Simultaneous differentiation of articular and transient cartilage: WNT-BMP interplay and its therapeutic implication

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ABSTRACT Limb skeleton forms through the process of endochondral ossification. This process of osteogenesis proceeds through an intermediate cartilage template and involves several stages of chondrocyte maturation and eventual bone formation. During the process of endochondral ossification, interplay between BMP and WNT signaling regulate simultaneous differentiation of articular and transient cartilage. In this review, we focus on the recent literature which explores the simultaneous differentiation of these two different types of cartilage. We discuss a new paradigm of developmental biology-inspired tissue engineering of bone and cartilage grafts and provide novel insight into treatment of osteoporosis.

KEY WORDS: *endochondral ossification, articular cartilage, transient cartilage, BMP signaling, WNT signaling*

Overview of endochondral ossification

Vertebrate limb skeleton is primarily formed by a process known as endochondral ossification (*endon* – within, *chondros* – cartilage), where bone is formed within a cartilaginous template. The process starts with condensation of a group of mesenchymal cells that undergo chondrogenic differentiation. This cartilage primordium undergoes branching and segmentation to give rise to all the distinct skeletal elements of vertebrate limbs. Concomitant to the formation of these distinct elements, the process of replacement of individual cartilage elements by bone through endochondral ossification initiates. Eventually most of the cartilage element is replaced by bone, barring a few layers of cartilage on either side of the plane of segmentation. The cartilage capping the distinct elements are maintained as cartilage forever and is varyingly referred to as permanent or articular or joint cartilage. In this article this cartilage will be mostly referred to as articular cartilage as they line the articulation surfaces of the skeletal elements. On the other hand, the cartilage within the primordia that is replaced by bone is referred to as transient cartilage, because of its transient existence. In this review we will use this term. In the literature, however, this cartilage is also referred to as growth plate cartilage.

It should be noted that both, (i) generation of distinct skeletal elements and (ii) replacement of the cartilage primordia by bone, are multistep processes and are temporally overlapping. Endochondral ossification takes its cue from the putative site of segmentation as soon as the process of formation of distinct skeletal elements begins. It should be noted here that at this pre-specified segmentation site, cells undergo dramatic change in shape and appear flattened.

The structure comprising the flattened cells is referred to as the interzone. The interzone demarcates each independent cartilage element. Once the interzone is specified, the cells in the middle of individual cartilage elements proceed through pre-hypertrophic and hypertrophic differentiation to finally give rise to bone formation. Hypertrophic cells, which are characterized by their large cellular volume, also secrete factors which induce invasion by the blood vessels. Blood vessels bring in osteoclasts and other remodeling factors which help in remodeling the cartilage matrix, eventually making it conducive for bone differentiation. Thus, after the invasion of blood vessels, the cartilage template is eventually replaced by bone. While interzone formation is a major intermediate landmark for cartilage segmentation, hypertrophic differentiation of cartilage cells is the major intermediate landmark for replacement of cartilage with bone.(Karsenty, 2001, Kronenberg, 2003, Pacifici *et*

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al., 2005, Shubin *et al.*, 1997, Shubin, 1986). Existing literature demonstrates that BMP and WNT signaling play critical role in limb skeletal development, both in endochondral ossification as well as articular cartilage differentiation.

In this review, we will first provide brief introductions of BMP and WNT signaling pathways in the context of skeletal differentiation. With that background, we will primarily focus on the interplay and relationship between the processes of cartilage segmentation, articular and transient cartilage differentiation, cross regulation of the two processes and how lessons from these developmental processes may be extrapolated to derive insights into the pathology of osteoporosis and osteoarthritis as well as for regenerative medicine.

Endochondral ossification and BMP signaling

Marshall Urist, in a seminal work published in 1965, first demonstrated the autoinduction activity of decellularised bone matrix. In this work he demonstrated that implantation of decellularised bone matrix, either subdermally or intramuscularly, was able to induce bone formation in otherwise non-skeletal tissues (Urist, 1965). Eventually, the activity resident in the decellularised matrix and capable of inducing ectopic bone formation was named as Bone Morphogenetic Protein (BMP) (Urist and Strates, 1971). The major part of the next two decades was spent in extraction and molecular characterization of the proteins belonging to the BMP family. An important role in identification and characterization of the active principle capable of bone induction was played by the laboratory of Hari Reddi (Reddi, 1983, Reddi, 1998a, Reddi, 1998b, Reddi *et al.*, 1989, Reddi and Reddi, 2009, Reddi *et al.*, 1987). They characterized the receptors responsible for transduction of the signal (ten Dijke *et al.*, 1994), first demonstrated pleotropic effects of BMPs, and elucidated the mechanism of action of BMP signaling in multiple contexts including development, differentiation and maintenance of various tissue types. Additionally, the Reddi laboratory described the role of BMP signaling in multiple pathologies of the skeletal system, cancer and various developmental defects, as well as their potential for skeletal tissue engineering (Reddi, 2001, Reddi *et al.*, 1989). The cloning of the gene(s) encoding BMP proteins was carried out by scientists of the Genetics Institute, Cambridge, MA, USA (Wozney and Rosen, 1998, Wozney *et al.*, 1988). The cloning and sequencing revealed that vertebrate BMPs are homologues of fly morphogen decapentaplegic (Dpp) (Gelbart, 1989, Irish and Gelbart, 1987, Padgett *et al.*, 1987, Spencer *et al.*, 1982). However, unlike in the flies, the human genome encodes around 12 different BMPs of which Bmp2, Bmp4, Bmp5, Bmp6 and Bmp7 are commonly referred to as osteogenic BMPs, based on their ability to induce ectopic bone formation (Lowery and Rosen, 2018).

Experiments by Urist's group, and subsequently by Hari Reddi's and other groups demonstrated that BMP proteins are sufficient for bone formation. However, due to the pleotropic nature of BMP action and because of the redundancy of activity among different osteogenic BMPs (Oxburgh *et al.*, 2005) it remained difficult to demonstrate that BMPs are also necessary for osteogenesis. Particularly, embryonic lethality of BMP loss of function mouse strains remained an insurmountable problem that awaited technological advancement. Development of tissue specific conditional knockout mouse technology allowed Bandyopadhyay et. al., to inactivate both Bmp2 and Bmp4 – two major osteogenic BMP ligands – specifically in the limb mesenchymal cells. It was observed that simultaneous knock out of Bmp2 and Bmp4 in limb mesenchymal cells largely spared chondrogenesis but completely abrogated osteogenesis. However, it should be noted that other osteogenic BMPs, namely Bmp5, Bmp6 and Bmp7 are also known to be expressed in the developing limb bud and could provide sufficient signaling for chondrogenesis. Bandyopadhyay et. al., did not investigate whether knockout of Bmp2 and Bmp4 completely eliminated BMP signaling activity in the developing limb skeletal elements. Thus, although this study demonstrated that BMP signaling is essential for osteogenesis, it could not rule out the possibility that BMP signaling may also be needed for chondrogenesis (Bandyopadhyay *et al.*, 2006). Moreover, the specific step(s) of endochondral ossification in which BMP signaling plays an essential role remains to be fully elucidated.

Downstream effectors of BMP signaling in endochondral ossification

One line of investigation that can help in uncovering the step(s) of endochondral ossification that are critically dependent on BMP signaling will be to identify the genes which are transcribed in skeletal progenitor cells as a result of active BMP signaling. However, despite the fact that Urist demonstrated the critical importance of BMP signaling in osteogenesis as early as in 1965, the first set of osteogenesis specific BMP downstream targets were identified only in 2012. This study utilized existing micro-array datasets available in the GEO database to perform a meta-analysis to shortlist an initial list of 14 candidate genes. All these micro-array experiments had similar design where mRNA expression was compared between osteogenic cells experiencing BMP signaling gain- or loss-of-function. Validation of the meta-analysis by expression screening of the candidate genes in the absence and presence of BMP signaling both *in vitro* and *in situ*, resulted in the identification of the first set of seven bone specific BMP downstream targets. This study by Prashar et. al. also identified Dpysl3 as a potential regulator of cell secretion (Prashar *et al.*, 2014). More recently, through proteomic study of BMP signaling depletable cell line, TVA-BMSC (Yadav *et al.*, 2016), Kumar et. al., identified yet another downstream target of BMP signaling in developing bone. Prdx1 is a Reactive Oxygen Species (ROS) scavenger molecule that is specifically expressed in the pre-hypertrophic cells of the developing skeletal element (Kumar *et al.*, 2018). The specific roles of Dpysl3 and Prdx1 in the context of endochondral ossification is discussed below. It is likely that many more targets of BMP signaling that are expressed during endochondral ossification, and are likely to play critical role(s) in the process, are yet to be identified. Further investigation using high throughput transcriptomic analysis of osteogenic cells during the course of bone formation, followed by *in situ* expression screening would be needed to identify a comprehensive set of BMP downstream genes involved in the process of endochondral bone formation.

Endochondral ossification and WNT signaling

WNT signaling is another critical regulator of endochondral ossification. Wnt pathway components were mostly discovered from

genetic studies conducted in *Drosophila*. Sharma and Chopra from Indian Agricultural Research Institute isolated a wingless *Drosophila* mutant (Sharma, 1973, Sharma and Chopra, 1976). This mutant was rediscovered by Nüsslein-Volhard and Eric Wieschaus in their Nobel prize winning study (Nusslein-Volhard and Wieschaus, 1980). The mutated gene in *wingless* codes for a secreted morphogen that affects polarity within *Drosophila* body segments. In parallel, Roel Nusse and Harold Varmus in their search for genetic loci, insertion in which caused cancerous changes, identified a gene which they named Int1 (Nusse and Varmus, 1982). Eventually it was found out that Int1 is the vertebrate homolog of *wingless* and the name of a gene family Wnt was coined. Investigations in flies, mice, frogs, fishes etc., helped uncover many members of the WNT signaling pathway (reviewed in, (Wiese *et al.*, 2018)).

Cliff Tabin's laboratory in Harvard Medical School conducted large scale expression screens in developing chicken embryos to identify developmental processes in which WNT signaling may play an important role. In one such screen Hartmann and Tabin discovered that Wnt14 (now called Wnt9a) is expressed in the limb cartilage interzone (Hartmann and Tabin, 2001). Their study, in conjunction with studies conducted by other groups, established that canonical WNT signaling is sufficient to induce articular cartilage differentiation (Guo *et al.*, 2004, Kan and Tabin, 2013, Spater *et al.*, 2006).

It is equally interesting to note that WNT signaling is also critically required for transient cartilage differentiation (Houben *et al.*, 2016, Rodda and McMahon, 2006). Thus it becomes important to understand how the same signaling pathway influences cartilage cells to adopt two completely different fates, at two different locations, within a skeletal anlagen. This in turn would require identification of transcripts that are expressed in these two distinct cells types as a result of active canonical WNT signaling. To the best of our knowledge, downstream effector genes that are specifically expressed in developing limb skeletal elements in response to WNT signaling and are needed for articular cartilage differentiation and/or hypertrophic differentiation are yet to be identified.
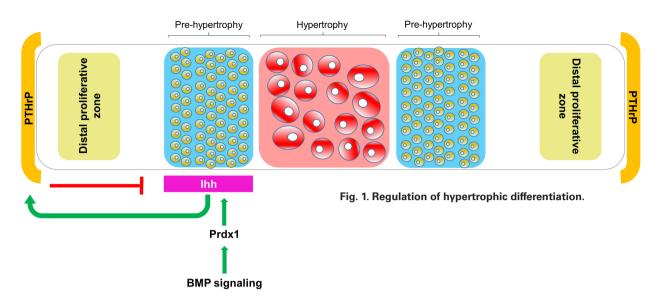
Molecular and cellular description of transient cartilage differentiation

Undifferentiated mesenchymal cells of the developing limb bud condense to form the cartilage primordia. Herein, adhesion molecules, many of which are regulated by BMP signaling, regulate the process of condensation (Hall and Miyake, 1992, Oberlender and Tuan, 1994a, Oberlender and Tuan, 1994b). The entire condensed mass of mesenchymal cells start expressing Sox9, which subsequently induces the expression of Col2a1 (Bell *et al.*, 1997, Ng *et al.*, 1997). Thus, the cartilage anlagen is initially a single unit of Col2a1 expressing chondrocytes. However, as mentioned earlier, most of the cartilage is eventually replaced with bone through the process of endochondral ossification. Only the terminal ends of each skeletal element remain cartilage permanently, that is articular cartilage. On the other hand, the cartilage which differentiates into bone is known as transient cartilage. It is important to note here that, BMP signaling promotes transient cartilage while WNT signaling promotes initiation of articular cartilage differentiation (Bandyopadhyay *et al.*, 2006, Bandyopadhyay *et al.*, 2013, Hartmann and Tabin, 2001, Jaswal, 2017). The transient cartilage differentiation initiates at the center of the Col2a1 expressing

cartilage anlagen and then radiates outward in each direction. It should be noted that the center of the anlagen is at the farthest distance from both the articular surfaces, the source of Parathyroid Hormone Related Peptide (PTHrP). PTHrP prevents hypertrophic differentiation of chondrocytes, the ultimate stage of transient cartilage differentiation. Before the chondrocytes reach hypertrophy, they pass through several different stages of maturation – resting, proliferative, pre-hypertrophic and finally hypertrophic chondrocytes (Karsenty, 2001, Kronenberg, 2003). The pre-hypertrophic cartilage cells express Indian Hedgehog (Ihh). PTHrP is a ligand secreted from the articular surface. As long as the chondrocytes are within the zone of active PTHrP signaling, they do not enter hypertrophy. However, due to growth of the anlagen, as soon as the chondrocytes exit the zone of influence of PTHrP signaling the cells turn on Ihh expression and pre-hypertrophic differentiation begins. In turn, Ihh negatively regulates PTHrP expression. This negative feedback loop that regulates expression of PTHrP and Ihh controls the rate and extent of transient cartilage differentiation (Dentice *et al.*, 2005, Lanske *et al.*, 1996, Vortkamp *et al.*, 1996).

Until recently, no gene that positively regulates the expression of Ihh in the prehypertrophic cells was known. BMP signaling is known to be essential for endochondral ossification but the precise step(s) of endochondral ossification that is regulated by BMP signaling remained unknown. A collaborative work between the laboratories of Arun Trivedi and ours recently identified Prdx1, a ROS scavenging enzyme, as a gene whose expression is dependent on active BMP signaling and is restricted to the pre-hypertrophic domain of the developing cartilage anlagen. Our work demonstrated that loss of BMP signaling dramatically increases ROS level in cells. Prdx1, presumably through downregulation of ROS level, positively is necessary for the expression of *Ihh* in the pre-hypertrophic cartilage. However, as Prdx1 expression maintains Ihh expression it does not allow the cells to undergo hypertrophy. In contrast, Morita et. al., demonstrated that increase in ROS level positively regulates hypertrophic differentiation (Morita *et al.*, 2007). Thus BMP signaling induced expression of Prdx1 critically regulates the transition from proliferating to pre-hypertrophic to hypertrophic chondrocytes (Fig. 1). This study is one of the rare instances where BMP signaling has been implicated to regulate a specific step of endochondral ossification (Kumar *et al.*, 2018).

In skeletal tissues, only a small fraction of the volume is occupied by the bone cells, the rest is ECM. Cartilage and bone have highly specialized ECM. Since BMP signaling is essential for development of cartilage and bone, it is expected that this signaling pathway would be involved in cartilage and bone differentiation. However, the link between BMP signaling and synthesis of ECM has not been extensively explored. We identified Dpysl3 as a BMP signaling target gene likely to be involved in regulating secretion in chondrocytes. Though its exact role in developing chondrocytes needs to be fully elucidated, a recent study has implicated Dpysl3 with regulation of actin assembly in osteoblast (Abdallah *et al.*, 2017). Following hypertrophy, extensive matrix degradation and remodeling ensues which is mediated primarily by matrix degrading enzymes belonging to the Matrix Metalloproteinase family and disintegrins of ADAMTS family (Kelwick *et al.*, 2015). This is followed by vascular invasion and angiogenesis which is marked by expression of VEGF-A in the transient cartilage domain (Gerber *et al.*, 1999). The immediate step following hypertrophic differentiation is osteoblast differentiation which is marked by the expression of

Fig. 1. Regulation of hypertrophic differentiation.

a transcription factor belonging to Runt family known as Runx2 (Komori, 2010). It appears that there are two primary sources of osteoblasts during embryonic development: a) Vascular invasion which facilitates entry of osteoblast precursors from the outer layer of the developing primary ossification center and b) Trans-differentiation of hypertrophic, ColX expressing cells into Runx2 expressing osteoblast precursors (Park *et al.*, 2015, Yang *et al.*, 2014). Following invasion into the matrix, osteoblast undergo differentiation into type I collagen (Col1a1) expressing cells which secrete mineral rich matrix leading to ossification (Kozhemyakina *et al.*, 2015).

Molecular and cellular description of articular cartilage differentiation

Cartilage interzone

Interzone is the first overt sign of segmentation in an erstwhile contiguous piece of cartilage anlagen. The cells of the interzone are arranged in three layers of densely packed cells having a conspicuous flattened morphology (Holder, 1977, Mitrovic, 1977). The interzone is divided histologically into two outer chondrogenous layers and an inner transitional layer (Ito and Kida, 2000). As mentioned above, cartilage anlagen expresses Sox9 and Col2a1 throughout the structure. At the site of interzone induction, expression of both Sox9 and Col2a1 is downregulated. Instead, interzone cells express a host of specific molecules such as Gdf5, Atx, Chordin etc., (Hartmann and Tabin, 2001). These molecules continue to be expressed in the articular cartilage cells as well. Earliest demonstration of the role of interzone in the context of joint formation was performed by Holder et. al., where he surgically removed the interzone from a developing chicken limb, clamped the stumps together and observed that no joint formed in the absence of the interzone (Holder, 1977). This classical experiment suggests that interzone is essential for joint formation. However, what remained unanswered is whether interzone plays a role in segmentation as well as articular cartilage differentiation. In other words, it was unclear whether segmentation and articular cartilage differentiation are coupled events.

In 2001 Hartmann and Tabin demonstrated that cells of the interzone express Wnt ligands such as Wnt4, Wnt9a etc. They also demonstrated that ectopic expression of Wnt9a ligands led to upregulation of expression of articular cartilage markers such as Gdf5, Atx, Chordin etc., in the chondrocytes. Though misexpression of Wnt9a led to an upregulation of molecular markers of articular cartilage and loss of type II collagen, but the characteristic flattened morphology of interzone cells was not attained. Thus, WNT signaling is capable of inducing articular cartilage fate in the chondrocytes but cannot induce ectopic joint formation as WNT signaling is not sufficient for inducing a neo-interzone. In this study, Hartmann and Tabin speculated that articular cartilage differentiation is not promoted by canonical WNT signaling rather by non-canonical WNT signaling (Hartmann and Tabin, 2001). Subsequently, however, Yingzi Yang's group at National Institute of Health, USA demonstrated that canonical WNT signaling can induce articular cartilage fate (Guo *et al.*, 2004). Recent reports describing loss of function mouse mutants of Wnt ligands (Wnt9a as well as Wnt9a; Wnt4 double knockout) demonstrated that these mice develop with minimal joint defects suggesting that though WNT signaling is sufficient to promote articular cartilage differentiation in cartilage cells, Wnt9a and/or Wnt4 are however, not necessary to induce interzone and subsequent joint formation in developing skeletal system (Spater *et al.*, 2006). The importance of WNT signaling in articular chondrocyte differentiation was demonstrated by Kan and Tabin through loss of function of cJun, a transcription factor that induces expression of several Wnt ligands in the articular surface (Kan and Tabin, 2013). Taken together, it is now clear that induction of the interzone sets up a Wnt producing tissue which in turn induces articular cartilage differentiation in the nearby cells.

Since the ratio of the length of each skeletal element forming as a result of segmentation is preserved across different individuals of a species, what can be easily deduced is that the site of interzone formation is regulated by a tight molecular mechanism. Though some theories attempting to explain the periodicity of digit phalanges have been proposed (Hiscock *et al.*, 2017, Kavanagh *et al.*, 2013), however, the molecular mechanism that dictates the site of interzone induction and the process of interzone formation *per se* remain completely unexplored.

Articular cartilage differentiation

Articular cartilage differentiation is the process by which cells in the immediate vicinity of the interzone or site of segmentation take up permanent cartilage fate. Till recently, other than WNT signaling no molecule capable of inducing articular cartilage fate was known. Particularly, the role of transcription factors critical for articular cartilage differentiation remains largely unexplored. Only a handful of transcription factors have been found which play a role in this process, the principal among which are the C-1-1 variant of the ERG family of transcription factors and two Odd-skipped related transcription factors, Osr1 and Osr2. C-1-1 has been shown to block transient cartilage differentiation. However, the overexpression of C-1-1 does not lead to ectopic expression of articular cartilage markers (Iwamoto *et al.*, 2001, Iwamoto *et al.*, 2000, Iwamoto *et al.*, 2007). OSR1/2 loss-of-function reduces the expression of Wnt ligands, thereby blocking joint formation and allowing transient cartilage differentiation in the putative joint region (Gao *et al.*, 2011). Since OSR1/2 misexpression studies have not been performed, it is still unknown whether OSR1/2 can induce articular cartilage fate.

Despite tremendous progress in the past two decades, our understanding of articular cartilage differentiation remains in infancy as we still lack a comprehensive knowledge of the transcription factors necessary for articular cartilage differentiation and how articular cartilage escapes hypertrophy and continues to remain as permanent cartilage (Karsenty and Wagner, 2002). These questions are of paramount importance as articular cartilage tissue gets affected and is suspected to undergo transient cartilage differentiation during the progression of the most prevalent skeletal disease, called osteoarthritis (Pitsillides and Beier, 2011).

In order to identify novel genes that are expressed in the articular cartilage, Singh *et al.*, conducted microarray-based transcriptome comparison of articular cartilage tissue against transient cartilage tissue derived from embryonic day 12 and 14 chicken embryos and identified 17 novel genes expressed in chicken interzone and articular cartilage cells (Singh *et al.*, 2016). Further, Singh et. al., identified a transcription factor, Gata3, which is exclusively expressed in the articular cartilage of a developing embryo. It is the first transcription factor to be identified which upon misexpression can initiate articular cartilage differentiation at the expense of transient cartilage differentiation, whereas abolishing Gata3 activity downregulated the expression of articular cartilage specific markers e.g., SFRP2 and Atx (Singh *et al.*, 2018b).

Simultaneous differentiation of articular cartilage and transient cartilage

As mentioned above, majority of the cells in the original cartilage primordia undergoes transient cartilage differentiation while only a small subset, adjoining the plane of cartilage segmentation, undergoes articular cartilage differentiation. The molecular basis of simultaneous differentiation of these two contrasting types of cartilage cells from a common pool of progenitors has been put forth in a hypothesis where it is asserted that the spatially and temporally separate domains of BMP and WNT signaling in the developing joints makes this simultaneous differentiation possible (Ray *et al.*, 2015). According to this model, there exists a bi-potential Col2a1 expressing cell population that is proliferative. This group of cells, due to proliferation, expands towards the site of segmentation (interzone) and away from it as well. Although interzone positioning is poorly understood, the site of segmentation or the interzone is the source of Wnt ligands. The cells that enter the zone of active WNT signaling differentiate as articular cartilage while the cells that expand towards the center of the cartilage anlagen are exposed to BMP signaling and undergo transient cartilage differentiation. There exists a band of Noggin expressing cells in the sub-articular zone which ensures that cells destined to form articular cartilage are not exposed to BMP signaling and remain competent to differentiate as articular cartilage upon exposure to WNT signaling. The critical importance of maintaining the sub-articular cartilage cells in BMP signaling deficient state became apparent when our group uncovered the molecular basis of immobilization-induced ectopic transient cartilage differentiation in the domain of articular cartilage. It turns out that immobilization of mouse or chicken embryos results in loss of expression of an intracellular inhibitor of BMP signaling – Smurf1/2. Loss of Smurf expression prevents the sub-articular cells to turn off BMP signaling and as a result they can no longer differentiate into articular cartilage fate in response to WNT signaling. Instead, they differentiate as transient cartilage under the influence of BMP signaling (Singh *et al.*, 2018a). Hence, the simultaneous differentiation of a common pool of cells is facilitated by the existence of a mechanism where two signaling pathways, operational at two distinct loci, promote opposite cell fates and generate two tissues from a single cell population (Fig. 2).

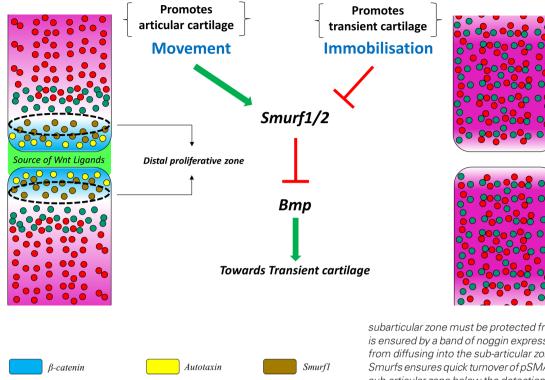### Coordination between articular cartilage and transient cartilage differentiation

Singh et. al., in their transcriptomic screen, identified a transcription factor, NFIA, which is exclusively expressed in the articular cartilage. Misexpression of NFIA in the presumptive transient cartilage cells maintains these cells in an immature cartilage state by negatively regulating transient cartilage differentiation. However, more interestingly, knockdown of NFIA using a miRNA blocked the transition from Col2a1 expressing cartilage cells to ColX expressing hypertrophic cells. The cells were arrested in a Col2a1 and Ihh expressing state. Considering that NFIA is not expressed in the transient cartilage cells, the only way knockdown of NFIA can affect transient cartilage differentiation is if there is a coordination between articular and transient cartilage differentiation and if NFIA plays a role in this process (Singh *et al.*, 2018b).

For now, it appears that there exists three networks at the articular surface controlling articular and transient cartilage differentiation: (1) Network involving GATA3, OSR1/2, c-JUN and Wnt ligands controlling articular cartilage differentiation; (2) Another network involving NFIA, GDF5 and ERG preventing transient cartilage differentiation in the articular cartilage domain, and (3) A network involving IHH, PTHrP, NFIA and C-1-1 controlling the coordination between articular cartilage differentiation and hypertrophy. However, the molecular mechanism governing the cross-talk between these networks remains to be explored.

Paradigm shift in tissue engineering approach

In the field of tissue engineering, designing articular cartilage grafts for cartilage defects have been a major focus of research. The need for tissue engineered grafts stems mainly from the fact that articular cartilage is non-proliferative and self-repair deficient. However, traditional cartilage engineering has failed to prevent *in vitro* grafts from proceeding towards cellular hypertrophy. One of

Fig. 2. Molecular mechanism regulating simultaneous differentiation of articular and transient cartilage. *Nascent cartilage is bi-potential. This anlagen can differentiate as articular cartilage in response to WNT signaling and as transient cartilage in response to BMP signaling. Within the anlagen, the only population of proliferative cells is confined within the distal proliferative zone. Due to proliferation this zone expands and cells that expand towards the interzone (source of Wnt ligand) experience WNT signaling, while the cells that expand away from the interzone experience BMP signaling. For successful articular cartilage differentiation, the cells in the subarticular zone must be protected from high level of BMP signaling. This is ensured by a band of noggin expressing cells that prevents BMP ligands from diffusing into the sub-articular zone. At the same time, expression of Smurfs ensures quick turnover of pSMAD1/5/8 to keep BMP signaling in the sub-articular zone below the detection level of pSMAD1/5/8 immunostaining. Smurf expression is turned off in the absence of mechanical loading.*

the problems with the current approaches in tissue engineered cartilages were its failure to consider the differences between articular and transient cartilage developmental path. It was in this context that Sourabh Ghosh's group and our laboratory collaborated to engineer a 3D bio-printed cartilage with a new approach – developmental biology-inspired tissue engineering. In this study, for the first time, articular cartilage was tissue engineered considering different developmental aspects observed *in vivo*, like the difference in cell density between embryonic and adult articular cartilage, expression of articular cartilage specific markers and resistance towards expression of hypertrophic differentiation markers (Chawla *et al.*, 2017). For this study TVA-BMSC cells were used. These are immortalized BMSCs derived from a mouse strain that can be conditionally depleted of both *Bmp2* and *Bmp4* upon induction by tamoxifen. Further, TVA-BMSCs express the receptor for avian leukosis virus - subgroup A, TVA which enables avian retroviral mediated gene transduction in these cells (Yadav *et al.*, 2016). The study showed that TVA-BMSCs when cultured at high density in 3D-bioprinted silk-gelatin scaffold in absence of TGFβ1, exhibit high resemblance to cartilage formed during *in vivo* articular cartilage differentiation. The engineered construct was assessed not only by the level of expression of articular cartilage markers and suppression of hypertrophic differentiation, but also taking into account cell viability. It should be noted that the silk scaffold led to a steady and sustained stimulation of WNT signaling within these constructs, thus, eliminating the need for addition of exogenous Wnt ligands, which is otherwise critical for articular cartilage differentiation.

A similar challenge existed in developing bone constructs for load-bearing applications. The long bones which typically perform load bearing functions are formed through endochondral ossification.

Thus it is likely that bone constructs developed following endochondral pathway would be better conformant as grafts for load-bearing bones. However, till recently, tissue engineering of bone involved direct ossification of mesenchymal cells through a process that is reminiscent of intramembranous ossification *in vivo*, the process which leads to formation of craniofacial bones. It should be noted that such tissue engineered constructs have never been tested *in vivo* for load-bearing ability. The laboratories of Sourabh Ghosh and ours collaborated again to engineer a 3D bone construct following a developmental biology-inspired strategy. In this work, TVA-BMSCs in 3D bio-printed silk-gelatin scaffold were initially made to undergo chondrogenic differentiation, followed by hypertrophic differentiation and finally osteogenic differentiation. Careful assessment revealed 3D bone construct made through this approach had massive up-regulation of osteocyte specific gene expression. It is thus likely to be better suited for transplantation in load-bearing bones as compared to the approaches taken by previous studies (Chawla *et al.*, 2018). Thus, both the studies highlight the importance of taking into consideration the developmental biology-perspective in rendering future tissue engineered bone and cartilage grafts.

BMP signaling in adult tissue homeostasis

The role of signaling pathways such as BMP and WNT have been extensively studied in the context of embryonic development. Adult animals have a steady turn-over of differentiated cells. The homeostasis is maintained majorly by the progenitor cells, which proliferate and replace the differentiated cells. Thus, it is expected that the signaling pathways that are important for embryonic differentiation of different cell types would continue to play a critical

role in adult animals in maintenance of homeostasis. BMP signaling has been heavily implicated in endochondral bone development, but not much is known about its role in adult bone tissue.

Limb specific Bmp2 knockout mouse embryos develop without any defects, both in terms of patterning as well as skeletal differentiation. However, after 13 weeks of birth, the Bmp2 deficient mice bones became severely fracture prone and lacked fracture repair capabilities (Tsuji *et al.*, 2006). This observation suggested a possible role of BMP signaling in adult skeletal maintenance. To test this directly, we developed a mouse strain in which Bmp2 and Bmp4 can be conditionally knocked-out upon tamoxifen administration. This temporally controlled knockout helped us in avoiding any adult stage phenotype arising out of embryonic complexities due to Bmp2 and Bmp4 loss. Also, with no a priori information on the tissue types in which BMP signaling might play a role in adult mice, Bmp2 and Bmp4 was knocked out ubiquitously (Nag *et al.*, 2017). Upon knockdown at the age of 6 weeks, 9 week old mice exhibited gross defects in hair follicles and intestine. It is interesting to note that both hair follicles and intestine need BMP signaling during development and both have high turn-over rate which perhaps explains rapidity with which these tissues degenerated upon loss of Bmp2 and Bmp4. These mice eventually also developed osteopenia. Ovariectomized mice, which serve as osteopenic mice model, when treated with teriparatide (PTH) – the only clinically used bone anabolic drug, exhibited skeletal preservation through stimulated skeletal growth. Interestingly, PTH treatment failed to rescue osteopenia developed in the Bmp2/Bmp4 knockout mice. This showed that PTH-induced rescue of osteopenia and stimulation of bone formation was mediated through BMP signaling. In fact, in line with this observation, we uncovered that when TVA-BMSCs were treated with PTH, both Bmp2 and Bmp4 mRNA levels increased (Khan *et al.*, 2016). Taken together, these studies exhibit how BMP signaling is not only essential for embryonic development, but also plays an important part in maintaining adult bone homeostasis. Further, this study indicates BMP agonists may serve as next-generation bone anabolic drugs.

Future research directions

We have attempted to highlight above that many fundamental aspects of skeletal differentiation remain yet to be explored. To summarize, BMP signaling is essential for transient cartilage differentiation. WNT signaling, on the other hand, though is essential for initiation of articular cartilage differentiation but is also needed for hypertrophic differentiation, a key step in transient cartilage differentiation. It remains unclear how the same signaling pathway, within the context of cartilage cells promotes two distinct processes. It is possible that the cellular context within which the pathway acts makes the difference. However, to understand these processes in molecular terms, the downstream targets of both BMP and WNT signaling in the context of articular and transient cartilage need to be identified.

We have highlighted the importance of thorough understanding of the developmental principles in order to apply the same for translational and regenerative medicine purposes. Discovering pathway modulators of BMP, WNT and other signaling pathways and devising delivery vehicles for these in different disease contexts can have significant clinical impact. Tissue engineering of bone and cartilage is pursued by many groups. However, there are very few groups that integrate principles of developmental biology in these approaches which in our opinion can significantly improve the outcome of such endeavours.

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