Review Article

Chondrocytes-osteoblast transition in endochondral ossification

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Abstract: The concern of chondrocyte-osteoblast transition in skeleton development has continued for more than one century. Even though many morphological and *in vitro* data clearly indicated osteoblast differentiation as an alternative fate choice of chondrocytes besides of undergoing apoptosis, absence of the solid *in vivo* observation led to this chondrocyte-osteoblast transition concept stagnant. Fortunately, the development of gene-manipulated strategy in biology assisted to resolve the century-problem by *in vivo* lineage tracing and the concept of chondrocyte-osteoblast transition was finally accepted until 2014, when three elegant studies published. These works done by three individual groups reached out similar conclusion about chondrocyte-osteoblast transdifferentiation in the process of endochondral bone formation. The acceptance of this concept shed light on skeletal biology and the endochondral ossification (EO) chapter of text book will be re-written with this finding. However, the different origins and the proposed functional discrepancy of skeletal osteoblasts also bring us to re-explain and think of previous data again. More in-depth studies will implement more curiosity on the underlying molecular mechanism of chondrocyte-osteoblast differentiation and the physiological function of chondrocyte-derived osteogenic cells in both skeleton homeostasis and bone marrow environment.

Keywords: Chondrocytes; Osteoblasts; transition; endochondral ossification (EO)

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Vertebrate skeleton is formed by two kinds of mechanisms: intramembranous ossification (IO) and endochondral ossification (EO). The remarkable difference between them is the formation of cartilage anlage in EO. In the intramembranous ossification, the mesenchymal cells condense and directly differentiate into bone-forming osteoblasts which can secrete bone extracellular matrix and ultimately differentiate into osteocytes. Some craniofacial bones are formed by this way (1). The axial and appendicular skeletons are formed by EO in which a cartilage intermediate is involved. Mesenchymal cells first condense where the future bone will form, and become osteochondroprogenitors. The osteochondroprogenitors in the outer layer of condensation progressively differentiate to osteogenic cells. At the same time, cells in the center of condensation undergo strict and sequential chondrogenic differentiation. At the later stage of chondrocyte development, the immature chondrocytes exit from cell cycle and hypertrophy. When cartilage matrix is mineralized and the vascular invasion occurs, osteoblast precursors differentiate into osteoblasts and deposit bone matrix on the degenerating cartilage scaffolding (*Figure 1*). However, the ultimate fate of HCs in this process is still controversial.

Origin and differentiation program of chondrogenic lineage

After mesenchymal cells migrate from the cranial neural

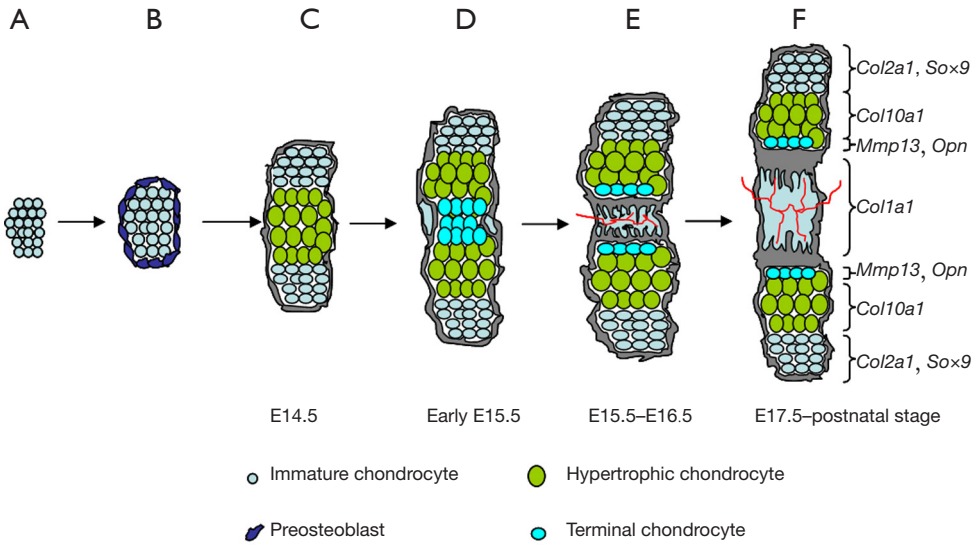

Figure 1 Schematic diagram of endochondral ossification (EO). (A) Mesenchymal cells condense; (B) cells in center of condensation become chondrocytes and the outer layer cells differentiate into osteogenic cells; (C) chondrocytes at the centre of cartilage anlage mature and hypertrophy; (D) at the onset of osteogenesis, HCs undergo terminal differentiation to LHCs; (E) the capillaries invade the cartilage anlage and the ossification center forms; (F) chondrocytes continue to proliferate, lengthening the bone (characteristic genes expressed by chondrocytes of different-stages are shown in the diagram).

crest, paraxial mesoderm and lateral plate mesoderm, they pack in the presumptive skeleton sites, prefiguring the future skeletal elements (2). Under the control of transcription factor, *Sox9*, osteochondroprogenitors in the center of condensation begin to express *Col2a1* and are committed to early chondrocytes. Chondrocyte is the only cell component that makes up the cartilage tissue. Chondrocyte differentiation process is concomitant with a strong increase in deposition of cartilage matrix. Matrix proteins such as type II collagen, type IX collagen, type XI collagen and aggrecan are highly produced. Besides *Sox9*, two other Sox members, *Sox5* and *Sox6*, also play critical roles in early chondrocyte differentiation. But whether other unidentified transcription factors are likely to work there together with the Sox trio is still unclear.

Early chondrocytes are small, round, and progressively become flattened and organized into parallel, longitudinal columns, forming columnar chondrocytes. Since these cells proliferate at high rate, they are also called proliferating chondrocytes. Once they exit from cell cycle and undergo irreversible growth arrest, they mature and differentiate into prehypertrophic chondrocytes (preHCs). At gene expression level, columnar chondrocytes distinguish themselves from early chondrocytes by quantitative changes, rather than qualitative changes. No specific molecular markers have been identified for these proliferating chondrocytes. There are some extracellular matrix genes up-regulating progressively concomitantly with the appearance of these cells: e.g., *Agc1*, and *Comp*. Many signaling pathways, such as IHH, PTHrP/PPR, FGFs, BMPs and WNT signaling, have been found to interact together to regulate the formation and maintenance of the proliferating chondrocytes.

When proliferating chondrocytes differentiate into prehypertrophic and hypertrophic chondrocytes (HCs), these cells undergo obvious phenotypic switch in some aspects: cell morphology, cell status, gene expression profile and so on. Proliferating chondrocytes exit from cell cycle and progressively increase their cytoplasmic volume up to about 10 times. PreHCs still express high level of *Col2a1*, *Agc1* and most other cartilage matrix genes. However, when chondrocytes are fully hypertrophic, they reduce, even stop, the expression of some cartilage markers and up-regulate *Col10a1*. It is well known that PTHrP/IHH feedback loop, FGFs, BMPs signaling pathways play essential roles in controlling the rate at which proliferating chondrocytes undergo hypertrophy (3-5).

Terminal differentiation of HCs

It is generally believed that HCs represent the terminal

stage of differentiation in chondrogenic cell lineage (6) and these post-mitosis chondrocytes finally undergo apoptosis in the process of EO (6-8).

However, some study showed that HC could undergo another phenotypic change and further differentiate into terminal chondrocyte (4). A histological photography of murine humerus stained with alcian blue showed the presence of some smaller cells called terminal chondrocytes. They were found to be located between proximal and distal HCs, adjacent to the bone collar. Furthermore, HCs were shown to only reach its late stage by down-regulating *Col10a1* expression and expressing some of osteoblast markers, e.g., alkaline phosphatase (*Alp*), *Mmp13* and *Opn* (6,9,10).

Owing to its negligible number and the bottom-most location in the postnatal hypertrophic zone, the terminal chondrocytes gain little attention for a long time. Hence, the regulative mechanism of terminal differentiation process and the function of these terminal chondrocyte are still poorly understood.

Besides many regulatory roles of *Runx2* in osteogenesis and chondrogenesis, *Runx2* was also found to regulate chondrocyte maturation. *Runx2* null mice died just after birth, due to a failure to breathe (11-13). These mice showed a lack of hypertrophy and terminal differentiation of chondrocyte in most of the skeleton. Ectopic expression of *Runx2* in non-HCs promoted their hypertrophic differentiation and disrupted joint formation (12,14). *VEGF*, which is normally expressed in hypertrophic chondrocyte, was not expressed in chondrocytes of *Runx2* null mutant. Furthermore, *VEGF* expression was up regulated by *Runx2* in fibroblasts in tissue culture (15). These data suggested that *Runx2* might be a direct regulator of *VEGF* expressed by HCs. Moreover, several studies have strongly suggested that *Runx2* might directly regulate and activate such genes as *Mmp13* and *Opn* (16-19). Therefore, one hypothesis is that *Runx2*, which is expressed in HCs, can activate markers of terminal chondrocytes, e.g., *Mmp13*, *Opn*.

Sox9 plays a vital and irreplaceable role in the cell fate determination of the chondrogenic lineage. The expression of *Sox9* is turned on in osteochondroprogenitor cells prior to condensation. *Sox9* maintains its high level of expression in chondrocyte until the beginning of prehypertrophy. The expression of *Sox9* is turned off when chondrocytes exit from cell cycle and undergo maturation. Previous gene regulation studies revealed that *Sox9* could bind to the enhancer region of some chondrocyte-specific markers such as *Col2a1* (20,21), *Col11a1* (22) and *aggrecan* (23), and increase their expression. Consistent with the vital role of *Sox9* in chondrocyte differentiation, overexpression of *Sox9* in HCs delayed endochondral bone formation with the decrease of *VEGFa*, *Mmp13* and *Rankl*, indicative of the necessity of "turn-off" of *Sox9* in the terminal differentiation of HCs.

Another transcription factor involving in regulating terminal differentiation of HCs is *c-Maf*, a member of Maf family. This family belongs to the basic leucine zipper (bZIP) superfamily which includes Fos, Jun and the CREB/ATF family. Although *c-Maf* is believed to act as a developmental regulator, there are few cellular targets of *c-Maf* identified so far. *In situ* hybridization (ISH) has shown that *c-Maf* was expressed specifically in HCs, terminal chondrocytes, primary spongiosa and the perichondrium (24). *c-Maf* deficient mutant mice displayed normal prehypertrophy and early hypertrophy, determined by normal onset and level of expression of *Ppr*, *Ihh* and *Col10a1* (24). However, the terminal differentiation of HCs was initially delayed in *c-Maf*$^{-/-}$ embryos, followed by a subsequent expansion of the hypertrophic chondrocyte domain in the growth plate of fetal and postnatal long bones. These results suggested that *c-Maf* facilitated the initiation of terminal differentiation of HCs and influenced the disappearance of HCs. The specific decrease of *Mmp13* expression in *c-Maf*$^{-/-}$ embryos at E15.5 suggested it was possible that *Mmp13* was a downstream target of *c-Maf*.

Up to now, our knowledge about terminal differentiation of chondrocytes is at its infancy. Further characterization of the terminally differentiated chondrocytes and investigation of the transcriptional regulation of terminal differentiation will help us understand more about the switch from hypertrophy to terminal differentiation and the ultimate cell fate of chondrocyte in EO.

Apoptotic fate *vs.* trans-differentiation fate of terminal HCs

Whether HCs undergo apoptosis or transdifferentiate to bone cells in EO is still unclear. This question is really controversial for more than a century mainly because that the transdifferentiation concept of HCs was difficult to be accepted. Instead, apoptosis detection using many separate techniques have showed strong evidences supporting HCs undergo programmed cell death in EO (8,25-29). And the in-depth investigation of apoptosis showed the specific microenvironment present in the cartilage-bone transition zone linked to the activation of apoptosis. Concomitant

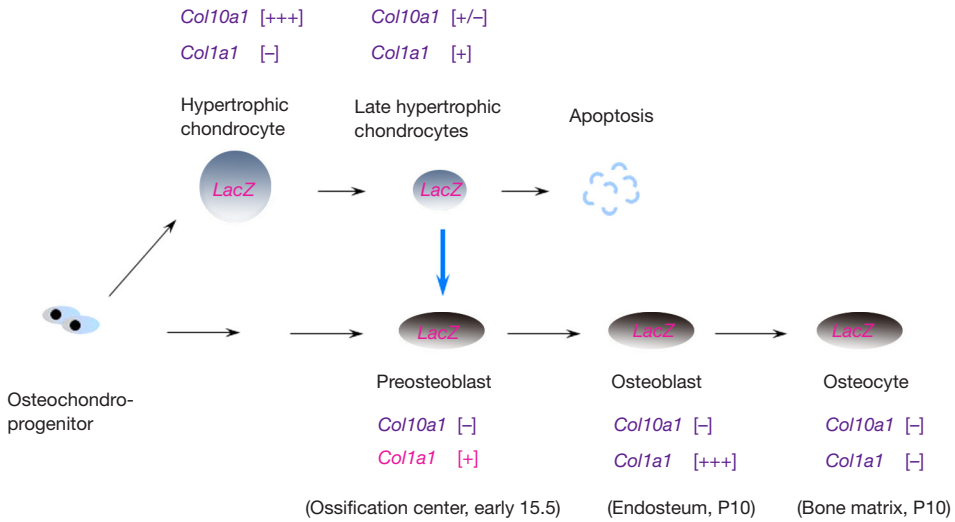

Figure 2 Transdifferentiation fate of hypertrophic chondrocytes (HCs). When *Col10a1*$^{+}$/*Col1a1*$^{-}$ HCs terminally differentiate into late HCs, *Col10a1* expression is down and slight *Col1a1* expression is on. The decrease of *Col10a1* and the increase of *Col1a1* are sustained at the following osteoblast differentiation stages until the final mature of osteoblasts to osteocytes, which *Col1a1* expression is turn off (*Col10a1*, molecular marker of hypertrophic chondrocytes; *Col1a1*, molecular marker of osteoblasts).

with the degeneration of mineralized matrix, the local microenvironment in HZ is changed and it liberates high local concentrations of O_2, ions, peptides and glycans, many of which are essential for the activation of apoptosis (30-32).

However, the finding of apoptosis in HCs shown in these studies could not exclude other fate of HCs. Apoptosis occurs frequently in normal tissues and organs. It is not surprising to find them in the process of cartilage-bone conversion. By TUNEL assay, the percentage of apoptotic HCs in all HCs was quantified and varied in different group's studies, 3.29% or 44%, suggesting that other HCs may have other final than "apoptosis" fate. Serial morphological studies from Crelin and Koch (33) have showed each chondrocyte could generate several small cells and form reticular tissue when undergoing ossification. This conclusion seems more striking, but their observation might give some hints. In 1996, Erenpreisa and Roach described an asymmetrical division of chondrocyte. Chondrocyte gave rise to two daughter cells: one undergoes apoptosis and the other one survives and re-enters cell cycle. This interesting finding provided new insight into the transdifferentiation hypothesis (34-37).

Moreover, the *in vitro* culture and transplantation experiments also brought more direct evidence to the transdifferentiation hypothesis. Chondrocyte in culture were found to be able to express the markers of osteoblast and the cell morphology also changed to osteoblast-like cells (38-40). And when growth plates from quail were transplanted into the chick chorioallantoic membrane (41), the osteoblasts in the resultant bone were entirely derived from quail, suggestive of transdifferentiation from HCs to osteoblasts.

Even so, some researchers also considered that transdifferentiation was more circumstantial. They believed that hypertrophic chondrocyte was proposed to have the potential to differentiate into osteogenic cells and initially contributed to bone formation. However, only the "borderline chondrocytes", which were exposed to the appropriate matrix and environment, could differentiate into osteogenic cells (42). The HCs residing in the core of the epiphyseal growth plate was suggested to undergo apoptosis (29). This scenario indeed gives another interesting explanation for the final fate of HCs.

This dispute is still going on until three elegant works published by different groups. The common strategy in these studies is *in vivo* lineage tracing used to address this challengeable question. The specificity of Cre recombination mouse is the key factor affecting us to draw such an overwhelming conclusion. Cheah's group first reported chondrocyte-to-osteoblast lineage continuum by using tamoxifen-inducible *Col10-Cre* knock-in mouse to label HCs temporally (10). Cre fragment is

transcriptional expressed by endogenous *Col10a1* promoter and Cre expression is strictly limited within the *Col10a1*-expressing region. This specific *Col10-Cre* knock-in mouse was adopted to trace the ultimate fate of HCs *in vivo* by crossing to reporter mice. This study clearly showed HCs, at least partially, survived in cartilage-bone transition and transdifferentiate into osteogenic cells (*Figure 2*). These HCs-derived osteoblasts (HCOB) are mainly located within the primary spongiosa at embryonic stage. At postnatal stage, these descendant cells are not evenly distributed in metaphyseal and they predominately resided in trabecular bone and endosteum (10). These HCOBs finally differentiated to osteocytes, mostly residing within metaphyseal bone. HCs-derived positive cells were not detected in periosteum and much less positive cells was found in diaphyseal cortical bone. The profound lesson from these discriminative locations of different ontogenies of osteoblasts and osteocytes remained unknown. It might be correlatively connected with some special physiological functions of HCOB. Crombrugghe's group (43) and Yang's group (44) also gave consistent conclusion by use of different Cre recombination mouse lines: *Col10a1-BAC-Cre*, *Col10a1*^{int2}*-Cre* and other tamoxifen-induced *Agc1-CreERT and Col2a1-CreERT*. Besides of osteogenic lineage, Yang's group also showed some data that HCs exhibited multiple destinies at postnatal stage: vascular endothelial cells, pericytes and adipocytes. These results indicated the *in vivo* multipotential cell fates of HCs (44). However, we could not jump to the final conclusion of the multi-differentiation potential of HCs descendants and need more solid evidences to support this striking hypothesis. It will overturn current concept of skeletal stem cells if this hypothesis is finally demonstrated in the future.

Molecular control of chondrocyte fates during ossification

Apart from the direct observation and study of chondrocyte-osteoblast transdifferentiation, we obtained more about the underlying molecular regulation of this process from analyzing genetically manipulated mice.

Even though the transdifferentiation concept has not been accepted before, there already have some reports showing the terminal differentiation and cell fate were impaired by genetically modifying some key regulators. *Osterix* (*Osx*), a Kruppel-like Sp-1 binding factor, is a novel osteoblast-specific transcription factor and belongs to the SP family. Compared to the absence of both of hypertrophic chondrocyte and bone marrow in *Runx2*$^{-/-}$ mice, *Osx* deficient mice blocked bone marrow formation only and hypertrophic zone is formed normally (45). This study indicated that *Osx* function followed downstream of *Runx2* and might play more strict role in chondrocyte-osteoblast differentiation. And the unregulated expression of *Osx* in late HCs of E15.0 also supported its possible function in cartilage-bone transition (10).

WNT proteins are secreted glycoproteins involved in various developmental and Wnt/β-catenin pathway regulates skeleton development at many key levels (46). The indispensable role of canonical Wnt signaling in osteogenic differentiation has been revealed by modifying β-catenin gene expression temporally and spatially. When Wnt/β-catenin pathway was ablated in osteochondroprogenitors, the osteogenic differentiation was arrested at osteochondroprogenitor stage and these cells exhibited chondrocyte characteristics, expressing *Sox9* and *Col2a1*. Further *in vivo* and *in vitro* studies suggested that β-catenin functions as a cell fate determinant factor driving osteochondroprogenitors into the osteoblast lineage through preventing chondrogenesis (47-49). Gain of function study of Wnt/β-catenin pathway in immature chondrocytes leads to their dedifferentiation and blockage of further hypertrophic differentiation. Wnt10b and Wnt7b has been considered as a ligand candidate regulating this process because it could induce the mesenchymal cells differentiating to osteogenic cells rather than adipocyte (50,51). After the concept of chondrocyte-to-osteoblast transdifferentiation was accepted, the role of Wnt/β-catenin pathway in this transition got much attention. Although similar work was reporter on 2013 by Klaus von der Mark team (52), Christine Hartmann's study emphasized the contribution of decreased chondrocyte-derived osteoblast differentiation when Wnt/β-catenin pathway was removed from HCs, other than its indirect regulation on osteoclast differentiation (53). This elegant work provides solid evidence indicating Wnt/β-catenin pathway is the key regulator of chondrocyte-osteoblast transdifferentiation.

Col10a1-13del transgenic mice expressing mutant collagen X as a consequence of a 13-base pair deletion in *Col10a1* (*13del*) elicited endoplasmic reticulum stress (ERS) in HCs because of the misfolded a1(X) chains accumulated within HCs (54). HCs under such situation altered their terminal differentiation and cell fate. They were found to re-enter cell cycle and re-express immature chondrocyte markers, *Sox9* and *Col2a1*. These cells did not show much apoptosis fate; therefore, the most possibility was that they changed

their transdifferentiation back to more immature status. Similar finding of ERS-induced cell fate determination was also observed in cartilage oligomeric matrix protein (COMP) deficient mice, indicative of the key role of ER stress and response on cell fate determination of HCs.

Besides of autonomous regulation by transcription factors and signaling pathways, the *in vivo* cell fate of HCs was also influenced by surrounding matrix environment. Matrix metalloproteases, such as MMP13 and MMP14 (also known as MT1-MMP), were synthesized by HCs and degraded cartilage matrix in cartilage-bone transition. Loss of *Mmp13* or *Mmp14* induced an expansion of hypertrophic zone due to less vascular invasion and bone resorption defects (55,56). Undoubtedly, the cartilage-bone transition process was interrupted by bone resorption defects. And the terminal differentiation and final fate of HCs could be predicted to have changed a lot because of an expanded hypertrophic zone. However because of the absence of detecting more molecular markers, the status and the ending of HCs are hardly determined in these two mutant mice. It will be more convincing if the fate of mutant HCs was explored and followed by further lineage tracing experiments.

Most of specific transcription factors and signaling pathways of osteoblast differentiation, such as *Osx* and Wnt/β-catenin pathway, are involved in the chondrocyte-osteoblast transdifferentiation. But whether there is any special regulator responsible for this lineage-crossing differentiation remains unclear. The finding of such a key lineage-crossing factor might give us more insight for many lineage studies in other organs.

Perspectives on physiological function of chondrocyte-derived osteogenitors

Up to now, the concept of chondrocyte-osteoblast transdifferentiation has been well known as an alternative fate of HCs besides of undergoing apoptosis. Therefore, there are two sources of osteogenitors in endochondral bone formation: perichondral cells and chondrocytes. Their locations in long bone are obviously different, with chondrocyte-derived osteoblasts in endosteum and chondrocyte-derived osteocyte mainly in metaphyseal bone, indicating they might have variant physiological functions in maintaining the homeostasis of bone marrow. And whether they exhibit differently in disease cases are also unknown. Upon aging and osteoporosis, these perichondrium-derived and chondrocyte-derived osteoblasts and osteocytes loss their biological function equally or not. All these answers will help us understand more about skeleton homeostasis and develop more suitable strategy to maintain bone mass.

Of articular cartilage, chondrocytes in calcified zone are positive for *Col10a1*, and these cells were also labelled in lineage tracing experiment by using of *Col10a1-Cre* mice. Unlike of the formation of primary ossification center in which the perichondrium-derived osteoprogenitors are much involved, the secondary ossification center is much clear and the involved of another origin of subchondral osteoblasts was suspected except for articular cartilage-derived. The ultimate fate of these articular chondrocytes is still absent and it need to be addressed urgently by solid *in vivo* data. Notably in osteoarthritis (OA), articular cartilage undergoes degeneration and calcified zone was expanded in some cases. Whether these degenerated cartilages produce more chondrocyte-derived osteoblasts for subchondral bone was still an open question. The answer will extend our knowledge on joint homeostasis and disease, helping us develop more effective drugs or interventions for future.

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Footnote

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