattened shape. Taken together, our data demonstrate that ectopic exposure to Wnt induces articular cartilage markers, and that BMP signaling induces transient cartilage markers in the chondrocytes of the developing cartilage anlagen.

Reduction of Nog activity leads to ectopic transient cartilage differentiation

Earlier, it has been demonstrated that inactivation of Nog in mice resulted in ectopic transient cartilage differentiation in the cells of the putative interzone (Brunet et al., 1998). We examined whether the band of noggin-expressing cells, uncovered in this study, protects the DPZ cells from BMP signaling. For this purpose, we reduced noggin activity by injecting anti-noggin neutralizing antibody at multiple joint sites of a HH34 chick hindlimb digit (Fig. 7B, D). The cells of the interzone of a 15.5 dpc interphalangeal joint and those flanking it on either side in a control (Col2a1Cre;Nogfl/fl) mouse hindlimb digit are devoid of pSMAD1/5/8 activity (Fig. 7I). On the other hand, the cells in the analogous region in a mutant (Col2a1-CRE;Nogfl/fl) are pSMAD1/5/8 immunoreactive (Fig. 7L), indicating that these cells are exposed to active BMP signaling due to the removal of Nog from the zone of transient cartilage. This ectopic activation of BMP signaling leads to ectopic expression of Col2a1 mRNA (compare panels Fig. 7H and K) and downregulation of Gdf5 mRNA in the interzone (compare panels Fig. 7G and J). These molecular changes in gene expression by abolition of Nog from the domain of transient cartilage region led to loss of joint formation. This became apparent histologically by Alcian Blue staining of section of an autopod at 15.5 dpc (compare Fig. 7M and N). At a later developmental time point, such as 18.5 dpc, we observed complete absence of joint structures in the digits and the metatarsus of the mutant hindlimb (compare Fig. 7O and P). These data suggest that the domain of noggin expression is necessary to insulate the cells around the interzone from BMP signaling, and in turn allow the development of embryonic articular cartilage.

DISCUSSION

Articular cartilage cells are derived from proliferative chondrocytes present at the DPZ of embryonic skeletal elements

Based on lineage tracing of Gdf5-expressing cells, it had been concluded previously that articular cartilage originates from a Gdf5-expressing population (Koyama et al., 2008). The observations made in that report have been subsequently interpreted as interzone
cells giving rise to articular cartilage (Kahn et al., 2009; Candela et al., 2014). By contrast, here we present data strongly indicating that the interzone cells are largely proliferation deficient and hence cannot support the growth of articular cartilage; instead, proliferative chondrocytes of the DPZ become part of the articular cartilage. Thus, our data demonstrate for the first time that these cells not only contribute to transient cartilage growth, but also contribute to embryonic articular cartilage growth. Our data also provide the mechanistic framework that allows contrasting differentiation events, e.g. embryonic transient and articular cartilage differentiation, to take place in juxtaposed cell populations.

To support the conclusion that embryonic articular cartilage must be derived from the DPZ, we first showed that, in developing chick embryos, there is a significant increase in cell number in the interzone/articular cartilage (Table 1); yet, the interzone cells of either chick or mouse do not proliferate significantly during the time window of articular cartilage specification (Fig. 1). These results suggest that cells from other source(s) contribute to the growth of interzone/embryonic articular cartilage. EdU/BrdU pulse-chase of proliferating chondrocytes in conjunction with lineage tracing of Col2a1 mRNA-expressing cells, presented in this study, show that cells of the DPZ contribute to the embryonic articular cartilage. Although we cannot rule out the possibility that other proliferating cell populations do not contribute to the growth of interzone, DPZ certainly contributes to the growth of embryonic articular cartilage. It should be noted that, at 15.5 dpc, the DPZ cells express Col2a1 mRNA, but at 18.5, these cells are detected in the interzone, where cells express Gdf5 mRNA but not Col2a1. Thus, the DPZ cells must undergo a transition before being incorporated in the interzone. In fact, we have identified such a zone between the DPZ and the interzone, where the cells express neither Col2a1 nor Gdf5 mRNA (Fig. 4). Furthermore, we could detect BrdU+ cells in the articular cartilage of six-week-old mice that were labeled at 13.5-15.5 dpc (Fig. 3), suggesting that the DPZ cells eventually become part of adult articular cartilage. Some of the BrdU−labeled cells at P21 or P42 are located in the superficial layer of articular cartilage, a flattened morphology (Fig. 3V,W). We suggest that these cells act as reservoir that might support postnatal articular cartilage growth (Dowthwaite et al., 2004). Our data is in agreement with recently published results (Candela et al., 2014).

**Model for simultaneous differentiation of transient and embryonic articular cartilage**

Ectopic activation of BMP signaling in developing cartilage resulted in the entire limb cartilage differentiating as transient cartilage, with...
no morphologically distinct articular joint visible (this study and Duprez et al., 1996; Zou et al., 1997; Brunet et al., 1998). We have also demonstrated that ectopic activation of BMP signaling suppresses Wnt signaling in the cells flanking the interzone. Therefore, for simultaneous differentiation of transient and articular cartilage in juxtaposed populations, Wnt-responsive cells must be insulated from BMP influence. We have demonstrated that a band of Nog-expressing cells (Fig. 5) serves this purpose. Thus, the absence of joint structures upon ectopic activation of BMP signaling should not be interpreted as fusion of cartilage elements (Duprez et al., 1996; Zou et al., 1997; Guo et al., 2004; Rountree et al., 2004). Rather, it should be interpreted as failure of articular cartilage formation and consequent failure to segment the cartilage anlagen. This is in keeping with the postulation of Brunet et al. (1998). We have demonstrated that ectopic activation of BMP or of Wnt even at late stage (HH34) (i.e. after specification/differentiation of interzone) turns on ectopic expression of transient or of articular cartilage markers, respectively (Fig. 6J-Q). Therefore, the domains of BMP and Wnt signaling must be kept spatially distinct to allow for simultaneous differentiation of embryonic articular and transient cartilage.

Based on our experimental data, we propose a model integrating concurrent longitudinal growth of transient cartilage and appositional growth of articular cartilage from the proliferating cells of the embryonic cartilage anlagen (Fig. 8). The proposed mechanism is likely to be conserved among vertebrates, as a majority of the postulates have been validated both in mouse and in chick.

**MATERIALS AND METHODS**

**Tissue**

Mouse experiments were conducted as per protocol approved by the Institute Animal Ethics Committee (registration number 810/03/ac/ CPCSEA, dated 15 October 2005). A Nog loss-of-function mutant (Col2a1-CRE;Nogfl/fl) was generated by mating Col2a1-CRE transgenic mice (Terpstra et al., 2003) with conditional Nogfl/fl mice (Stafford et al., 2011). Col2a1-CreER<sup>TM1TmG</sup> was generated by crossing Col2a1-CreER<sup>T2</sup> (Nakamura et al., 2006) and RosamTmG (Muzumdar et al., 2007) (Jackson Laboratories) mouse strains. A single tamoxifen injection (Sigma-Aldrich, 2.5 mg/20 g body weight) was administered intraperitoneally to pregnant females at 15.5 dpc. Embryos were harvested at 18.5 dpc for further analysis.

**Processing of tissue, histology and in situ hybridization**

Post harvesting, tissues were fixed in 4% paraformaldehyde overnight at 4°C. Mouse tissues harvested postnataally were decalcified in 8% EDTA solution (pH 7.5) before further processing. Fixed tissues were embedded in paraffin and sectioned at 5 μm thickness.
Fig. 8. Model for articular cartilage development. (A) Schematic of landmark events in cartilage differentiation, resulting in simultaneous formation of transient and articular cartilage in adjacent populations. (B,C) According to our model, all newly formed chondrocytes are capable of differentiating along the transient as well as articular cartilage fates. When a morphologically distinct interzone is first visible, Nog mRNA is expressed in a broad domain in the developing cartilage outside the interzone. During this early cartilage development, BMP signaling is restricted to the perichondrium (I). As development progresses, the Nog domain becomes restricted to the epiphyseal ends of the skeletal elements, a few cell layers away from the interzone. As a result, the BMP signaling domain expands but the cells of the DPZ (blue area in B,C) remain insulated. Wnt ligands that had been produced in the interzone signal the cells flanking the interzone to promote articular cartilage fate (II). During the even later developmental period, these expression domains are further consolidated. The progeny of the DPZ cells that expand towards the interzone turn off Col2α1 mRNA expression but continue to express the prechondrogenic marker Sox9. These prechondrogenic cells, sandwiched between the BMP and Wnt signaling domains (III), are capable of undergoing transient as well as articular cartilage differentiation. When these cells come under the influence of Wnt signaling, they differentiate as articular cartilage. On the other hand, the progeny of DPZ cells that expand towards the hypertrophic region come within the zone of BMP signaling influence and differentiate as transient cartilage.

Skeletal sections were stained with Alcian Blue 8GX (Sigma-Aldrich) solution (pH 2.5) for 2 min and washed. Labeled antisense-RNA probes were synthesized using ChEST clones (Geneservice) for Atx (ChEST520a5) and PCNA (ChEST631f8). The templates for Gdf5 and Col2α1 (Hartmann and Tabin, 2001) and for Nog (Pathi et al., 1999) have been described previously. Digoxigenin- or biotin-labeled anti-sense probes were generated as described, and mRNA in situ hybridization on paraffin sections for chondrogenic detection was performed essentially as described (Murtaugh et al., 1999). Fluorescent double mRNA in situ hybridization was carried out as described (Roy et al., 2013).

Cell proliferation assay
200 μg of EdU (E10415)/BrdU (B23151) (Invitrogen) in 200 μl of PBS was injected on top of chicken embryos, irrespective of the stage of development. For EdU/BrdU labeling in mice, pregnant females were injected intraperitoneally with 100 mg/kg body weight of EdU and/or BrdU. In the EdU competition assay, five times molar excess of dTTP (Thermo Fisher Scientific) was used.

EdU detection was carried out as per manufacturer’s instruction (C10083, Invitrogen). BrdU or double-labeling of EdU-BrdU was performed as per Zeng et al. (2010).

For postnatal stages, we could not get either EdU or DAPI to work on P21 and P42 samples. Moreover, we observed non-specific staining in fluorescence detection of BrdU. Therefore, we developed BrdU with DAB reagents to detect it chromogenically for these stages.

Immunohistochemistry
Sections were deparaffinized and were rehydrated in PBS, followed by post-fixation in 4% PFA. Sections were incubated overnight at 4°C with the following primary antibodies: anti-ph3 (Sigma-Aldrich, H0412; 1:100), anti-BrdU (Sigma-Aldrich, B8434; 1:150), anti-β-catenin-Cy3 (Sigma-Aldrich, C7738; 1:200), anti-GFP (Invitrogen, A6455; 1:300), anti-GAG (3C2; Potts et al., 1987), anti-pSMAD1/5/8 (Cell Signaling, 9511; 1:100) and anti-noggin (R&D Systems, AF719; 1:100). Following this step, tissues were washed in PBT and incubated with respective secondary antibodies, such as Dylight 549 AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, 111-505-003; 1:200); Alexa Fluor 594-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, 115-585-003; 1:200); anti-goat HRP antibody (Vector Labs, PI-9500; 1:100) for noggin. These secondary antibodies were later detected using a TSA kit (T30955, T20932, Invitrogen) and a Vectastain ABC kit (PK6100).

Viral misexpression
RCAS-caBMPR1B (Zou et al., 1997) and RCAS-mBMP4 (Duprez et al., 1996) virus particles were generated as described (Logan and Tabin, 1998).

Organ culture
H34 chick embryonic hindlimbs were cultured for 24 h (Bandyopadhyay et al., 2008). rhBMP4 protein (peproTech, 120-05) was dissolved in DMEM and 2% BSA to a working concentration of 100 ng/μl along with the vital dye Fast Green, and injected in HH34 MTP joints prior to organ culture. The control included the buffer without protein. Wnt5a (R&D Systems, 1324-WN-002) protein injection was carried out in a similar manner at a working concentration of 100 ng/μl.

For injection, the anti-noggin antibody (R&D Systems, AF719) was dissolved at 0.1 mg/ml concentration in glycerol. Control was an isotype-matched antibody, i.e. an anti-WGA antibody (Vector Labs, AS2024) was used as an isotype-matched control.

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Competing interests
The authors declare no competing or financial interests.

Author contributions

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Supplementary material
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References


Supplementary Figures

Supplementary Figure S1

Figure S1. Number of cells in the interzone/articular cartilage of MTP joint of digit 3 in chick increases during development. (A) To estimate the change in dimensions of MTP joint, DAPI- (A1-A4, A7, A10 and A13) and Alcian Blue (A5, A6, A8, A9, A11 and A12)-stained sections of chick hind limb from indicated developmental stages were used. The lines in A1, A3, A5, A8 and A11 represent the diameter and the depth of developing interzone/articular cartilage, as marked in A11. The green and red boxes (A2, A4, A7, A10 and A13) have identical dimensions (100Px × 100Px) and placed randomly in the developing interzone/articular cartilage and transient cartilage areas, respectively to estimate the average number of cells in the interzone/articular cartilage region versus development.
the transient cartilage region. D, diameter; H, height (depth). (B) Average radius (blue bars) and depth (orange bars) of chick interzone/articular cartilage of the digit 3 MTP joint during different stages of development show that dimensions of the articular cartilage rises steadily at successive stages of development. \( r = \text{radius} = 0.5 \times D \) (from panel A). (C) An estimate of fold change in average volume of the interzone/articular cartilage in chick MTP joint of digit 3 during successive stages of development. It should be noted that HH28 is the first data point used. It can be observed that the volume steadily increases to eightfold between HH28 and HH38. (D) An estimate of the average number of cells per unit area of the interzone/articular cartilage (blue bars) and transient cartilage (orange bars) in and around chick MTP joint at the indicated developmental stages demonstrate the compact nature of interzone/articular cartilage versus the transient cartilage. (E) An estimate of fold change in average cell number of the interzone/articular cartilage of the chick MTP joint during successive stages of development. It is seen that the average number of cells in the interzone increases 12-fold between HH28 and HH38.

**Method for estimation of growth of interzone/articular cartilage in terms of increase in volume and cell number.**

We generated longitudinal sections of chick hindlimb from three independent embryos for each of the stages investigated. We measured the diameter and depth of the interzone/articular cartilage in three different sections corresponding to each stage (HH28, 31, 34, 36 and 38 of the chick embryonic development (Supplementary Figure 1A). We calculated the average radius and depth/height for interzone/articular cartilage in each of these stages (Supplementary Figure 1B). Assuming the developing interzone/articular cartilage to be perfect cylinders we employed the formula \( \pi r^2 H \) to obtain the volume of this tissue, where \( r \) is the radius and \( H \) is the depth/height of the tissue. Using unitary method we calculated the fold change in volume of interzone/articular cartilage between successive stages (Supplementary Figure 1C). We created a box of \( 100 \times 100 \) px and placed six such boxes in each of the sections either in the interzone/articular cartilage region (green colored boxes in Supplementary Figure 1A) or in the transient cartilage region (red colored boxes in Supplementary Figure 1A). We counted the number of DAPI-stained nuclei in each of these boxes in each of the sections, as mentioned above. The average number of cells in these boxes for a given stage was used to obtain the number of cells in a given arbitrary unit area. We used a constant imaginary third dimension (C) to calculate the number of cells in an arbitrary constant volume of interzone/articular cartilage as (number of cells per unit area \( \times C \)). Following which, we employed unitary method to calculate the percentage increase in number of cells (in the entire volume of interzone/articular cartilage) between two successive stages as well as fold increase in number of cells with respect to HH28. It should be noted that C, the imaginary third dimension used, would be cancelled out between the numerator and the denominator in these calculations.
**Figure S2.** Cells of the interzone/articular cartilage are non-proliferative (A,B) mRNA in situ hybridization (ISH) of PCNA in chick hind limb digit from HH28 (A). B shows a magnified view of the marked region taken from tibiotarsus joint, from panel A. (C) Cells of the interzone are largely devoid of phospho-histone H3 (Ser10) immunoreactivity. phospho-histone H3 immunofluorescence (red) in sections of chick MTP joint at distinct stages show that they are absent from the interzone/articular cartilage region. (D,E) Chick HH31 (D) and mouse 14.5 dpc (E) digits co-stained for Gdf5 mRNA and BrdU. Purple arrowheads mark the distal interzone, whereas the green arrowheads mark proximal interzone. It is interesting to note that in the distal interzone Gdf5 is expressed in a broader domain, and that many cells outside the interzone have incorporated BrdU and also express Gdf5 (the superposition of the Gdf5 and BrdU panels are presented in Fig. 2A,D). Scale bar: 100 μm.
Figure S3. Proliferating cells of DPZ are incorporated in the interzone and eventually become part of articular cartilage. (A-D) These panels are supplementary to Fig. 3D,E. Articular cartilage and transient cartilage are marked by the expression of *Gdf5* and *Col2a1*, respectively. The tissue
sections used in panels A and C are adjacent to the ones used in Fig. 3D, whereas the sections used in panels B and D are adjacent to the ones used in Fig. 3E. (A, B) Gdf5 mRNA expression and (C, D) Col2a1 mRNA expression. Pulse chase for 24 h show that most of the EdU-incorporated cells are present in the interzone (compare panels B,D with Fig. 3E). (E,F) These panels are supplementary to Fig. 3J-M. During EdU-BrdU double-pulse and chase, tissues were harvested 24 h post-EdU administration and 2 h post-BrdU administration. We observe that most of the EdU-incorporated cells are detected in the domain of interzone marked by the expression of (E) Gdf5, whereas many (F) BrdU-immunoreactive cells are detected in the domain of transient cartilage region as seen by the expression of Col2a1. (F') Superposed image using Fig. 3K,M and supplementary material Fig. 3F, in which Col2a1 mRNA ISH is pseudo-colored in green and BrdU incorporation is in red. Here, Fig. 3K,M and supplementary material Fig. 3F are on adjacent sections. (G-J) Schematic (G) of time course of BrdU pulse and chase for 3 (H), 4 (I) and 5 (J) hours. BrdU-positive cells (red) are detected primarily outside of the interzone of MTP joints of 15.5 dpc mouse embryos. MT, metatarsus; PH, phalangeal elements. (K,O) Lineage tracing with Col2a1-CreERT2;Rosa<sup>mT/mG</sup> mouse embryos is demonstrated in M. In this study, pregnant females bearing Col2a1-CreERT2;Rosa<sup>mT/mG</sup> embryos were injected with tamoxifen at 15.5 dpc. After 5 h, these females were injected with EdU. At 18.5 dpc, the embryos were harvested and analysed for GFP-expressing cells in the distal joints of the hindlimbs. L,M show many GFP-expressing cells detected by anti-GFP antibody (green) in the domain of the embryonal articular cartilage that do not express Col2a1 (red). (N,O) On adjacent section, EdU was detected (red) along with GFP. Images in L-O are used for the analysis in Fig. 3P-R. (P) Schematic of long-term pulse-chase of BrdU incorporated cells in mice. Pregnant females were injected with three consecutive doses of BrdU or EdU from 13.5 to 15.5 dpc and embryos were harvested at postnatal days P0, P3, P7, P21 and P42. The data for P0, P7, P21 and P42 are presented in Fig. 3S-W. (Q) MTP joint articular cartilage at P3 shows EdU-labeled cells. White arrowheads mark the cells that incorporated BrdU and are a part of the articular cartilage. A magnified region is provided as an inset. (R,S) Magnified region MTP joint articular cartilage at P21 (R) and P42 (S) shows magnified region from Fig. 3V and 3W, respectively.
Supplementary Figure S4

Figure S4. Simultaneous detection of *Gdf5* and *Col2a1* as markers of interzone/articular cartilage and transient cartilage, respectively, in both chick and mice. (A) Simultaneous detection of *cGdf5* (green) and *cCol2a1* (red) in chick HH36 digit by fluorescent ISH show that distal phalangeal regions co-express both interzone (*cGdf5*) and transient cartilage (*cCol2a1*) markers as opposed to the proximal phalanges. (B,C) mRNA ISH of *Col2a1* (B) and *Gdf5* (C) on adjacent sections from HH36 chick hind limb digit. These panels have been pseudocolored, superposed and presented in Fig. 4J. (D,E) mRNA ISH of *Col2a1* (D) and *Gdf5* (E) on adjacent sections from 16.5 dpc mouse hind limb digit. These panels have been pseudocolored, superposed and presented in Fig. 4M. (F,G) mRNA ISH of *Col2a1* (F) and *Gdf5* (G) on adjacent sections from 16.5 dpc mouse metatarsus. These panels have been pseudocolored, superposed and presented in Fig. 4N. Scale bar: 100 μm.
Supplementary Figure S5

Figure S5. Expression of Noggin near the distal ends of the skeletal anlagen insulates the interzone/articular cartilage region from BMP signaling. (A,B) Noggin mRNA ISH in the MTP joint at HH28 (A) and HH38 (B). At HH38, Noggin-expressing cells in the MTP joint show a tight band of expression and black arrowheads mark the extent of Noggin mRNA expression domains towards the interzone/articular cartilage. The region devoid of Noggin mRNA expression domain in the developing epiphyseal ends of cartilage is marked by asterisks. (C-G) Active BMP signaling domain detected by pSMAD 1/5/8 immunoreactivity (red) at HH28 tibia (C,D), HH31 phalangeal and MTP joint (E, F and G). (D) Magnified view of the marked region from C. (F,G) Magnified views of
the marked regions from E. F is an MTP joint, whereas G is the proximal-most phalangeal joint from digit 3. White arrowhead, pSMAD1/5/8-immunoreactive cells in the perichondrium; white asterisk, region flanking the interzone devoid of pSMAD1/5/8-immunoreactive cells. (H-K) Relative domains of Noggin protein expression (green, H) and pSMAD1/5/8 immunoreactivity (red, I) in a 14.5 dpc mouse MTP joint. (J) After antibody detection the section was stained with DAPI. (K) Merged image of H, I and J. Asterisks in I and K mark the domains that are devoid of pSMAD1/5/8 immunoreactivity. (L) Section through RCAS-caBMPRIB-infected putative phalangeal joint of HH36 chick hind limb. The uninfected cells continue to express Gdf5 mRNA. This section is adjacent to the sections used in Fig. 6E-G. (M) Representative image for BMP4 or WNT3a protein injection at the phalangeal joint of HH38 digit hind limb in organ culture. (N,O) Immunohistochemistry of Noggin in skeletal tissues from conditional Noggin knockout mice (Col2a1-CRE;Noggin\textsuperscript{fl/fl}) in panel O as opposed to the control, Col2a1-CRE;Noggin\textsuperscript{fl/+}, in panel N. Noggin-expressing cells are present in the cartilage anlagen in the control as opposed to the mutant (compare white arrowheads in N,O). N shows adjacent section from that shown in Fig. 7G-I, and O shows adjacent section from that shown in Fig. 7J-L. Scale bar: 100 μm.
## Supplementary Material Table 1

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