1

Early Skeletal Morphogenesis in Embryonic Development

Yingzi Yang

Harvard School of Dental Medicine, Harvard Stem Cell Institute, Boston, MA, USA

INTRODUCTION

Formation of the skeletal system is one of the hallmarks that distinguish vertebrates from invertebrates. In higher vertebrates (ie, birds and mammals), the skeletal system contains mainly cartilage and bone that are mesoderm-derived tissues and formed by chondrocytes and osteoblasts, respectively, during embryogenesis. A common mesenchymal progenitor cell also referred to as the osteochondral progenitor gives rise to both chondrocytes and osteoblasts. Skeletal development starts from mesenchymal condensation, during which mesenchymal progenitor cells aggregate at future skeletal locations. Because mesenchymal cells in different parts of the embryo are derived from different cell lineages, the locations of initial skeletal formation determine which of the three mesenchymal cell lineages contribute to the future skeleton. Neural crest cells from the branchial arches contribute to the craniofacial bone, the sclerotome compartment of the somites gives rise to most axial skeletons, and lateral plate mesoderm forms the limb mesenchyme, from which limb skeletons are derived (Fig. 1.1). Ossification is one of the most critical processes in skeletal development and this process is controlled by two major mechanisms: intramembranous and endochondral ossification. Osteochondral progenitors differentiate into osteoblasts to form the membranous bone during intramembranous ossification, whereas during endochondral ossification, osteochondral progenitors differentiate into chondrocytes instead to form a cartilage template of the future bone. The location of each skeletal element also determines its ossification mechanism and unique anatomic properties such as the shape and size. Importantly, the positional identity of each skeletal element is acquired early in embryonic development, even before mesenchymal condensation, through a process called pattern formation.

Cell–cell communication that coordinates cell proliferation, differentiation, and polarity plays a critical role in pattern formation. Patterning of the early skeletal system is controlled by several major signaling pathways that also regulate other pattern formation processes. These signaling pathways are mediated by morphogens including Wnts, Hedgehogs (Hhs), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and Notch/Delta. Recently, the Turing model [1] of pattern formation that determines skeletal formation spatially and temporally has drawn increasing attention. In his seminar paper [1], Turing proposed an ingenious hypothesis that the patterns we observe during embryonic development arise in response to a spatial prepattern in morphogens. Cells would then respond to this prepattern by differentiating in a threshold-dependent way. Thus, Turing hypothesized that the patterns we see in nature, such as skeletal structures, are controlled by a self-organizing network of interacting morphogens. The Turing model has been successfully tested in limb skeletal patterning with combined computational modeling and experimental approaches [2–5].

EARLY SKELETAL PATTERNING

In the craniofacial region, neural crest cells are major sources of cells establishing the craniofacial skeleton [6]. It is the temporal and spatial-dependent reciprocal signaling between and among the neural crest cells and the epithelial cells (surface ectoderm, neural ectoderm,

Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, Ninth Edition. Edited by John P. Bilezikian.

Companion website: www.wiley.com/go/asbmrprimer

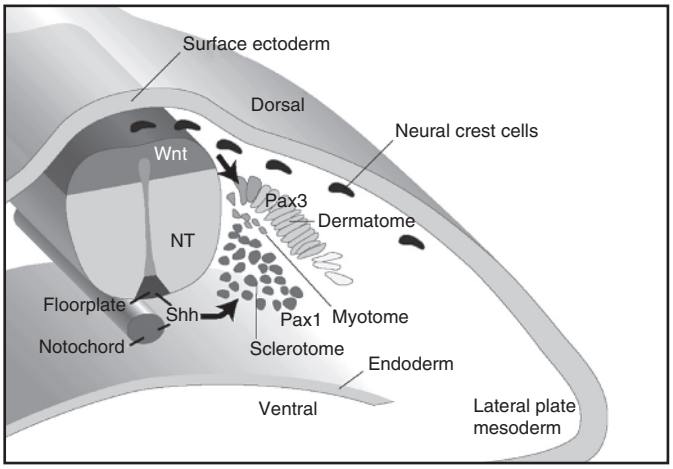

Fig. 1.1. Cell lineage contribution of chondrocytes and osteoblasts. Neural crest cells are born at the junction of dorsal neural tube and surface ectoderm. In the craniofacial region, neural crest cells from the branchial arches differentiate into chondrocytes and osteoblasts. In the trunk, axial skeletal cells are derived from the ventral somite compartment, sclerotome. Shh secreted from the notochord and floor plate of the neural tube induces the formation of sclerotome which expresses Pax1. Wnts produced in the dorsal neural tube inhibits sclerotome formation and induces dermomyotome that expresses Pax3. Cells from the lateral plate mesoderm will form the limb mesenchyme, from which limb skeletons are derived. Source: [16,17]. Reproduced with permission of Elsevier.

or endodermal cells) that ultimately establish the pattern of craniofacial skeleton formed by neural crest cells [7].

The most striking feature of axial skeleton patterning is the periodic organization of the vertebral columns along the anterior–posterior (A–P) axis. This pattern is established when somites, which are segmented mesodermal structures on either side of the neural tube and the underlying notochord, bud off at a defined pace from the anterior tip of the embryo's presomitic mesoderm (PSM) [8]. Somites give rise to axial skeleton, striated muscle, and dorsal dermis [9]. The repetitive and left–right symmetrical patterning of axial skeleton is controlled by a molecular oscillator or the segmentation clock that act in the PSM (Fig. 1.2*A*). The segmentation clock is operated by a traveling wave of gene expression (or cyclic gene expression) along the embryonic A–P axis, which is generated by an interacting molecular network of the Notch, Wnt/β-catenin, and FGF signaling pathways (Fig. 1.2*B*). Understanding molecular control of vertebrate segmentation has provided a conceptual framework to explain human diseases of the spine, such as congenital scoliosis [10].

The Notch signaling pathway mediates short-range communication between contacting cells [11]. The majority of cyclic genes are downstream targets of the Notch signaling pathway and code for Hairy/Enhancer of split (Hes) family members, Lunatic fringe (Lfng), and the Notch ligand Delta. The Wnt/β-catenin and FGF signaling pathways mediate long-range signaling across several cell diameters. Upon activation, β-catenin is stabilized and translocates to the nucleus where it binds Lef/Tcf factors and activates expression of downstream genes. Axin2, Dkk1, Dac1, and Nkd1 are Wnt-activated negative regulators that are rhythmically expressed in the PSM. The FGF signaling pathway is also activated periodically in the posterior PSM, indicated by the dynamic phosphorylation of ERK in the mouse PSM. FGF-negative feedback inhibitors, such as Sprouty homolog 2 and 4 (Spry2 and Spry4) and Dual specificity phosphatase 4 and 6 (Dusp4 and 6), are cyclically expressed. There are extensive cross-talks among these major oscillating signaling pathways. However, current studies suggest that none of the three signaling pathways individually acts as a global pacemaker. If there is no unidentified master pacemaker, it likely that each of the three pathways has the capacity to generate its own oscillations, while interactions among them allow efficient coupling and entrain them to each other.

The retinoic acid (RA) signaling controls somitogenesis by regulating the competence of PSM cells to undergo segmentation via antagonizing FGF signaling (Fig. 1.2*A*) [12]. RA signaling has an additional role in maintaining left–right bilateral symmetry of somites by buffering asymmetric signals that establish the left–right axis of the body, particularly Fgf8 [13].

The functional significance of the segmentation clock in human skeletal development is highlighted by congenital axial skeletal diseases. Abnormal vertebral segmentation (AVS) in humans is a relatively common malformation. For instance, mutations in NOTCH signaling components cause at least two human disorders, spondylocostal dysostosis (SCD, #277300, #608681, and #609813) and Alagille syndrome (AGS, OMIM #118450, and #610205), both of which exhibit vertebral column defects. However, the identified mutations explain only a minor fraction of congenital scoliosis cases. More work needs to be performed to elucidate the pathological mechanism underlying congenital and idiopathic scoliosis in human.

The formed somite is also patterned along the dorsal–ventral axis by cell signaling from the surface ectoderm, neural tube, and the notochord (Fig. 1.1). Ventralizing signals such as Sonic hedgehog (Shh) from the notochord and ventral neural tube is required to induce sclerotome formation on the ventral side [14,15], whereas Wnt signaling from the surface ectoderm and dorsal neural tube is required for the formation of dermomyotome on the dorsal side of the somite (Fig. 1.1) [16,17]. The sclerotome gives rise to the axial skeleton and the ribs. In the mouse mutant that lacks Shh function, the vertebral column and posterior ribs fail to form. The paired domain transcription factor Pax1 is expressed in the sclerotome and Shh is required to regulate its expression [18,19]. However, axial skeletal phenotypes in Pax1 mutant mice [20] were far less severe than those in the Shh mutants.

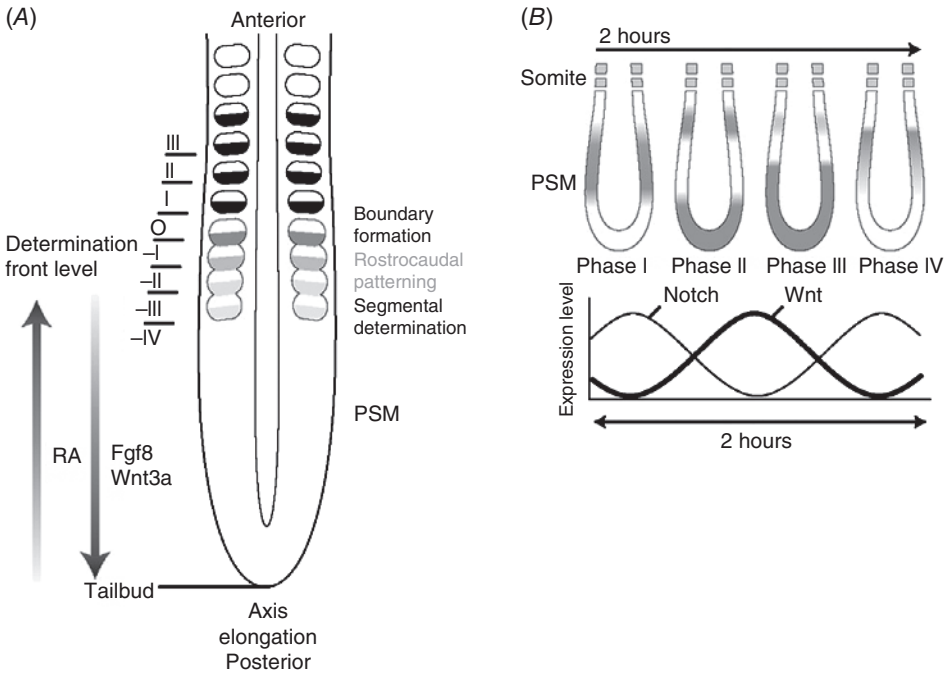

Fig. 1.2. Periodic and left–right symmetrical somite formation is controlled by signaling gradients and oscillations. (*A*) Somites form from the presomitic mesoderm (PSM) on either side of the neural tube in an anterior to posterior (A–P) wave. Each segment of the somite is also patterned along the A–P axis. Retinoic acid signaling controls the synchronization of somite formation on the left and right side of the neural tube. The most recent visible somite is marked by "0," whereas the region in the anterior PSM that is already determined to form somites is marked by a determination front that is determined by Fgf8 and Wnt3a gradients. This FGF signaling gradient is antagonized by an opposing gradient of retinoic acid. (*B*) Periodic somite formation (one pair of somite/2 hours) is controlled by a segmentation clock, the molecular nature of which is oscillated expression of signaling components in the Notch and Wnt pathway. Notch signaling oscillates out of phase with Wnt signaling.

Limb skeletons are patterned along the proximal–distal (P–D, shoulder to digit tip), anterior–posterior (A–P, thumb to little finger) and dorsal–ventral (D–V, back of the hand to palm) axes (Fig. 1.3) [21,22]. Along the P–D axis, the limb skeletons form three major segments: humerus or femur at the proximal end, radius and ulna or tibia and fibula in the middle and carpal/tarsal, metacarpal/metatarsal, and digits in the distal end. Along the A–P axis, the radius and ulna have distinct morphological features, as do each of the five digits. Patterning along the D–V limb axis also results in characteristic skeletal shapes and structures. For instance, the sesamoid processes are located ventrally whereas the knee patella forms on the dorsal side of the knee. The three-dimensional limb patterning events are regulated by three signaling centers in the early limb primodium, known as the limb bud, before mesenchymal condensation.

The apical ectoderm ridge (AER), a thickened epithelial structure formed at the distal tip of the limb bud, is the signaling center that directs P–D limb outgrowth (Fig. 1.3). Canonical Wnt signaling activated by Wnt3 induces AER formation, whereas BMP signaling leads to AER regression. FGF family members Fgf4, Fgf8, Fgf9, and Fgf17 are expressed specifically in the AER and Fgf8 alone is sufficient to mediate the function of AER. Fgf10, expressed in the presumptive limb mesoderm, is required for limb initiation and it also controls limb outgrowth by maintaining Fgf8 expression in the AER. It is interesting that exposure to the combined activities of distal signals (Wnt3a and Fgf8) and the proximal signal (RA) in the early limb bud or in culture maintains the potential to form both proximal and distal structures. As the limb bud grows, the proximal cells fall out of range of distal signals (Wnt3a and Fgf8) that act, in part, to keep the cells undifferentiated. Cells closer to the flank therefore differentiate and form proximal structures under the influence of proximal signals such as RA. The potential of distal mesenchymal cells becomes restricted over time to distal fates as they grow beyond the range of proximally produced RA [23,24]. Patterning of the limb bud progenitor cells into distinct segments along the P–D axis may also result in region-specific unique cellular properties such as cell sorting and aggregation behaviors that may direct their contribution toward specific skeletal elements such as the humerus or digits [25].

The second signaling center is the zone of polarizing activity (ZPA) which is a group of mesenchymal cells located at the posterior distal limb margin and immediately adjacent to the AER (Fig. 3.3*B*). When ZPA tissue is grafted to the anterior limb bud under the AER, it leads to digit duplication in mirror image of the endogenous ones [26]. Shh is expressed in the ZPA and is

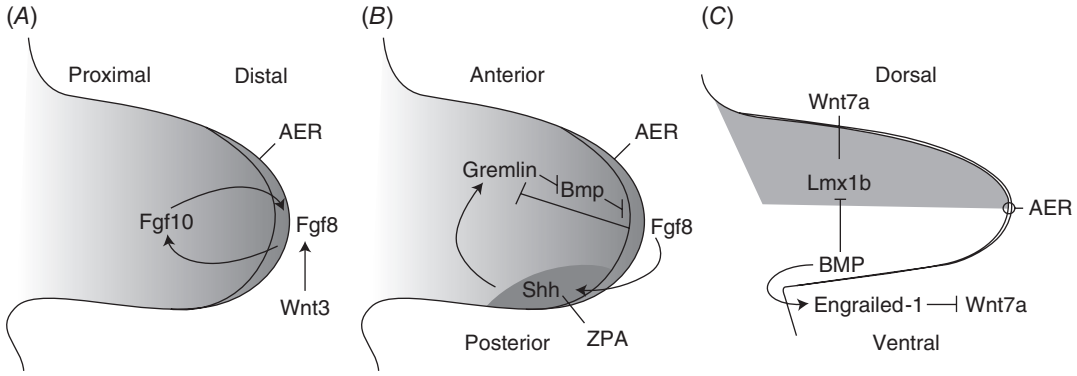

Fig. 1.3. Limb patterning and growth along the proximal–distal (P–D), anterior–posterior (A–P), and dorsal–ventral (D–V) axes are controlled by signaling interactions and feedback loops. (*A*) A signaling feedback loop between Fgf10 in the limb mesoderm and Fgf8 in the AER is required to direct P–D limb outgrowth. Wnt3 is required for AER formation. (*B*) Shh in the ZPA controls A–P limb patterning. A–P and P–D limb patterning and growth are also coordinated through a feedback loop between Shh and FGFs expressed in the AER. FGF signaling from the AER is required for Shh expression. Shh also maintains AER integrity by regulating Gremlin expression. Gremlin is a secreted antagonist of BMP signaling which promotes AER degeneration. The inhibitory feedback loop between Gremlin in the limb mesenchyme and FGFs in the AER is critical in terminating limb bud outgrowth. (*C*) D–V patterning of the limb is determined by Wnt7a and BMP signaling through regulating the expression of Lmx1b in the limb mesenchyme.

both necessary and sufficient to mediate ZPA activity in patterning digit identity along the A–P axis [27]. However, the A–P axis of the limb is established before Shh signaling. This pre-Shh A–P limb patterning is controlled by combined activities of Gli3, Alx4, and basic helix-loop-helix (bHLH) transcription factors dHand and Twist1. The Gli3 repressor form (Gli3R) and Alx4 establish the anterior limb territory by restricting dHand expression to the posterior limb, which in turn activates Shh expression [28,29]. The activity of dHand in the posterior limb is also antagonized by Twist1 via a dHand-Twist1 heterodimer. Recently, the zinc finger factors Sall4 and Gli3 have been found to cooperate for proper development of the A–P skeletal elements and also function upstream of Shh-dependent posterior skeletal element development [30].

Mutations in the human *TWIST1* gene cause Saethre–Chotzen syndrome (SCS, OMIM #101400), one of the most commonly inherited craniosynostosis conditions. The hallmarks of this syndrome are premature fusion of the calvarial bones and limb abnormalities. Mutations in the *GLI3* gene also cause limb malformations including Greig cephalopolysyndactyly syndrome (GCPS, OMIM #175700) and Pallister–Hall syndrome (PHS, OMIM #146510).

The third signaling center is the non-AER limb ectoderm that covers the limb bud. It sets up the D–V polarity of not only the ectoderm but also the underlying mesoderm (Fig. 1.3*C*) (review by [21,31]). Wnt and BMP signaling are required to control D–V limb polarity. *Wnt7a* is expressed specifically in the dorsal limb ectoderm and it activates the expression of *Lmx1b*, which encodes a dorsal-specific LIM homeobox transcription factor that determines the dorsal identity. *Wnt7a* expression in the ventral ectoderm is suppressed by En-1, which encodes a transcription factor that is expressed specifically in the ventral ectoderm. The BMP signaling pathway is also ventralizing in the early limb (Fig. 1.3*C*). It appears that the effects of BMP signaling are mediated by Msx1 and Msx2, two transcription factors that are also transcriptionally regulated by BMP signaling. The function of BMP signaling in the early limb ectoderm is upstream of En-1 in controlling D–V limb polarity [32]. However, when BMPRIA is specifically inactivated only in the mouse limb bud mesoderm, the distal limb is dorsalized without altering the expression of Wnt7a and En-1 in the limb ectoderm [33]. Thus, BMPs also have En-1-independent ventralization activity by directly signaling to the limb mesenchyme to inhibit *Lmx1b* expression.

Limb development is a coordinated three-dimensional event. Indeed, the three signaling centers interact with each other through interactions of the mediating signaling molecules. First, there is a positive feedback loop between Shh and FGFs expressed in the AER, which connects A–P limb patterning with P–D limb outgrowth (Fig. 1.3*B*) [21,22]. This positive feedback loop is antagonized by an FGF/Grem1 inhibitory loop that attenuates strong FGF signaling and terminates limb outgrowth signals in order to maintain a proper limb size [34]. Second, the dorsalizing signal Wnt7a is required for maintaining the expression of Shh that patterns the A–P axis [35,36]. Third, Wnt/β-catenin signaling has been found to be both distalizing and dorsalizing [37–39].

Identification of these interacting signaling networks in early limb patterning has provided a fertile ground to test the self-organizing Turing models [1] that simulate the pattern of digit formation in the limb. By combining experiments and modeling, a self-organizing Turing network implemented by BMP, Sox9, and Wnt has been found to drive digit specification. When modulated by morphogen gradients, the network is able to recapitulate

the expression patterns of Sox9 in the wild type and in perturbation experiments [2]. Interestingly, the Turing model is also found to explain the dose effects of distal *Hox* genes in modulating the digit period or wavelength [3]. Progressive reduction in *Hoxa13* and *Hoxd11-Hoxd13* genes from the Gli3-null background results in progressively more severe polydactyly, displaying thinner and densely packed digits.

Recently, the generality and contribution of this Turing network implemented by BMP, Sox9, and Wnt to the morphological diversity of fins and limbs has been further explored [5]. Is has been suggested that the skeletal patterning of the catshark *Scyliorhinus canicula* pectoral fin is likely driven by a deeply conserved BMP–Sox9–Wnt Turing network. Therefore, the union of theory and experimentation is a powerful approach to not only identify and validate the minimal components of a network regulating digit pattern, but also to ask a new set of questions that will undoubtedly be answered as a result of the continued merging of disciplines.

EMBRYONIC CARTILAGE AND BONE FORMATION

The early patterning events determine where and when the mesenchymal cells condense, though the mechanism remains to be elucidated. Subsequently, osteochondral progenitors in the condensation form either chondrocytes or osteoblasts. Sox9 and Runx2, master transcription factors that are required for the determination of chondrocyte and osteoblast cell fates respectively [40,41], are both expressed in osteochondral progenitor cells, but Sox9 expression precedes that of Runx2 in the mesenchymal condensation in the limb [42]. Early Sox9-expressing cells give rise to both chondrocytes and osteoblasts regardless of ossification mechanisms [43]. Loss of Sox9 function in the limb leads to loss of mesenchymal condensation and Runx2 expression [42]. Coexpression of Sox9 and Runx2 is terminated upon chondrocyte and osteoblast differentiation when Sox9 and Runx2 expression is quickly segregated into chondrocytes and osteoblasts respectively. The mechanism controlling lineage-specific Sox9 and Runx2 expression is fundamental to the regulation of chondrocyte and osteoblast differentiation and the determination of ossification mechanisms. It is clear that cell–cell signaling, particularly those mediated by Wnts and Indian hedgehog (Ihh), are required for cell fate determination of chondrocytes and osteoblasts by controlling the expression of Sox9 and Runx2.

Active Wnt/β-catenin signaling is detected in the developing calvarium and perichondrium where osteoblasts differentiate through either intramembranous or endochondral ossification. Indeed, enhanced Wnt/β-catenin signaling enhanced bone formation and Runx2 expression, but inhibited chondrocyte differentiation and Sox9 expression [44–46]. Conversely, removal of β-catenin in osteochondral progenitor cells resulted in ectopic chondrocyte differentiation at the expense of osteolasts during both intramembranous and endochondral ossification [46–48]. Therefore, during intramembranous ossification, Wnt/β-catenin signaling levels in the condensation are higher, which promotes osteoblast differentiation while inhibiting chondrocyte differentiation. During endochondral ossification, however, Wnt/β-catenin signaling in the condensation is initially lower, such that only chondrocytes can differentiate. Later, when Wnt/β-catenin signaling is upregulated in the periphery of the cartilage, osteoblasts will differentiate. It is likely that by manipulating Wnt signaling, mesenchymal progenitor cells, and perhaps even mesenchymal stem cells, can be directed to form only chondrocytes, which are needed to repair cartilage damage in osteoarthritis, or only form osteoblasts, which will lead to new therapeutic strategies to treat osteoporosis. These studies have provided new insights into tissue engineering that aims to fabricate cartilage or bone in vitro using mesenchymal progenitor cells or stem cells.

Ihh signaling is required for osteoblast differentiation by activating Runx2 expression only during endochondral bone formation [49]. Ihh is expressed in newly differentiated chondrocytes and Ihh signaling does not seem to affect chondrocyte differentiation from mesenchymal progenitors. However, when Hh signaling is inactivated in the perichondrium cells, they ectopically form chondrocytes that express Sox9 at the expense of Runx2. This is similar to what has been observed in the Osterix (Osx) mutant embryos, except that in the $Osx^{-/-}$ embryos, ectopic chondrocytes express both Sox9 and Runx2 [50], suggesting that Runx2 is not sufficient to inhibit Sox9 expression and chondrocyte differentiation. It is still not clear what controls Ihh-independent Runx2 expression during intramembranous ossification. One likely scenario is that the function of Ihh is compensated by Shh in the developing calvarium or Hh signaling is activated in a ligand-independent manner in the developing calvarium. Indeed, it has been recently found that in the rare human genetic disease progressive osseous heteroplasia (POH), which is caused by null mutations in GNAS that encodes Gαs, Hedgehog signaling is upregulated. Such activation of Hh signaling is independent of Hh ligands and is both necessary and sufficient to induce ectopic osteoblast cell differentiation in soft tissues [51]. Importantly, GNAS gain-of-function mutations upregulate Wnt/β-catenin signaling in osteoblast progenitor cells, resulting in their defective differentiation and fibrous dysplasia [52]. Therefore, studies of human genetic diseases identify Gαs as a key regulator of proper osteoblast differentiation through its maintenance of a balance between the Wnt/β-catenin and Hedgehog pathways.

Both Wnt/β-catenin and Ihh signaling pathways are required for endochondral bone formation. To understand which one acts first, a genetic epistatic test was carried out [53]. These studies found that β-catenin is required downstream of not just Ihh, but also Osx in promoting osteoblast maturation. By contrast, Ihh signaling is not required after Osx expression for osteoblast differentiation [54]. The sequential actions of Hh and Wnt signaling in

osteoblast differentiation and maturation suggest that Hh and Wnt signaling need to be manipulated at distinct stages during fracture repair and tissue engineering.

BMPs are the transforming growth factor (TGF) superfamily members that were identified as secreted proteins able to promote ectopic cartilage and bone formation [55]. Unlike Ihh and Wnt signaling, BMP signaling promotes the differentiation of both osteoblast and chondrocyte differentiation from mesenchymal progenitors. The mechanisms underlying these unique activities of BMPs have been under intense investigation for the past two decades. During this time, our understanding of BMP action in chondrogenesis and osteogenesis has benefited greatly from molecular studies of BMP signal transduction [56]. Reducing BMP signaling by removing BMP receptors leads to impaired chondrocyte and osteoblast differentiation and maturation [57].

FGF ligands and FGF receptors (FGFR) are both expressed in the developing skeletal system. The significant role of FGF signaling in skeletal development was first identified by the discovery that achondroplasia (ACH, OMIM #100800), the most common form of skeletal dwarfism in humans, was caused by a missense mutation in FGFR3. Later, hypochondroplasia (HCH, OMIM #146000), a milder form of dwarfism and thanatophoric dysplasia (TD, OMIM #187600, and 187601), a more severe form of dwarfism, were also found to result from mutations in FGFR3. FGFR3 signaling acts to regulate the proliferation and hypertrophy of the differentiated chondrocytes. However, the function of FGF signaling in mesenchymal condensation and chondrocyte differentiation from progenitors remains to be elucidated as complete genetic inactivation of FGF signaling in mesenchymal condensation has not been achieved. Nevertheless, it is clear that FGF signaling acts in mesenchymal condensation to control osteoblast differentiation during intramembranous bone formation. Mutations in FGFR 1, 2 and 3 cause craniosynostosis (premature fusion of the cranial sutures). The craniosynostosis syndromes involving FGFR 1, 2, 3 mutations include Apert syndrome (AS, OMIM #101200), Beare-Stevenson cutis gyrata (OMIM #123790), Crouzon syndrome (CS, OMIM #123500), Pfeiffer syndrome (PS, OMIM #101600), Jackson-Weiss syndrome (JWS, OMIM #123150), Muenke syndrome (MS, OMIM #602849), crouzonodermoskeletal syndrome (OMIM #134934) and osteoglophonic dysplasia (OGD, OMIM #166250), a disease characterized by craniosynostosis, a prominent supraorbital ridge, and a depressed nasal bridge, as well as rhizomelic dwarfism and nonossifying bone lesions. All these mutations are autosomal dominant and many of them are activating mutations of FGF receptors. FGF signaling can promote or inhibit osteoblast proliferation and differentiation depending on the cell context. It does so either directly or through interaction with the Wnt and BMP signaling pathways.

Apart from having the right types of cells and proper size, cartilage and bone also have distinct morphologies which are required for their function. For example, the limb and long bones preferentially elongate along the P–D axis. It is well understood that Wnts can act as morphogens by forming gradients that specify distinct cell types in distinct spatial orders by inducing the expression of different target genes at threshold concentrations. In this regard, morphogen gradients provide quantitative information to generate a distinct pattern by coordinating cell proliferation and differentiation. Because the limbs are elongated organs instead of a three-dimensionally symmetrical ball, directional information has to be provided during limb and long bone elongation.

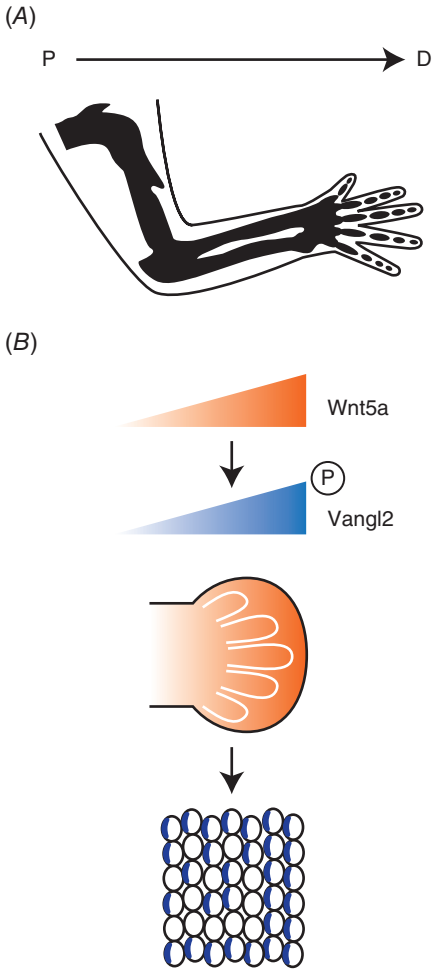

Fig. 1.4. Wnt5a gradient controls directional morphogenesis by regulating Vangl2 phosphorylation and asymmetrical localization. (*A*) Schematics of skeletons in a human limb that preferentially elongates along the proximal–distal (P–D) axis. (*B*) A model of a Wnt5a gradient controlling P–D limb elongation by providing a global directional cue. Wnt5a is expressed in a gradient (orange) in the developing limb bud and this Wnt5a gradient is translated into an activity gradient of Vangl2 by inducing different levels of Vangl2 phosphorylation (blue). In the distal limb bud of an E12.5 mouse embryo showing the forming digit cartilage, the Vangl2 activity gradient then induces asymmetrical Vangl2 localization (blue) and downstream polarized events.

Although the molecular mechanism underlying such directional morphogenesis was poorly understood in the past, there is evidence that alignment of the columnary chondrocytes of the growth plate might be regulated by planar cell polarity (PCP) during directional elongation of the formed cartilage [58]. PCP is an evolutionarily conserved pathway that is required in many directional morphogenetic processes including left–right asymmetry, neural tube closure, body axis elongation and brain wiring [59]. Recently, a major breakthrough has been made by demonstrating that newly differentiated chondrocytes in the developing long bones in the limb are polarized along the P–D axis. For the first time it was found with a definitive molecular marker, Vangl2 protein, a core regulatory component in the PCP pathway. Vangl2 protein is asymmetrically localized on the proximal side of the Sox9 positive chondrocytes, not in Sox9 negative interdigital mesenchymal cells [60]. Importantly, Vangl2 protein asymmetrical localization requires a Wnt5a signaling gradient. In the $Wnt5a^{-/-}$ mutant limb, the cartilage forms a ball-like structure and Vangl2 is symmetrically distributed on the cell membrane (Fig. 1.4). PCP mutations in the *WNT5a* and *ROR2* genes have been found in skeletal malformations such as the Robinow syndrome and brachydactyly type B1, which both exhibit short-limb dwarfisms [61–65]. In addition, mutations in PCP signaling components such as VANGL1 has been found in adolescent idiopathic scoliosis (AIS).

CONCLUSION

Skeletal formation is a process that has been perfected by nature in embryos during vertebrate evolution. Understanding the underlying molecular mechanisms of cartilage and bone formation in embryonic development will advance our knowledge of vertebrate embryonic morphogenesis in general. This knowledge will allow us to develop the strategy to promote skeletal tissue repair by endogenous cells or rejuvenate old skeletal tissues without having to use cells cultured in vitro. In addition, to use autologous cells and tissues or iPS (induced pluripotent stem) cells to repair bone and cartilage damaged during injury and disease, we require a more complete knowledge of skeletal development so that cartilage or bone can be fabricated using the body's own cells. Understanding skeletal development is indispensable for understanding pathological mechanisms of skeletal diseases, finding therapeutic targets, promoting consistent cartilage or bone repair in vivo, and eventually growing functional cartilage or bone in vitro.

ACKNOWLEDGMENT

I apologize to those authors whose work could not be cited directly because of space restrictions.

REFERENCES

1. Turing AM. The chemical basis of morphogenesis. Phil Trans R Soc Lond B. 1952;237(641):37–72.
2. Raspopovic J, Marcon L, Russo L, et al. Modeling digits. Digit patterning is controlled by a Bmp-Sox9-Wnt Turing network modulated by morphogen gradients. Science. 2014;345(6196):566–70.
3. Sheth R, Marcon L, Bastida MF, et al. Hox genes regulate digit patterning by controlling the wavelength of a Turing-type mechanism. Science. 2012;338(6113):1476–80.
4. Marcon L, Diego X, Sharpe J, et al. High-throughput mathematical analysis identifies Turing networks for patterning with equally diffusing signals. Elife. 2016;5.
5. Onimaru K, Marcon L, Musy M, et al. The fin-to-limb transition as the re-organization of a Turing pattern. Nature Comm. 2016;7:11582.
6. Santagati F, Rijli FM. Cranial neural crest and the building of the vertebrate head. Nat Rev Neurosci. 2003;4(10):806–18.
7. Helms JA, Cordero D, Tapadia MD. New insights into craniofacial morphogenesis. Development. 2005;132(5):851–61.
8. Hubaud A, Pourquie O. Signalling dynamics in vertebrate segmentation. Nat Rev Mol Cell Biol. 2014;15(11):709–21.
9. Bothe I, Ahmed MU, Winterbottom FL, et al. Extrinsic versus intrinsic cues in avian paraxial mesoderm patterning and differentiation. Dev Dyn. 2007;236(9):2397–409.
10. Pourquie O. Vertebrate segmentation: from cyclic gene networks to scoliosis. Cell. 2011;145(5):650–63.
11. Ilagan MX, Kopan R. SnapShot: notch signaling pathway. Cell. 2007;128(6):1246.
12. Moreno TA, Kintner C. Regulation of segmental patterning by retinoic acid signaling during Xenopus somitogenesis. Dev Cell. 2004;6(2):205–18.
13. Duester G. Retinoic acid regulation of the somitogenesis clock. Birth Defects Res C Embryo Today. 2007;81(2):84–92.
14. Fan CM, Tessier-Lavigne M. Patterning of mammalian somites by surface ectoderm and notochord: evidence for sclerotome induction by a hedgehog homolog. Cell. 1994;79(7):1175–86.
15. Johnson RL, Laufer E, Riddle RD, et al. Ectopic expression of Sonic hedgehog alters dorsal-ventral patterning of somites. Cell. 1994;79(7):1165–73.
16. Fan CM, Lee CS, Tessier-Lavigne M. A role for WNT proteins in induction of dermomyotome. Dev Biol. 1997;191(1):160–5.
17. Capdevila J, Johnson RL. Endogenous and ectopic expression of noggin suggests a conserved mechanism for regulation of BMP function during limb and somite patterning. Dev Biol. 1998;197(2):205–17.
18. Koseki H, Wallin J, Wilting J, et al. A role for Pax-1 as a mediator of notochordal signals during the dorsoventral specification of vertebrae. Development. 1993;119(3):649–60.
19. Chiang C, Litingtung Y, Lee E, et al. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature. 1996;383(6599):407–13.

20. Wallin J, Wilting J, Koseki H, et al. The role of Pax-1 in axial skeleton development. Development. 1994;120(5):1109–21.
21. Niswander L. Pattern formation: old models out on a limb. Nat Rev Genet. 2003;4(2):133–43.
22. Zeller R, Lopez-Rios J, Zuniga A. Vertebrate limb bud development: moving towards integrative analysis of organogenesis. Nat Rev Genet. 2009;10(12):845–58.
23. Cooper MK, Porter JA, Young KE, et al. Teratogen-mediated inhibition of target tissue response to Shh signaling. Science. 1998;280(5369):1603–7.
24. Rosello-Diez A, Ros MA, Torres M. Diffusible signals, not autonomous mechanisms, determine the main proximo-distal limb subdivision. Science. 2011;332(6033):1086–8.
25. Barna M, Niswander L. Visualization of cartilage formation: insight into cellular properties of skeletal progenitors and chondrodysplasia syndromes. Dev Cell. 2007;12(6):931–41.
26. Saunders JWJ, Gasseling MT. Ectoderm-mesenchymal interaction in the origin of wing symmetry. In: Fleischmajer R, Billingham RE (eds) *Epithelia-Mesenchymal Interactions*. Baltimore: Williams and Wilkins, 1968, pp. 78–97.
27. Riddle RD, Johnson RL, Laufer E, et al. Sonic hedgehog mediates the polarizing activity of the ZPA. Cell. 1993;75(7):1401–16.
28. Charite J, McFadden DG, Olson EN. The bHLH transcription factor dHAND controls Sonic hedgehog expression and establishment of the zone of polarizing activity during limb development. Development. 2000;127(11):2461–70.
29. Fernandez-Teran M, Piedra ME, Kathiriya IS, et al. Role of dHAND in the anterior-posterior polarization of the limb bud: implications for the Sonic hedgehog pathway. Development. 2000;127(10):2133–42.
30. Akiyama R, Kawakami H, Wong J, et al. Sall4-Gli3 system in early limb progenitors is essential for the development of limb skeletal elements. Proc Natl Acad Sci U S A. 2015;112(16):5075–80.
31. Tickle C. Patterning systems – from one end of the limb to the other. Dev Cell. 2003;4(4):449–58.
32. Lallemand Y, Nicola MA, Ramos C, et al. Analysis of Msx1; Msx2 double mutants reveals multiple roles for Msx genes in limb development. Development. 2005;132(13):3003–14.
33. Ovchinnikov DA, Selever J, Wang Y, et al. BMP receptor type IA in limb bud mesenchyme regulates distal outgrowth and patterning. Dev Biol. 2006;295(1):103–15.
34. Verheyden JM, Sun X. An Fgf/Gremlin inhibitory feedback loop triggers termination of limb bud outgrowth. Nature. 2008;454(7204):638–41.
35. Parr BA, McMahon AP. Dorsalizing signal Wnt-7a required for normal polarity of D-V and A-P axes of mouse limb. Nature. 1995;374(6520):350–3.
36. Yang Y, Niswander L. Interaction between the signaling molecules WNT7a and SHH during vertebrate limb development: dorsal signals regulate anteroposterior patterning. Cell. 1995;80(6):939–47.
37. Ten Berge D, Brugmann SA, Helms JA, et al. Wnt and FGF signals interact to coordinate growth with cell fate specification during limb development. Development. 2008;135(19):3247–57.
38. Hill TP, Taketo MM, Birchmeier W, et al. Multiple roles of mesenchymal beta-catenin during murine limb patterning. Development. 2006;133(7):1219–29.
39. Cooper KL, Hu JK, ten Berge D, et al. Initiation of proximal-distal patterning in the vertebrate limb by signals and growth. Science. 2011;332(6033):1083–6.
40. Zelzer E, Olsen BR. The genetic basis for skeletal diseases. Nature. 2003;423(6937):343–8.
41. Nakashima K, de Crombrugghe B. Transcriptional mechanisms in osteoblast differentiation and bone formation. Trends Genet. 2003;19(8):458–66.
42. Akiyama H, Chaboissier MC, Martin JF, et al. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev. 2002;16(21):2813–28.
43. Akiyama H, Kim JE, Nakashima K, et al. Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors. Proc Natl Acad Sci U S A. 2005;102(41):14665–70.
44. Hartmann C, Tabin CJ. Dual roles of Wnt signaling during chondrogenesis in the chicken limb. Development. 2000;127(14):3141–59.
45. Guo X, Day TF, Jiang X, et al. Wnt/beta-catenin signaling is sufficient and necessary for synovial joint formation. Genes Dev. 2004;18(19):2404–17.
46. Day TF, Guo X, Garrett-Beal L, et al. Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev Cell. 2005;8(5):739–50.
47. Hill TP, Spater D, Taketo MM, et al. Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes. Dev Cell. 2005;8(5):727–38.
48. Hu H, Hilton MJ, Tu X, et al. Sequential roles of Hedgehog and Wnt signaling in osteoblast development. Development. 2005;132(1):49–60.
49. St-Jacques B, Hammerschmidt M, McMahon AP. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes Dev. 1999;13(16):2072–86.
50. Nakashima K, Zhou X, Kunkel G, et al. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell. 2002;108(1):17–29.
51. Regard JB, Malhotra D, Gvozdenovic-Jeremic J, et al. Activation of Hedgehog signaling by loss of GNAS causes heterotopic ossification. Nat Med. 2013;19(11):1505–12.
52. Regard JB, Cherman N, Palmer D, et al. Wnt/beta-catenin signaling is differentially regulated by Galpha proteins and contributes to fibrous dysplasia. Proc Natl Acad Sci U S A. 2011;108(50):20101–6.
53. Mak KK, Chen MH, Day TF, et al. Wnt/beta-catenin signaling interacts differentially with Ihh signaling in controlling endochondral bone and synovial joint formation. Development. 2006;133(18):3695–707.

54. Rodda SJ, McMahon AP. Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. Development. 2006;133(16):3231–44.
55. Wozney JM. Bone morphogenetic proteins. Prog Growth Factor Res. 1989;1(4):267–80.
56. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature. 2003;425(6958):577–84.
57. Yoon BS, Ovchinnikov DA, Yoshii I, et al. Bmpr1a and Bmpr1b have overlapping functions and are essential for chondrogenesis in vivo. Proc Natl Acad Sci U S A. 2005;102(14):5062–7.
58. Li Y, Dudley AT. Noncanonical frizzled signaling regulates cell polarity of growth plate chondrocytes. Development. 2009;136(7):1083–92.
59. Yang Y, Mlodzik M. Wnt-Frizzled/planar cell polarity signaling: cellular orientation by facing the wind (Wnt). Annu Rev Cell Dev Biol. 2015;31:623–46.
60. Gao B, Song H, Bishop K, et al. Wnt signaling gradients establish planar cell polarity by inducing Vangl2 phosphorylation through Ror2. Dev Cell. 2011;20(2):163–76.
61. Minami Y, Oishi I, Endo M, et al. Ror-family receptor tyrosine kinases in noncanonical Wnt signaling: their implications in developmental morphogenesis and human diseases. Dev Dyn.239(1):1–15.
62. Person AD, Beiraghi S, Sieben CM, et al. WNT5A mutations in patients with autosomal dominant Robinow syndrome. Dev Dyn. 2010;239(1):327–37.
63. van Bokhoven H, Celli J, Kayserili H, et al. Mutation of the gene encoding the ROR2 tyrosine kinase causes autosomal recessive Robinow syndrome. Nat Genet. 2000;25(4):423–6.
64. Schwabe GC, Tinschert S, Buschow C, et al. Distinct mutations in the receptor tyrosine kinase gene ROR2 cause brachydactyly type B. Am J Hum Genet. 2000;67(4):822–31.
65. DeChiara TM, Kimble RB, Poueymirou WT, et al. Ror2, encoding a receptor-like tyrosine kinase, is required for cartilage and growth plate development. Nat Genet. 2000;24(3):271–4.