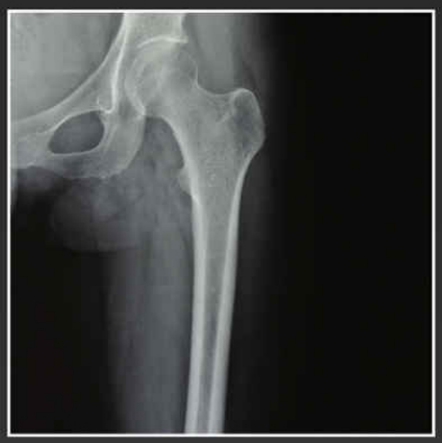
Principles of Bone Biology

FOURTH EDITION



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Principles of Bone Biology

Fourth Edition

Volume 1

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Volume 2

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Dedication of Fourth Edition to Lawrence G. Raisz

By the end of the 1970s, when the bone research community felt that it was ready for its own scientific society, Larry Raisz was one of the leaders of the group that founded the American Society for Bone and Mineral Research (ASBMR). The ASBMR had its first annual conference in 1979, with Larry serving as its second president. As the first editor of the *Journal of Bone and Mineral Research*, for a decade, Larry set the highest scientific standards for quality and integrity. That standard remains untarnished today.

Larry's knowledge of the facts in our field was prodigious. His expertise and experience in basic elements of bone biology were exceptional. He had great understanding and wisdom in interpretation of the clinical implications of basic bone biology. But he always wanted to know more. At ASBMR and other annual meetings, it was always Larry who rose to the microphone after a presentation to ask, not only the first question, but typically the best one! Remarkably, Larry could translate basic bone biology to the clinical arena. Few in our field then or now could so smoothly integrate clinical aspects of metabolic bone diseases with the burgeoning knowledge of underlying pathophysiological mechanisms. Adding to these talents was a collegiality and an exuberant enthusiasm that pervaded all venues of Larry Raisz's world. As osteoporosis became more widely recognized to be a medical scourge, then and now, Larry quickly grasped the need to speak about the burden of the disease and contributed to the international dialogue, raising awareness among us all. This awareness was a major factor in the recognition among countries that we are dealing with a disease that needs greater understanding at all levels. And, indeed, at all levels, Larry contributed so much.

These qualities made Larry Raisz a wonderfully effective coeditor of the first three editions of *Principles of Bone Biology*. Much more than that, though, he was a pleasure to work with as a colleague and friend, exceptionally efficient and with unfailing humor and optimism when faced with any adversity. Larry would share the highs and lows with you, but the lows were rare and short lived.

We remember him constantly and dedicate to Lawrence G. Raisz, MD, this fourth edition of what he called "Big Gray."

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Preface to the Fourth Edition

The first edition of *Principles of Bone Biology* was published about 24 years ago, in 1996. Our field was ready then for a compendium of the latest concepts in bone biology. Now, several decades and two editions later, we are pleased to welcome you to the fourth edition of "Big Gray." Since the third edition was published in 2008, our field has continued to undergo sea changes of knowledge and insights. As a result of these advances since then, all chapters have undergone major revisions. In addition, areas not previously covered in depth are featured, such as vascular and nerve interactions with bone, interorgan communicants of bone, hematopoietic−bone cell interactions, nonskeletal aspects of vitamin D and RANK ligand, newly recognized signaling molecules and systems, and advances in methodological aspects of skeletal research. Illustrative of the vibrancy of our field, over 50% of the authors in this edition are new to it. Our returning and new authors are the very best.

We remember Larry Raisz. He, along with Gideon Rodan, constituted the triumvirate of coeditors for the first and second editions. We dedicated the third edition to the memory of Gideon. We dedicate *Principles of Bone Biology*, fourth edition, to the memory of Larry. In these front pages, we reprint our dedication to Gideon and remember Larry with a separate dedication for this edition. We miss them both very much.

We want to acknowledge Jasna Markovac, who has served as our liaison to our authors and our publisher. Given the nature of the times, this book would not have been completed without her dedication, perseverance, and single-minded purpose not to let anything disrupt our publishing goals. She accomplished this feat with an even handedness and a professionalism that was remarkable and remarkably effective. We are grateful to you, Jasna.

Finally, we are grateful to our authors, who have made this book what it is, namely a repository of knowledge and concepts in bone biology and a resource for us all in the years to come.

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Chapter 1

Molecular and cellular regulation of intramembranous and endochondral bone formation during embryogenesis

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Chapter outline

Introduction

The skeletal system performs vital functions: support, movement, protection, blood cell production, calcium storage, and endocrine regulation. Skeletal formation is also a hallmark that distinguishes vertebrate animals from invertebrates. In higher vertebrates (i.e., birds and mammals), the skeletal system contains mainly bones and cartilage, as well as a network of tendons and ligaments that connects them. During embryonic development, bones and cartilage are formed by osteoblasts and chondrocytes, respectively, both of which are derived from common mesenchymal progenitor cells called osteochondral progenitors. Skeletal development starts from mesenchymal condensation, during which mesenchymal progenitor cells aggregate at future skeletal locations. As 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 of the axial skeleton, and lateral plate mesoderm forms the limb mesenchyme, from which limb skeletons are derived.

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How osteoblast cells are induced during bone development is a central question for understanding the organizational principles underpinning a functional skeletal system. Abnormal osteoblast differentiation leads to a broad range of devastating skeletal diseases. Therefore, it is imperative to understand the cellular and molecular mechanisms underlying temporal and spatial controls of bone formation. Bone formation occurs by two essential processes: intramembranous ossification and endochondral ossification during embryonic development. Osteochondral progenitors differentiate into osteoblasts directly to form the membranous bone during intramembranous ossification, whereas during endochondral ossification, they differentiate into chondrocytes instead to form a cartilage template of the future bone. Both ossification processes are essential during the natural healing of bone fractures. In this chapter, we focus on current understanding of the molecular regulation of endochondral and intramembranous bone formation and its implication in diseases.

Intramembranous ossification

Intramembranous ossification mainly occurs during formation of the flat bones of the skull, mandible, maxilla, and clavicles. The mammalian cranium, or neurocranium, is the upper and back part of the skull. It protects the brain and supports the sensory organs, such as the ear, and the viscerocranium, which supports the face. The neurocranium can be divided into calvarium and chondrocranium, which grow to be the cranial vault that surrounds the brain and the skull base, respectively. The calvarium is composed of flat bones: frontal bones, parietal bones, the interparietal part of the occipital bone, and the squamous parts of the temporal bone (Jin et al., 2016). In mice, the calvarium consists of frontal bones, parietal bones, interparietal bone, and squamous parts of the temporal bone, all going through intramembranous ossification (Ishii et al., 2015). By lineage analysis in mouse models, frontal bones show a major contribution from neural crest and a small contribution from head mesoderm, while parietal bones entirely originate from head mesoderm (Jiang et al., 2002; Yoshida et al., 2008; Deckelbaum et al., 2012). Neural crest–derived and head mesoderm–derived cells coalesce to form calvarial bone primordia (Jiang et al., 2002; Yoshida et al., 2008). The mandible and maxilla are derived from the neural crest cells originating in the mid- and hindbrain regions of the neural folds that migrate ventrally, while the clavicles are formed from mesoderm.

The process starts from mesenchymal condensation and progresses through formation of the ossification center, ossification expansion, trabecula formation, and compact bone formation and the development of the periosteum (Fig. 1.1).

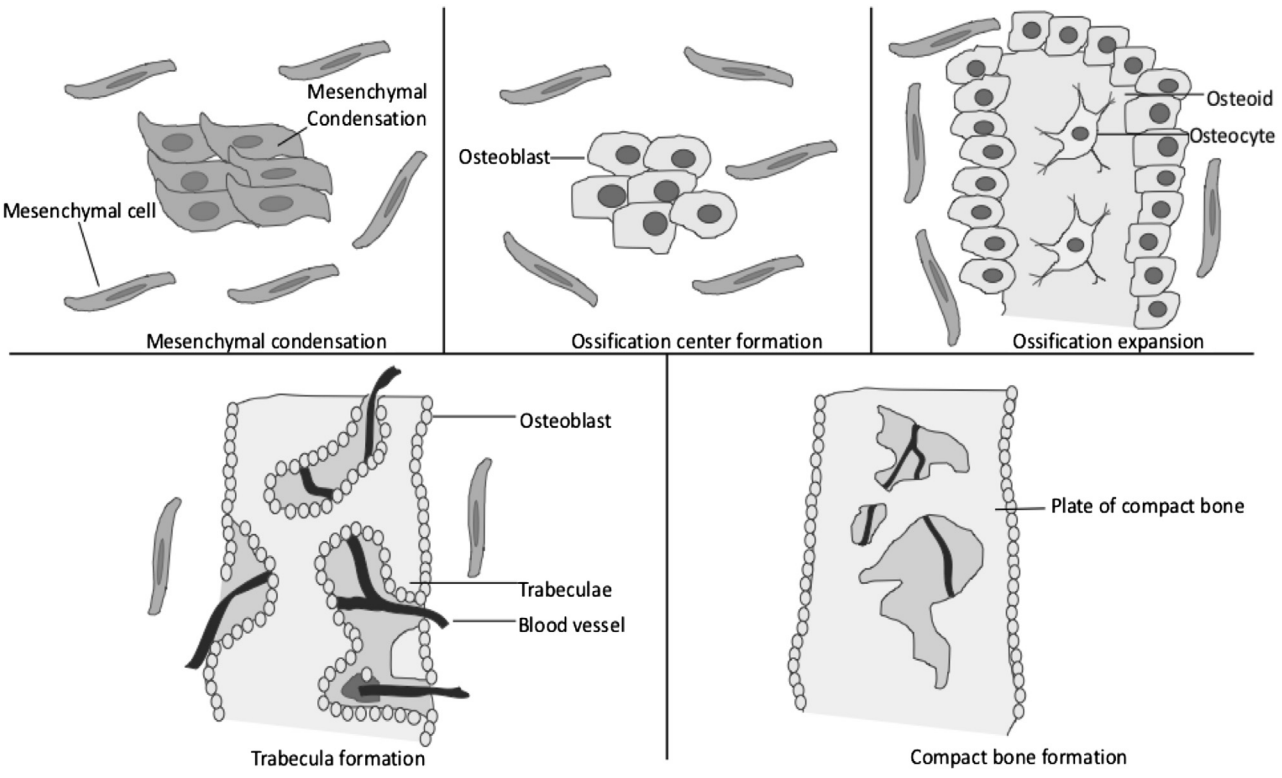

FIGURE 1.1 Schematics of intramembranous cranial bone formation. See text for details.

Condensation of mesenchymal progenitor cells is the first step for both intramembranous and endochondral ossification. During intramembranous ossification, mesenchymal progenitor cells differentiate into osteoblasts instead of chondrocytes as occurs during endochondral ossification. The osteoblasts that appear first in the condensation secrete bone matrix and form the ossification center. The early osteoblasts secrete osteoid, uncalcified matrix, which calcifies soon after, while the osteoblasts mature and terminally differentiate into osteocytes that are entrapped in the osteoid. As osteoblasts differentiate into osteocytes, more mesenchymal progenitors surrounding the osteoid differentiate into new osteoblast cells at the osteoid surface to expand the calcification center. Osteoid expansion around the capillaries results in a trabecular matrix of the spongy bone, while osteoblasts on the superficial layer become the periosteum. The periosteum is a layer that also contains mesenchymal progenitor cells, osteoblast differentiation of which contributes to the formation of a protective layer of compact bone. The blood vessels along with other cells between the trabecular bone eventually form the red marrow. Intramembranous ossification begins in utero during fetal development and continues on into adolescence. At birth, the skull and clavicles are not fully ossified. Sutures and fontanelles are unossified cranial regions that allow the skull to deform during passage through the birth canal. Sutures are joints between craniofacial bones, which are composed of two osteogenic fronts with suture mesenchyme between them (Fig. 1.2). Fontanelles are the space between the skull bones where the sutures intersect and are covered by tough membranes that protect the underlying soft tissues and brain. In humans, cranial sutures normally fuse between 20 and 30 years of age and facial sutures fuse after 50 years of age (Badve et al., 2013; Senarath-Yapa et al., 2012). Most sutures in mice remain patent throughout the animal's lifetime. Sutures and fontanelles allow the craniofacial bones to expand evenly as the brain grows, resulting in a symmetrically shaped head. However, if any of the sutures close too early (fuse prematurely), in the condition called craniosynostosis, there may be no growth in that area. This may force growth to occur in another area or direction, resulting in an abnormal head shape.

Apart from craniofacial bone development, intramembranous ossification also controls bone formation in the perichondral and periosteal regions of the long bone, where osteoblasts directly differentiate from mesenchymal progenitor cells. Yet, this requires a signal from the cartilaginous element. Furthermore, intramembranous ossification is an essential mechanism underlying bone repair and regeneration in the following processes: fracture healing with rigid fixation; distraction osteogenesis, a bone-regenerative process in which osteotomy followed by gradual distraction yields two vascularized bone surfaces from which new bone is formed (Ai-Aql et al., 2008); and blastemic bone creation, which occurs in children with amputations (Fernando et al., 2011).

Intramembranous ossification is tightly regulated at both molecular and cellular levels. Cranial malformations are often progressive and irreversible, and some of them need aggressive surgical management to prevent or mitigate severe impairment such as misshapen head or abnormal brain growth (Bronfin, 2001). For instance, craniosynostosis is a common congenital disorder that affects 1 in 2500 live births. It is characterized by premature cranial suture fusion, which may result in severe conditions such as increased intracranial pressure, craniofacial dysmorphism, disrupted brain development, and mental retardation. Craniosynostosis is generally considered a developmental disorder resulting from a disrupted balance of cellular proliferation, differentiation, and apoptosis within the suture (Senarath-Yapa et al., 2012; Levi et al., 2012; Slater et al., 2008; Lattanzi et al., 2012; Ciurea and Toader, 2009). Surgical correction followed by reshaping of the calvarial bones remains the only treatment available for craniosynostosis patients (Martou and Antonyshyn, 2011; Posnick et al., 2010; Hankinson et al., 2010). In contrast to craniosynostosis, cleidocranial dysplasia (CCD) is caused by reduced intramembranous bone formation, underdeveloped or absent clavicles (collarbones) as well as delayed maturation of the skull, manifested by delayed suture closure and larger than normal fontanelles that are noticeable as "soft spots" on the heads of infants (Farrow et al., 2018). Severe cases of CCD require surgical intervention. Identifying molecular pathways that control intramembranous ossification is critically important in the mechanistic understanding of craniofacial bone diseases and their targeted therapeutic development.

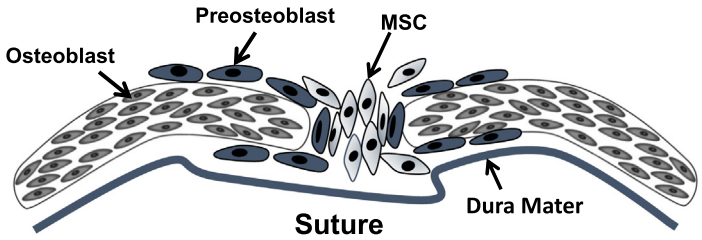

FIGURE 1.2 Schematics of cellular composition of the suture. In the suture, mesenchymal stem cells (MSCs) are located in the middle. They may first become committed preosteoblasts and then finally mature osteoblasts.

Studies of both developmental biology and rare genetic diseases have led to the identification of critical regulators of intramembranous ossification. Transcriptional regulation of the osteoblast lineage is considered in detail in Chapter 7. The runt-related transcription factor 2, RUNX2 (also known as CBFA1), and a zinc finger transcription factor, Osterix (OSX), are osteoblast lineage−determining factors required for both intramembranous and endochondral ossifications. *Runx2* is expressed in osteogenic progenitor cells and required for osteoblast cell fate determination by driving osteoblast-specific gene expression (Ducy et al., 1997; Otto et al., 1997). *Runx2* loss-of-function mutations are found in both mice and humans and cause CCD (Otto et al., 1997; Mundlos et al., 1997; Lee et al., 1997). RUNX2 induces the expression of *Osx*, which is required for osteoblast cell fate commitment, as loss of *Osx* leads to conversion from osteoblasts to chondrocytes (Nakashima et al., 2002). Under the control of RUNX2 and OSX, osteoblast cells produce osteoblast-specific collagen I together with a variety of noncollagenous, extracellular matrix (ECM) proteins that are deposited along with an inorganic mineral phase. The mineral is in the form of hydroxyapatite, a crystalline lattice composed primarily of calcium and phosphate ions.

Cell−cell communication that coordinates cell proliferation and differentiation also plays a critical role in intramembranous ossification. The WNT and Hedgehog (HH) signaling activities are required for cell fate determination of osteoblasts by controlling the expression of *Runx2*. Active WNT/β-catenin signaling is detected in the developing calvarium and perichondrium, where osteoblasts differentiate through intramembranous ossification. Indeed, enhanced WNT/β-catenin signaling enhances bone formation and *Runx2* expression, but inhibits chondrocyte differentiation and *Sox9* expression (Hartmann and Tabin, 2000; Guo et al., 2004; Day et al., 2005). *Sox9* is a master transcription factor that determines chondrocyte cell fate (Bi et al., 1999; Akiyama et al., 2002). Conversely, removal of *β-catenin* in osteochondral progenitor cells resulted in ectopic chondrocyte differentiation at the expense of osteoblasts during both intramembranous and endochondral ossification (Hill et al., 2005; Hu et al., 2005; Day et al., 2005). Therefore, during intramembranous ossification, WNT/β-catenin signaling levels in the mesenchymal condensation are higher, which promotes osteoblast differentiation while inhibiting chondrocyte differentiation. In addition, upregulated WNT/β-catenin signaling in the perichondrium also promoted osteoblast differentiation. In contrast to the WNT/β-catenin signaling, Indian hedgehog (IHH) signaling is not required for osteoblast differentiation of intramembranous bones in the skull (St-Jacques et al., 1999). It is still not clear what controls *Ihh*-independent *Runx2* expression during intramembranous ossification and it is important to understand further the differential regulation of intramembranous versus endochondral ossification by cell signaling. As removing Smoothened, which mediates all HH ligand-dependent signaling, does not abolish intramembranous ossification either (Jeong et al., 2004), HH signaling is likely to be activated in a ligand-independent manner in the developing calvarium. Indeed, it has been found that in the rare human genetic disease progressive osseous heteroplasia, which is caused by null mutations in *Gnas*, which encodes $G\alpha_s$, HH 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 (Regard et al., 2013). Importantly, *Gnas* gain-of-function mutations upregulate WNT/β-catenin signaling in osteoblast progenitor cells, resulting in their defective differentiation and in fibrous dysplasia that also affects intramembranous ossification (Regard et al., 2011). Therefore, $G\alpha_s$ is a key regulator of proper osteoblast differentiation through its maintenance of a balance between the WNT/β-catenin and the HH pathways. The critical role of WNT and HH signaling in intramembranous ossification is also shown in the suture. Mesenchymal stem cells that give rise to the cranial bone and regulate cranial bone repair in adult mice have been identified in the suture. These cells are either $GLI1^+$ or $AXIN2^+$ (Zhao et al., 2015; Maruyama et al., 2016), which marks cells that receive HH or WNT signaling, respectively (Bai et al., 2002; Leung et al., 2002; Jho et al., 2002).

Other signaling pathways, including those mediated by transforming growth factor (TGF) superfamily members, Notch, and fibroblast growth factors (FGFs), are also important in intramembranous ossification. Mutations in the FGF receptors FGFR1, FGFR2, and FGFR3 cause craniosynostosis. The craniosynostosis syndromes involving FGFR1, FGFR2, and FGFR3 mutations include Apert syndrome (OMIM 101200), Beare−Stevenson cutis gyrata (OMIM 123790), Crouzon syndrome (OMIM 123500), Pfeiffer syndrome (OMIM 101600), Jackson−Weiss syndrome (OMIM 123150), Muenke syndrome (OMIM 602849), crouzonodermoskeletal syndrome (OMIM 134934), and osteoglophonic dysplasia (OMIM 166250), a disease characterized by craniosynostosis, prominent supraorbital ridge, and 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 interactions with the WNT and bone morphogenetic protein (BMP) signaling pathways.

Apart from RUNX2 and OSX, other transcription factors are also important, as mutations in them cause human diseases with defects in intramembranous ossification. Mutations in the human *TWIST1* gene cause Saethre−Chotzen syndrome (OMIM 101400), one of the most commonly inherited craniosynostosis conditions. In addition, mutations in the homeobox

genes *MSX1*, *MSX2*, and *DLX* are also associated with human craniofacial disorders (Cohen, 2000; Kraus and Lufkin, 2006). *MSX2* haploinsufficiency decreases proliferation and accelerates the differentiation of calvarial preosteoblasts, resulting in delayed suture closure, whereas its "overexpression" results in enhanced proliferation, favoring early suture closure (Dodig and Raos, 1999). It is likely that *MSX2* normally prevents differentiation and stimulates proliferation of preosteoblastic cells at the osteogenic fronts of the calvariae, facilitating expansion of the skull and closure of the suture. It would be critical to understand further how these transcription factors interact with one another and the signaling pathways to regulate intramembranous bone formation, maintenance, and repair.

The axial skeleton

The axial skeleton consists of the occipital skull bones, the elements of the vertebral column, and the rib cage (ribs and sternum). With the exception of the sternum, the axial skeleton is derived from the paraxial mesoderm, which is segmented into somites during early embryonic development. The occipital skull bones are generated from the fused sclerotomes of the cranial-most 4.5 somites (Goodrich, 1930). The bilateral anlagen of the sternum originate from the lateral plate mesoderm and fuse at the ventral midline in the course of the formation of the rib cage (Chen, 1952).

Somitogenesis

The basic body plan of vertebrates is defined by the metameric segmentation of the musculoskeletal and neuromuscular systems, which originates during embryogenesis from the segmentation of the paraxial mesoderm (for reviews see Winslow et al., 2007; Pourquie, 2000). The paraxial mesoderm is laid down during gastrulation, appearing as bilateral strips of unsegmented tissue (referred to as segmental plate in the avian embryo and presomitic mesoderm in the mouse). It flanks the centrally located neural tube and notochord and gives rise to the axial skeleton (head and trunk skeleton) and all trunk and limb skeletal muscles, as well as the dermis, connective tissue, and vasculature of the trunk. During development, the paraxial mesoderm is segmented through a series of molecular and cellular events in an anterior to posterior (craniocaudal) sequence along the body axis, the anterior-most somites being the more mature ones. The posterior, unsegmented part of the paraxial mesoderm is also referred to as the presomitic mesoderm (PSM), and the sequentially arising, paired tissue blocks are called somites. The PSM is a loose mesenchymal tissue. The cells reaching the anterior border of the PSM progressively undergo a mesenchymal-to-epithelial transition (Christ et al., 2007). Newly formed somites are epithelial balls with a mesenchymal core. As the somites mature, accompanied by the commitment of the cells to the different lineages, this organization changes. In response to signals from the notochord and the ventral floor plate of the neural tube (Sonic Hedgehog [SHH] and the BMP antagonist Noggin), cells on the ventral margin undergo an epithelial−mesenchymal transition, scatter, and move toward the notochord (Christ et al., 2004; Cairns et al., 2008; Yusuf and Brand-Saberi, 2006). These cells will express the transcription factors PAX1, NKX3.1, and NKX3.2 and form the sclerotome, giving rise to the vertebrae and ribs. The dermomyotome is specified by WNT ligands secreted from the dorsal neural tube and the ectoderm covering the dorsal somite. Low levels of SHH signaling are, in combination with WNT signaling, required to maintain the expression of dermomyotomal and myotomal markers (Cairns et al., 2008). The dermomyotome remains epithelial and eventually gives rise to the epaxial muscles of the back and vertebrae, the hypaxial muscles of the body wall and limb, the dermis underneath the skin of the trunk, and the brown adipose tissue (Scaal and Christ, 2004; Atit et al., 2006). Tendons and ligaments of the trunk arise from the fourth somitic compartment, the syndetome, which is induced by the newly formed sclerotome and dermomyotome (Brent et al., 2003; Dubrulle and Pourquie, 2003).

The molecular mechanism driving somitogenesis at the anterior end of the PSM is intrinsic to the PSM, while new cells are continuously added to the PSM from a posteriorly located progenitor pool (Martin, 2016). The so-called segmentation clock, a molecular oscillator coordinating the rhythmic activation of several signaling pathways and the oscillatory expression of a subset of genes in the PSM, is thought to be at the molecular heart of somite formation (Hubaud and Pourquie, 2014). One of the main signaling pathways with oscillatory gene expression is the Notch/Delta/DELTA pathway. This pathway also synchronizes the oscillations between the individual cells (Hubaud and Pourquie, 2014). Also, members of the WNT/β-catenin and the FGF signaling pathway display cyclic gene expression (Aulehla and Pourquie, 2008). The oscillatory expression of these genes appears to go like a wave from the caudal end, sweeping anteriorly through the PSM (Fig. 1.3A). Another molecular system involved in somite formation is the wave front, which is defined by opposing signaling gradients in the PSM (Fig. 1.3B). Here, a posterior−anterior gradient of FGF8 and nuclear β-catenin is opposed by an anterior−posterior gradient of retinoic acid (RA) activity (Mallo, 2016). Despite the fact that the existence of an RA gradient is debated, there is clear genetic evidence that a gradient of WNT signaling activity interacts with the

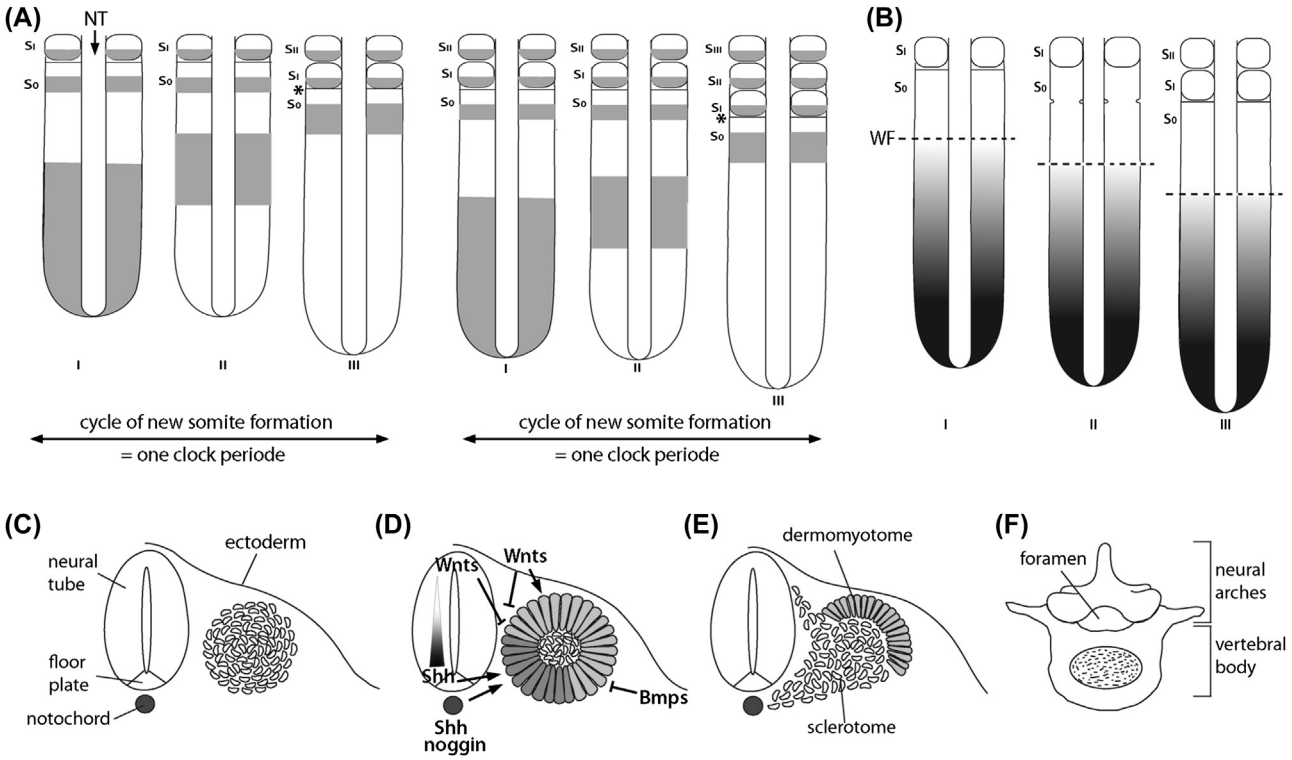

FIGURE 1.3 Somite formation and differentiation. (A) Cyclic gene expression during somite formation. The *asterisk* marks the position of new boundary formation. *NT*, neural tube; S_0, somite stage 0; S_I, somite stage I; S_{II}, somite stage II; S_{III}, somite stage III. (B) Signal gradient within the presomitic mesoderm (PSM), with the *dashed line* marking the position of the wave front (*WF*). (C–E) Schematic representations of the different somite stages. (C) Loose mesenchymal PSM, (D) epithelial ball stage (the ventral darker colored region marks the PAX1-positive sclerotomal region) and factors involved in the somite compartmentalization, (E) sclerotome differentiation. (F) Superior view of a vertebral element derived from the posterior and anterior sclerotomal compartments of two adjacent somites.

segmentation clock to determine the posterior border of a newly forming somite (Mallo, 2016). The morphological changes that eventually lead to the formation of a new somite at the anterior end of the PSM are triggered by Notch activity in combination with the T-box transcription factor, TBX6, and start with the expression of the basic helix–loop–helix transcription factor mesoderm posterior 2 (MESP2) (Saga, 2007; Sasaki et al., 2011). In cells posterior to the determination front, *Mesp2* is repressed by FGF signaling (Sasaki et al., 2011). In addition, *Mesp2* expression becomes restricted to the anterior half of the newly formed somite, as TBX6-mediated transcription of *Mesp2* is suppressed by the RIPPLY1/2 proteins expressed in the posterior part of the somite (Morimoto et al., 2007; Takahashi et al., 2007). MESP2 activity is essential for establishing somite polarity, which is in turn vital for the later formation of the vertebral bodies from the caudal/posterior part of one somite and the rostral/anterior part of the neighboring somite (Christ et al., 2007).

The positional identity of a somite defines the type of vertebral element (occipital, cervical, thoracic, lumbar, or sacral) it will eventually contribute to, and this is controlled, in part, by the regional code of *Hox* genes along the rostral–caudal body axis (for review see Wellik, 2007). Humans and all other bilateral animals have multiple *Hox* genes, encoding transcription factors with a homeobox DNA-binding domain, which are clustered together (Krumlauf, 1992). Through duplication events, the ancestral cluster of originally eight *Hox* genes has been multiplied to four gene clusters (*HoxA*, *HoxB*, *HoxC*, and *HoxD*) of 13 paralogous *Hox* genes in vertebrates. A particular feature of *Hox* gene expression from one cluster is that they are expressed in a temporal and spatial order that reflects their order on the chromosome, with the most 3′ *Hox* gene being expressed first and in the most anterior region. It is thought that the *Hox* genes provide a sort of positional code through their overlapping expression domains, which are characterized by a relatively sharp anterior border. For example, the expression of the *Hox5* paralogs (*HoxA5*, *HoxB5*, and *HoxC5*) correlates in different species such as mouse and chicken, always with the position of the last cervical vertebra, while the anterior domains of the *Hox6* paralogs lie close to the boundary between cervical and thoracic vertebrae (Burke et al., 1995; Burke, 2000). Yet, this correlation is not maintained at the levels of the somites, as mouse and chicken differ in their numbers of cervical elements. Changes in the HOX code can lead to homeotic transformation, which reflects a shift in the regional borders and axial identities.

Members of the polycomb family (*Bmi* and *Eed*) and the TALE class of homeodomain transcription factors are involved in further refining the positional identity provided by the *Hox* code. BMI and EED are transcriptional repressors limiting the rostral (anterior) transcription boundary of individual *Hox* genes (Kim et al., 2006). The TALE proteins, encoded by the *Pbx* and *Meis* genes, further modify the transcriptional activity of the Hox proteins through heterodimerization (Moens and Selleri, 2006).

Sclerotome differentiation

The earliest sclerotomal markers are the transcription factors *Pax1*, *Nkx3.1*, and *Nkx3.2/Bapx1*, which become expressed under the influence of SHH and Noggin signaling in the ventral somite region (Kos et al., 1998; Ebensperger et al., 1995; Murtaugh et al., 2001). *Pax9* expression appears slightly later in the sclerotome and overlaps in part with *Pax1* (Muller et al., 1996). Both genes act redundantly in the ventromedial region of the sclerotome, as in the *Pax1/Pax9* double-mutant mice the development of the ventral vertebra is strongly affected (Peters et al., 1999). NKX3.2 appears to act downstream of *Pax1/Pax9* and can be ectopically induced by PAX1 (Tribioli and Lufkin, 1999; Rodrigo et al., 2003). Although the initial *Pax1* expression is not affected by the loss of *Nkx3.2*, the vertebral differentiation also depends on the function of NKX3.2 (Tribioli and Lufkin, 1999). *Nkx3.1* mutant mice, on the other hand, do not display any skeletal defects (Schneider et al., 2000). As PAX1 is able to activate the expression of early chondroblast markers in vitro, it has been suggested that the activation of PAX1 is the key event that triggers sclerotome formation (Monsoro-Burq, 2005).

After their induction, the sclerotomal cells undergo epithelial−mesenchymal transition and migrate toward the notochord, around the neural tube, and in the thoracic segments also laterally, and then condense to form the vertebral bodies and the intervertebral discs, neural arches, and proximal part of the ribs, respectively (Fig. 1.3C−F). Some notochordal cells surrounded by sclerotomal cells die, while others become part of the intervertebral disc and form the nucleus pulposus (McCann and Seguin, 2016). The neural arches and spinous processes are derived from the mediolateral regions of the sclerotomes and from sclerotomal cells that migrated dorsally. The activity of PAX1/PAX9 is not required for these two compartments (Peters et al., 1999). The dorsally migrating sclerotomal cells contributing to the dorsal part of the neural arches and spinous processes do not express *Pax1* but another set of transcription factors, *Msx1* and Msx2 (reviewed in Monsoro-Burq, 2005; Rawls and Fischer, 2010). Other transcription factors, such as the winged-helix factor, MFH1 (FOXC2), are possibly required for the clonal expansion of cells taking place within the individual sclerotome-derived populations, as they migrate ventrally, laterally, and medially and then condense (Winnier et al., 1997). In addition, the homeodomain transcription factors *Meox1* and *Meox2* have been implicated in vertebral development and may even act upstream of PAX1/PAX9 (Mankoo et al., 2003; Skuntz et al., 2009). Within the individual sclerotomal condensations the chondrogenic and osteogenic programs are then initiated to eventually form the vertebral elements.

The limb skeleton

Overview of limb development

The mesenchymal cells contributing to the skeleton of the appendages (limbs) originate from the bilaterally located lateral plate mesoderm. The lateral plate mesoderm is separated from the somitic mesoderm by the intermediate mesoderm, which gives rise to the kidney and genital ducts. Our knowledge about limb development during embryogenesis is primarily based on two experimental model systems, chick and mouse. In all tetrapods, forelimb development precedes hindlimb development. The axial position of the prospective limb field is in register with the expression of a specific set of *Hox* genes within the somites (Burke et al., 1995). The limb fields are demarcated by the expression of two T-box transcription factors, *Tbx5* in the forelimb and *Tbx4* in the hindlimb field (Petit et al., 2017; Duboc and Logan, 2011). Yet, the identity of the limb is conveyed by the activity of another transcription factor, PITX1, which is expressed specifically in the hindlimb region and specifies hindlimb identity (Logan and Tabin, 1999; Minguillon et al., 2005). In mouse, the forelimb bud starts to develop around embryonic day (E) 9 and the hindlimb around E10. In chick, forelimb development starts on day 2½ (Hamburger Hamilton stage 16) with a thickened bulge (Hamburger and Hamilton, 1992). In humans, the forelimb is visible at day 24 of gestation. Experimental evidence from the chick suggests that WNT signaling induces FGF10 expression and the FGF-dependent initiation of the limb outgrowth (Kawakami et al., 2001). For continuous limb outgrowth the expression of *Fgfs* in the mesenchyme and in an epithelial ridge called the apical ectodermal ridge (AER) is essential (Benazet and Zeller, 2009; Martin, 2001) (Fig. 1.4A). Patterning of the outgrowing limb occurs along all three axes, the proximal−distal, the anterior−posterior, and the dorsal−ventral (Niswander, 2003). For example, in the human arm, the proximal−distal axis runs from the shoulder to the fingertips and can be subdivided into the stylopod (humerus),

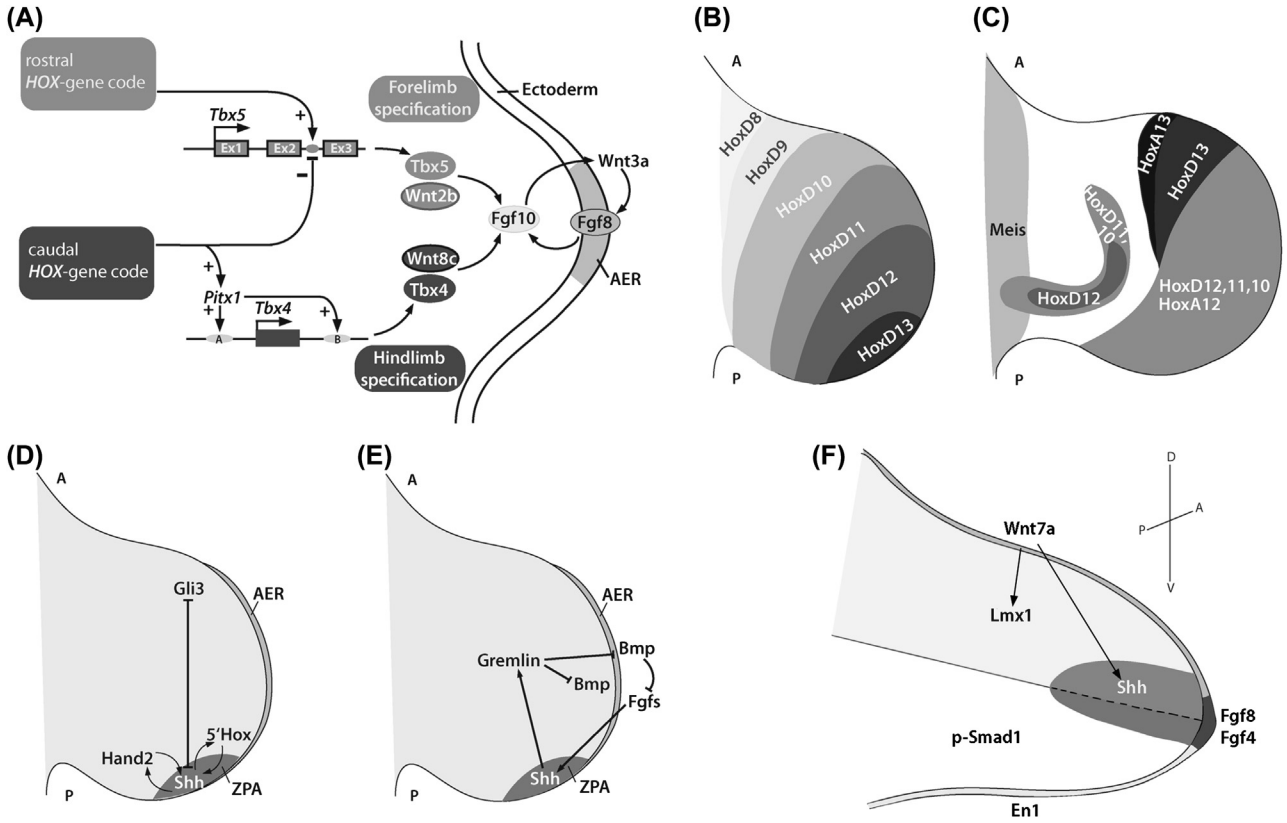

FIGURE 1.4 Limb development overview. (A) Early events in limb bud development: factors involved in the establishment of the limb identity and signals required for the initiation of limb outgrowth. *Hox* genes in the lateral plate mesoderm define the positions where the limbs will develop and activate or repress via specific enhancers the expression of *Pitx1* and the *Tbx4/5* genes. Together with the activity of limb field−specific WNTs an FGF10/WNT3a/FGF8 loop is established, which drives proximal−distal limb outgrowth. *AER*, apical ectodermal ridge. (B) Early nested expression of the HOXD cluster in the limb. *A*, anterior; *P*, posterior. (C) Late expression of the *HoxA* and *HoxD* genes in the autopod stage and expression of the proximal determinant *Meis1*. (D) Factors involved in anterior−posterior patterning of the limb, with *Shh* expressed in the zone of polarizing activity (*ZPA*) under the positive control of the transcription factors HAND2 and the 5′HOX proteins, while its activity in the anterior is opposed by the repressor GLI3. (E) Molecules involved in the interregulation of the anterior−posterior and proximal−distal axes. (F). Molecules involved in the specification of the dorsal−ventral axis: *Wnt7a* expressed in the dorsal ectoderm activates *Lmx1* expression in the dorsal mesenchyme specifying dorsal fate, while EN1 in the ventral ectoderm and phospho-SMAD1 in the ventral mesenchyme specify ventral fate. WNT7a also positively enforces the expression of *Shh*. *(A) Adapted from Fig. 1.2, Petit, F., Sears, K.E., Ahituv, N., 2017. Limb development: a paradigm of gene regulation. Nat. Rev. Genet. 18, 245−258.*

zeugopod (radius and ulna), and autopod regions (wrist and digits of the hand) (Fig. 1.5A). The anterior−posterior axis runs from the thumb to the little finger and the dorsal−ventral axis extends from the back of the arm/hand to the underside of the arm/palm. These three axes are established very early in development, and specific signaling centers, which will be briefly discussed in the following, coordinate the outgrowth and patterning of the limb.

Proximal−distal axis

As already mentioned, during the initiation stage, a positive FGF feedback loop is established between the *Fgfs* expressed in the mesenchyme (*Fgf10*) and the *Fgfs* in the AER (*Fgf8, Fgf4, Fgf9, Fgf17*). Mesenchymal FGF10 activity is essential for the formation of the AER (Sekine et al., 1999). In the positive feedback loop, FGF10 induces *Fgf8* expression in the AER, which is probably mediated by a *Wnt* gene's expression (*Wnt3a* in chick and *Wnt3* in mouse) (Kawakami et al., 2001; Kengaku et al., 1998; Barrow et al., 2003). The AER plays a critical role in the limb outgrowth. Removal of the AER at different time points of development leads to successive truncation of the limb (Saunders, 1948; Summerbell, 1974; Rowe and Fallon, 1982). The *Fgf* genes expressed in the AER confer proliferative and antiapoptotic activity on the distal mesenchyme and maintain the cells in an undifferentiated state (Niswander et al., 1994; Niswander et al., 1993; Fallon et al., 1994; Ten Berge et al., 2008). This is further supported by genetic studies showing that FGF4 and FGF8 are both required for the maintenance of the AER (Boulet et al., 2004; Sun et al., 2002). The most proximal part

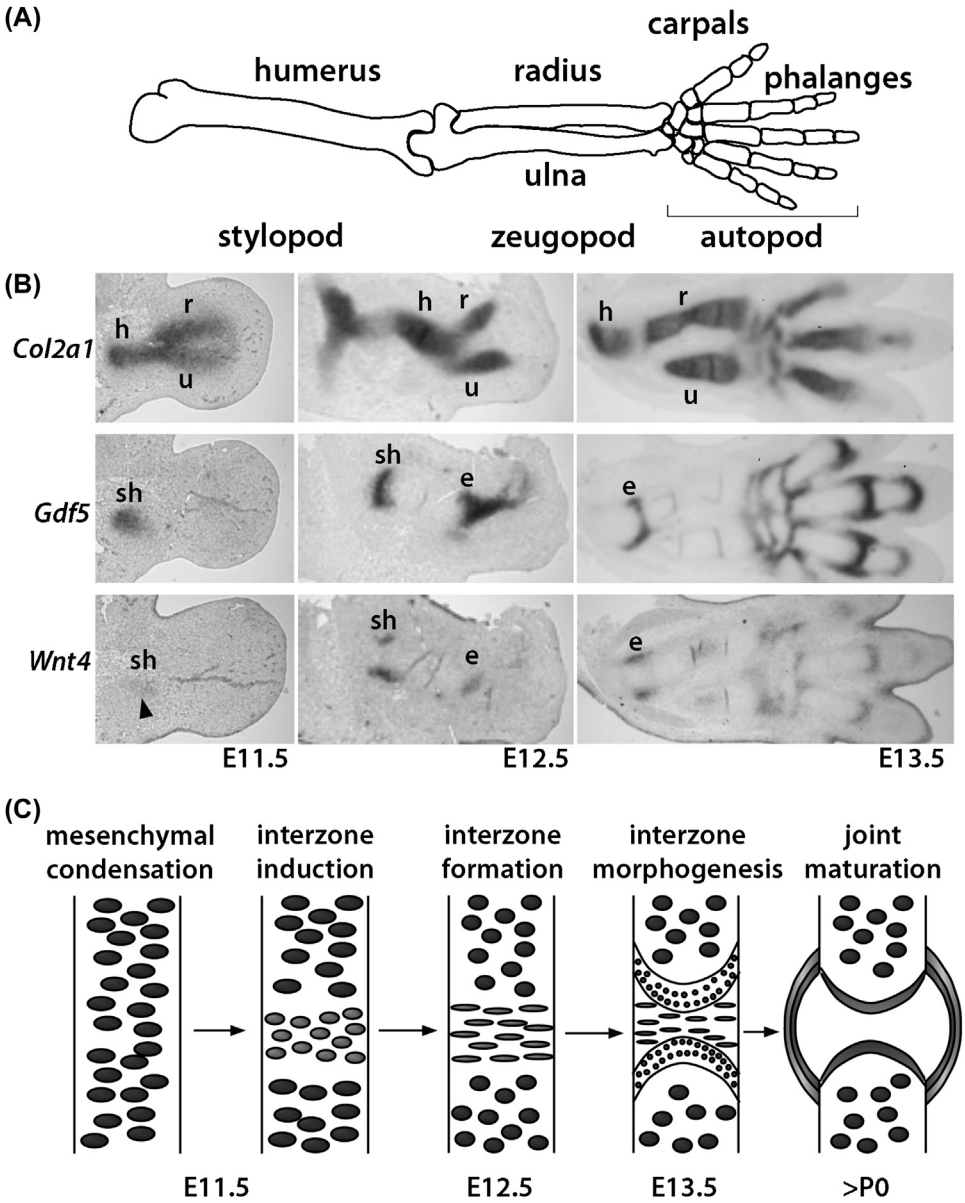

FIGURE 1.5 Patterning of the appendicular skeleton. (A) Schematic overview of the skeletal elements in a human arm. (B) In situ hybridizations on adjacent sections of a mouse forelimb (embryonic stages E11.5, E12.5, and E13.5), showing the branched structure of an early cartilaginous template (*Col2a1* expressing) consisting of the humerus (*h*), radius (*r*), and ulna (*u*). Note that at E11.5 markers of the joint interzone (*Gdf5* and *Wnt4*) are expressed in cells that also express the chondrogenic marker *Col2a1*. At E12.5, during interzone formation, *Col2a1* becomes downregulated in the shoulder (*sh*) and elbow (*e*) region, while the expression patterns of *Gdf5* and *Wnt4* undergo refinement. At E13.5, *Col2a1* is no longer expressed in the joint areas and the expression domains of *Gdf5* and *Wnt4* become distinct. (C) Schematic representation of the major steps during synovial joint formation.

of the limb expresses the TALE homeobox transcription factor MEIS1 under the control of opposing RA and FGF signaling (Mercader et al., 2000). MEIS1 alone is sufficient to proximalize the limb in the chick and mouse systems (Mercader et al., 1999, 2009). Along the proximal–distal axis, the 5′*Hox* genes, which are expressed early in a nested pattern (see Fig. 1.4B), are thought to provide positional cues for growth. As such, members of the group 11 paralogs (HOXA11 and D11 in the forelimb and HOXA11, C11, and D11 in the hindlimb) are required for the growth of the zeugopod, while the autopod establishment depends on the function of group 13 paralogs (Zakany and Duboule, 2007). *Hox* genes are also involved in connective tissue patterning in the limb (Pineault and Wellik, 2014). In addition to their role with regard to the proximal–distal axis, *Hox* genes also play an important role in establishing the signaling center within the limb bud regulating the anterior–posterior axis.

Anterior–posterior axis

Classical embryologic transplantation experiments uncovered the existence of a region present in the posterior limb bud conveying patterning information along the anterior–posterior axis (Saunder and Gasseling, 1968). Transplantation studies also revealed that this region, which was referred to as the zone of polarizing activity (ZPA), must contain some kind of positional information in the form of a secreted morphogen that specifies digit identity along the anterior–posterior axis (Tickle, 1981; Tickle et al., 1975; Wolpert, 1969). The molecular identity of this morphogen was uncovered only in 1993 with the cloning of a vertebrate homolog of the *Drosophila hh* gene, called *Shh*. *Shh* expression overlaps with the ZPA, and Shh-producing cells transplanted into the anterior mesoderm of the limb bud could reproduce mirror-image duplications of ZPA grafts (Riddle et al., 1993). Genetic experiments confirmed that *Shh* is required to establish posterior structures of the limb (Chiang et al., 1996). The *Shh* expression domain is established by the activity of positive and negative regulators. The transcription factor HAND2 (dHAND) is expressed in a posterior domain preceding and encompassing the *Shh* domain and acts as a positive regulator of SHH, which feeds back positively on the expression of HAND2 (Charite et al., 2000; Fernandez-Teran et al., 2000). Early in limb development, *Hand2* is expressed complementary to the transcription factor *Gli3* and GLI3 represses *Hand2* in the anterior (Wang et al., 2000). HAND2, on the other hand, represses *Gli3* in the posterior (Te Welscher et al., 2002). SHH signaling in the posterior prevents the cleavage of the full-length activator GLI3 into the GLI3 repressor (GLI3R) form. Hence, the GLI3R form is restricted to the anterior of the limb bud. The *5′Hox* genes and SHH signaling are also connected by a positive feed-forward regulatory loop (Tarchini et al., 2006; Ros et al., 2003), which may also involve FGF signaling (Rodrigues et al., 2017) (Fig. 1.4D). There is also an interconnection between the anterior–posterior and the proximal–distal axis: SHH signaling upregulates the BMP antagonist Gremlin in the posterior half of the limb. Gremlin antagonism of BMP signaling is required to maintain the expression of *Fgf4*, *Fgf9*, and *Fgf17* in the AER, and FGF signaling feeds positively onto *Shh* (Khokha et al., 2003; Laufer et al., 1994) (Fig. 1.4E).

Dorsal–ventral axis

The third axis that needs to be established is the dorsal–ventral axis. Here, the WNT ligand WNT7a is expressed in the dorsal ectoderm and regulates the expression of the LIM homeobox transcription factor LMX1 (LMX1B in the mouse) in the dorsal mesenchyme (Riddle et al., 1995; Vogel et al., 1995). LMX1B is required to maintain the dorsal identity of structures such as tendons and muscles in the limb (Chen et al., 1998). The ventral counterplayer is the transcription factor Engrailed 1 (EN1), which is expressed in the ventral ectoderm and the ventral half of the AER, and is essential for the formation of ventral structures (Davis et al., 1991; Gardner and Barald, 1992; Cygan et al., 1997; Loomis et al., 1996). BMP signaling appears also to be required for establishment of the dorsal–ventral axis, as the activated downstream component, phospho-SMAD1, is detected throughout the ventral ectoderm and mesenchyme (Ahn et al., 2001) (Fig. 1.4F). Deletion of a BMP receptor gene, *Bmpr1a*, from the limb bud ectoderm results in an expansion of *Wnt7a* and *Lmx1b* into ventral territories, an almost complete loss of *En1*, and severe malformation of the limbs missing the ventral flexor tendons (Ahn et al., 2001).

Mesenchymal condensation and patterning of the skeleton

Patterning of the somitic tissue and the limbs along the different axes is a prerequisite for the mesenchymal condensations to take place. In the craniofacial skeleton, epithelial–mesenchymal interactions occur during the precondensation phase (Hall and Miyake, 1995). Mesenchymal condensations are pivotal for intramembranous and endochondral bone formation. They define the positions and the basic shapes of the future skeletal elements. They can be visualized in the sclerotome, developing skull, and limbs in vivo and in micromass cell cultures in vitro by the presence of cell surface molecules that bind peanut agglutinin (Stringa and Tuan, 1996; Milaire, 1991; Hall and Miyake, 1992). During the prechondrogenic and preosteogenic condensation phase ECM molecules, such as the glycoproteins Fibronectin, Versican, and Tenascin; cell–cell adhesion molecules, such as N-CAM and N-cadherin; the gap-junction molecule Connexin43 (CX43); and Syndecans (type I transmembrane heparan sulfate proteoglycan) become upregulated, but their expression often changes dynamically during the subsequent differentiation process (for review see Hall and Miyake, 2000; DeLise et al., 2000). Cell adhesion and ECM proteins promote the formation of the condensations by establishing cell–cell contacts and cell–matrix interactions. Yet, through genetic studies, their functional requirement for the condensation process has not been demonstrated so far. For the cell–matrix interactions, integrins also play an important role as they act as receptors for Fibronectin (α5β1; αVβ3), types II and VI collagen (α1β1, α2β1, α10β1), Laminin (α6β1), Tenascin (α9β1, αVβ3, α8β1, αVβ6), and Osteopontin (OPN) (αVβ1; αVβ3; αVβ5; α8ßβ1) (Loeser, 2000, 2002; Tucker and Chiquet-Ehrismann, 2015; Docheva et al., 2014).

Various growth factors, such as members of the TGFβ superfamily, regulate the condensation process (reviewed in Moses and Serra, 1996). This has also been elegantly demonstrated in vitro for a subclass of this superfamily of growth factors, the BMP family (Barna and Niswander, 2007). For the proximal elements (femur, tibia, and fibula) in the hindlimb, genetics revealed a dual requirement for the zinc finger transcription factors GLI3 and PLZF to establish the correct temporal and spatial distribution of chondrocyte progenitors (Barna et al., 2005).

Mesenchymal cells within the condensations can differentiate into either osteoblasts (intramembranous ossification) or chondrocytes (endochondral ossification). WNT/β-catenin signaling is essential for the differentiation of osteoblasts, as no osteoblasts develop in conditional mouse mutants in which the β-catenin-encoding gene *Ctnnb1* was deleted in mesenchymal precursor cells of the limb and/or skull (Hu et al., 2005; Hill et al., 2005; Day et al., 2005). Instead, the precursor cells differentiate into chondrocytes (Day et al., 2005; Hill et al., 2005). Hence, β-catenin activity is not essential for chondrogenesis. WNT/β-catenin signaling is most likely acting as a permissive pathway at this early step of differentiation, as too high levels of WNT/β-catenin signaling block osteoblast as well as chondrocyte differentiation (Hill et al., 2005). WNT/β-catenin signaling in perichondrial cells is amplified by SOXC protein family members to further secure the nonchondrogenic fate of these cells (Bhattaram et al., 2014). For osteoblast differentiation to occur, the transcription factor RUNX2 needs to be upregulated within the preosteogenic condensations, while the HMG-box transcription factor SOX9 is required for the further differentiation of cells within the condensations along the chondrocyte lineage and probably also for the condensation process itself (Bi et al., 1999; Akiyama et al., 2002; Karsenty, 2001; Lian and Stein, 2003). The latter aspect has been challenged by the results of in vitro experiments by Barna and Niswander (2007) showing that *Sox9*-deficient mesenchymal cells compact and initially form condensations, yet the cells within the condensations do not differentiate into chondroblasts (Barna and Niswander, 2007).

The skeletal elements in the limbs, which are formed by the process of endochondral ossification, develop in part as continuous, sometimes bifurcated (pre)chondrogenic structures, such as, e.g., the humerus branching into the radius and ulna in the forelimb (Fig. 1.5B), being subsequently segmented by the process of joint formation (Shubin and Alberch, 1986; Hinchliffe and Johnson, 1980; Oster et al., 1988). Furthermore, studies have shown that the cartilage morphogenesis of the developing long bones also occurs in a modular way, with two distinct pools of progenitor cells contributing to the primary structures and the bone eminences (Blitz et al., 2013; Sugimoto et al., 2013). Cells within the bifurcated, $SOX9^{+}$ primary structures express the gene *Col2a1*, characteristic of chondroblasts/chondrocytes. Although they appear during early limb development (E11.5) to be morphologically uninterrupted, the region where a joint (here the shoulder joint) will be formed can be visualized using molecular joint markers, such as *Gdf5* (growth differentiation factor 5) or *Wnt4* (see Fig. 1.5B). Interestingly, the cartilage matrix protein Matrilin-1 is never expressed in the interzone region, nor in the adjacent chondrogenic region, which possibly gives rise to the articular cartilage (Hyde et al., 2007). How the position of joint initiation within the limb is determined is not completely understood as of this writing. A limb molecular clock operating in the distal region may be involved in this process. It has been proposed that two oscillation cycles of the gene *Hairy 2* (*Hes2*) are required to make one skeletal element in the zeugopod and stylopod region of the limb (Sheeba et al., 2016). As the joints develop sequentially along the proximal−distal axis at a certain distance from each other, secreted factors produced by the joint itself may provide some kind of self-organizing mechanism (Hartmann and Tabin, 2001; Hiscock et al., 2017). WNT/β-catenin signaling is also required for joint formation (Hartmann and Tabin, 2001; Guo et al., 2004; Spater et al., 2006a, 2006b). Yet, again, it may act in this process also as a permissive pathway, repressing the chondrogenic potential of the joint interzone cells. However, as WNT/β-catenin signaling also induces the expression of *Gdf5*, it may also play an active role in joint induction by inducing cellular and molecular changes required for joint formation. The AP1-transcription factor family member c-JUN acts upstream of WNT signaling in joint development regulating the expression of *Wnt9a* and *Wnt16*, which are both expressed in the early joint interzone (Kan and Tabin, 2013). Numerous other genes, including *Noggin*, *Hif1α*, *Gdf5*, *Gdf6*, *Gli3*, *Ihh*, *PTH/PTHrPR1*, *Tgfβ*, *Mcp5*, and *Crux1*, have been implicated in a variety of cellular processes during joint formation based on genetic or misexpression experiments (Brunet et al., 1998; Amano et al., 2016; Spagnoli et al., 2007; Longobardi et al., 2012), for review see (Archer et al., 2003; Pacifici et al., 2006).

Endochondral bone formation

Overview

The axial and appendicular skeletal elements are formed by the process of endochondral bone formation starting with a cartilaginous template (Fig. 1.6A−E). This process starts with the condensation of mesenchymal cells at the site of the future skeleton. As mentioned already, this involves alterations in cell−cell adhesion properties and changes in the ECM

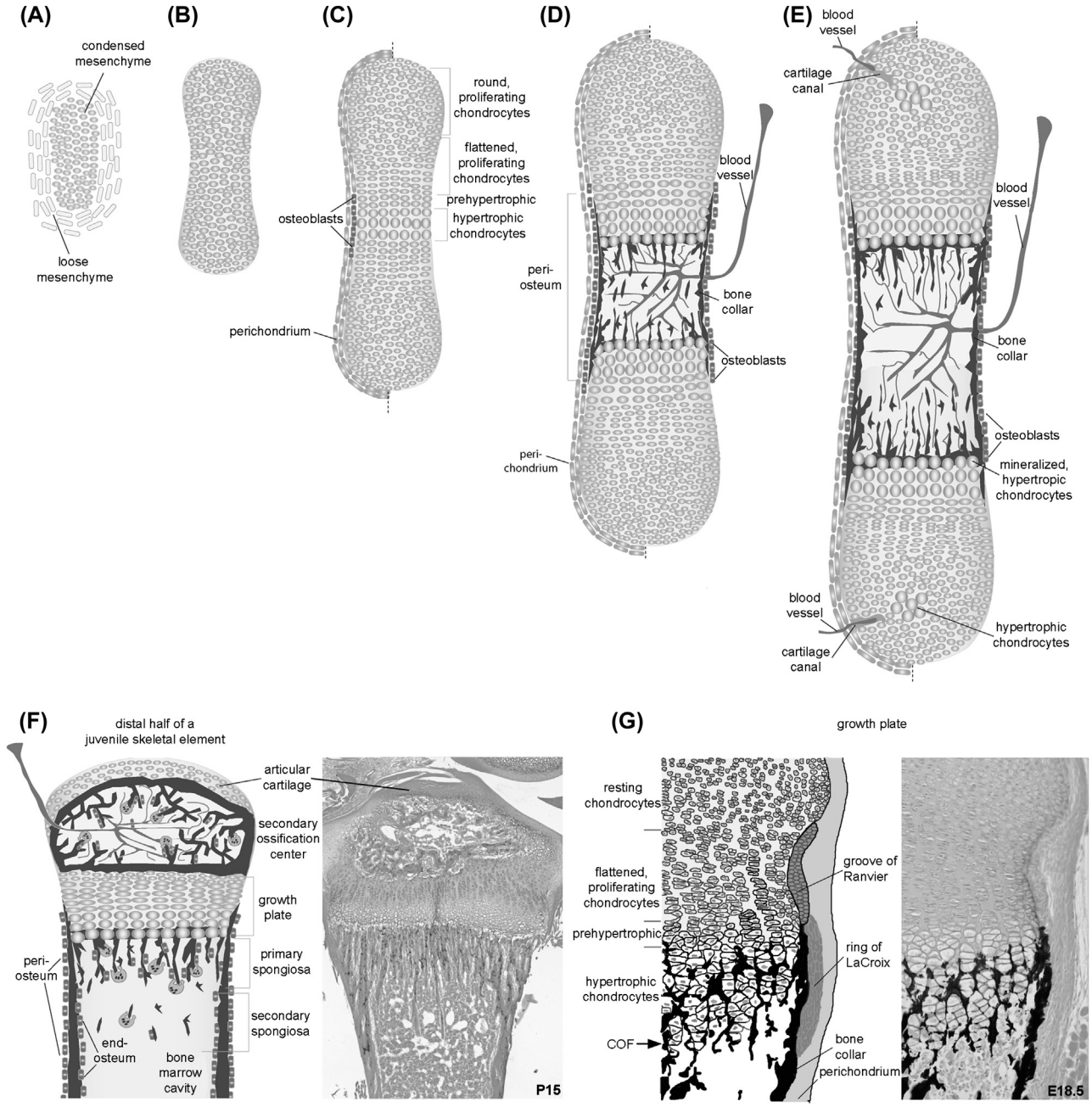

FIGURE 1.6 Schematic representation of the formation and growth of long bones by endochondral ossification. (A) Mesenchymal condensation with surrounding loose mesenchymal cells. (B) Cartilaginous template prefiguring the future skeletal element. (C) Chondrocyte differentiation within the cartilaginous template and differentiation of osteoblasts within a region of the perichondrium, which is then referred to as the periosteum. (D) Blood vessel invasion and onset of bone marrow cavity formation. (E) Onset of the formation of the secondary ossification center with differentiation of hypertrophic chondrocytes in the central region of the epiphysis and blood vessel invasion from the perichondrium through the cartilage canals. (F) Schematic representation on the left and corresponding Alcian blue/eosin−stained image of the proximal end of a postnatal day 15 (P15) mouse tibia on the right. (G) Schematic representation of the different features of a mouse growth plate based on the von Kossa/Alcian blue−stained proximal end of a mouse humerus at embryonic day 18.5 (E18.5). *COF*, chondro-osseous front.

(DeLise and Tuan, 2002a; Delise and Tuan, 2002b; Hall and Miyake, 1995; Bhat et al., 2011). Mesenchymal cells within the condensations start to express chondro-osteogenic markers, such as the transcription factors *Sox9* and *Runx2* (Hill et al., 2005; Akiyama et al., 2005; Wright et al., 1995). Next, the prechondrogenic precursor population of chondroblasts differentiates into chondrocytes, which produce an ECM rich in the proteoglycan aggrecan and fibrillar collagen of type II.

Cartilaginous template formation prefigures the future skeletal element and is surrounded by the so-called perichondrium, a layer of mesenchymal cells. As cartilage is avascular, limb vasculature regression needs to occur where cartilaginous structures form (Hallmann et al., 1987). Yet, interestingly, the chondrogenic condensation does express vascular endothelial growth factor (VEGF) (Eshkar-Oren et al., 2009). The outgrowth of vertebrate limbs occurs progressively along the proximal−distal axis (Newman et al., 2018; Zeller et al., 2009). Concomitantly, the skeletal elements develop in a proximodistal sequence, with the anlagen of the proximal elements (humerus in the forelimb and femur in the hindlimb) forming first, branching into more distal elements, and then being segmented into individual elements as the limb grows (Hinchliffe, 1994). The cartilaginous template increases in size by appositional and interstitial growth (Johnson, 1986). Interstitial growth by dividing chondrocytes allows the cartilage to grow rapidly along the longitudinal axis. The width of the cartilage element is controlled by appositional growth, whereby the perichondrium surrounding the cartilage template serves as the primary source of chondroblasts. Early on, all chondrocytes are still proliferating. As development progresses, the chondrocytes distant to the articulations in the central diaphysis will start to undergo a differentiation program. First, they flatten and rearrange into proliferative stacks of chondrocytes forming the zone of columnar proliferating chondrocytes. The elongation of these columns occurs internally through oriented cell division followed by intercalation movements of the daughters (Ahrens et al., 2009; Li and Dudley, 2009). A 2014 study showed that the daughter cells maintain intimate contact after cell division, preserving cadherin-mediated cell−cell interaction until the end of the rotational movement (Romereim et al., 2014). Interfering with cadherin-mediated cell−cell adhesion stalls the rotation process in vitro (Romereim et al., 2014). A similar rotation defect was observed in mice lacking integrin β1 (Aszodi et al., 2003). Chondrocytes at the lower end of the columns will then exit the cell cycle and become prehypertrophic; a stage that is not morphologically distinct but can be visualized using molecular markers such as the expression of the genes *Ihh* and parathyroid hormone/parathyroid hormone-like peptide receptor 1 (*Pthr1*). Next, the prehypertrophic chondrocytes increase dramatically in volume and become hypertrophic (Cooper et al., 2013; Hunziker et al., 1987). The almost 10-fold increase in volume occurs in parts by true cellular hypertrophy and swelling and significantly contributes to the longitudinal expansion of the skeletal elements as the cells are laterally restricted by matrix channels (Cooper et al., 2013). Hypertrophic chondrocytes (HCCs) are distinct in their ECM producing type X instead of type II collagen. Furthermore, they produce VEGF, which in this context attracts blood vessels to the diaphysis region (Gerber et al., 1999). The ECM of mature HCCs mineralizes and the cells produce matrix metalloproteinase 13 (MMP13) as well as OPN/SSP1. MMP13 (collagenase 3) breaks up the matrix of HCCs for the subsequent removal by osteoclasts (Inada et al., 2004; Stickens et al., 2004), while SSP1 has multiple functions; it regulates mineralization, serves as a chemoattractant for osteoclasts, and is functionally required for their activity (Franzen et al., 2008; Rittling et al., 1998; Boskey et al., 2002; Chellaiah et al., 2003). The final fate of HCCs has long been believed to be apoptotic cell death (Shapiro et al., 2005). Yet, ex vivo and in vitro experiments already hinted at an alternative fate, with HCCs transdifferentiating into osteoblasts (Shapiro et al., 2005). Lineage tracing experiments have confirmed this alternative fate, proposing a model of dual osteoblast origin (Zhou et al., 2014; Yang et al., 2014a, 2014b; Park et al., 2015). At least during embryonic development, about 20% of osteoblasts are chondrocyte derived and about 80% are derived from the perichondrium/periosteum. The latter population migrates into the bone marrow cavity along the invading blood vessels (Maes et al., 2010). This invasion originates from the periosteal collar, the area of the perichondrium in which osteoblasts differentiate and the bone collar is being formed (Colnot et al., 2004). In addition, monocytic osteoclast precursors as well as macrophages, both of which are of hematopoietic origin, enter the remodeling zone via the vascular system, which is attracted by VEGF (Henriksen et al., 2003; Engsig et al., 2000). Blood vessels have additional roles during trabecular bone formation in the primary spongiosa, which will be further discussed in the following. Endothelial cells, chondroclasts, and osteoclasts act together to erode the bone marrow cavity by removing HCC remnants. Interestingly, a bone marrow cavity can form in mouse mutants lacking osteoclasts or even macrophages and osteoclasts (Ortega et al., 2010). In these mutants, MMP9-positive cells are still present at the chondro-osseous junction and may be in part responsible for bone marrow cavity formation (Ortega et al., 2010). With the formation of the marrow cavity in the diaphysis, the two growth plates become separated from each other. The growth plates serve as a continual source of cartilage being converted into bone at the chondro-osseous front during the late stages of development and postnatally. In most species, a second ossification center appears during postnatal development within the epiphyseal cartilage. The onset differs between species for the individual bones and even within one bone for the two epiphyses (Adair and Scammin, 1921; Shapiro, 2001; Zoetis et al., 2003). Here, cartilage canals containing mesenchymal cells and blood vessels enter from the surrounding perichondrium, reaching eventually the hypertrophic center of the epiphysis (Blumer et al., 2008; Alvarez et al., 2005). After the formation of the secondary ossification center, the epiphyseal articular cartilage becomes distinct and the metaphyseal growth plate is sandwiched between the epiphyseal secondary ossification center and the primary ossification center in the diaphysis (Fig. 1.6F).

The growth plate

The cellular organization within the growth plate (schematically depicted in Fig. 1.6F) of a juvenile bone resembles the different zones in embryonic skeletal elements (Fig. 1.6G). There is a zone of small round chondrocytes, some of which are mitotically inert, that is often referred to as the resting zone. Stemlike or progenitor cells are thought to reside in this zone and require the activity of β-catenin for their maintenance (Candela et al., 2014). Concomitant with the growth plate closure that occurs in most vertebrates, with the exception of rodents, these progenitor cells eventually become senescent at the end of puberty and lose their proliferative potential, putting an end to long bone growth (Nilsson and Baron, 2004). The zone next to the resting zone contains flattened, stacked chondrocytes, which are mitotically active and form fairly regular columns. Eventually, the chondrocytes at the lower end of the zone will begin to enlarge, becoming first prehypertrophic and then HCCs (Ballock and O'Keefe, 2003). As already mentioned, some of the HCCs will undergo apoptosis (programmed cell death), while others survive and eventually differentiate into osteoblasts or other cells of the bone marrow cavity (Farnum and Wilsman, 1987; Shapiro et al., 2005; Tsang et al., 2015). The exact cellular and molecular mechanism of the transdifferentiation process of the surviving HCCs is not understood as of this writing. Earlier experiments suggested that this involves asymmetric cell division (Roach et al., 1995). According to the lineage tracing experiments, the transdifferentiating cells express at one point the gene *Col10a1*, encoding the α chain of type X collagen, but were they truly hypertrophic cells? If so, how was their cellular volume adjusted? Or alternatively, is there a pool of "stem cells" residing within the hypertrophic zone? So far, expression of stem cell markers has not been reported in HCCs of a normal growth plate. Yet, cells originating from the hypertrophic zone expressing the lineage tracer also express stem cell markers such as *Sca1* and *Sox2* in vitro (Park et al., 2015). Furthermore, a 2017 publication reported that during fracture healing HCCs express the stem cell markers *Sox2*, *Nanog*, and *Oct4* and that this is triggered by the invading vasculature (Hu et al., 2017). Other experiments such as one in rabbits, in which transdifferentiation was observed after physically preventing vascular invasion at the lower hypertrophic zone, suggest that the vasculature is not required for the transdifferentiation process to occur (Enishi et al., 2014). So far there are only a few molecules known to be required for the chondrocyte-derived differentiation of osteoblasts. One of them is β-catenin (Houben et al., 2016) and the other one SHP2, a protein tyrosine phosphatase (Wang et al., 2017). Mice lacking SHP2 activity in HCCs display a slight reduction in chondrocyte-to-osteoblast differentiation, and the mechanism behind this blockade is the persistence and/or upregulation of SOX9 protein in HCCs (Wang et al., 2017). Mice lacking β-catenin activity in HCCs display an even more severe reduction of chondrocytes differentiating into osteoblasts and its absence also affects in part the transdifferentiation of chondrocytes into other cell types (Houben et al., 2016). The mechanism by which β-catenin affects this transdifferentiation process is unknown as of this writing. Unlike what has been shown in perichondrial osteoblast precursors or in the case of SHP2, persistence of SOX9 protein was not observed (Houben et al., 2016). Furthermore, the loss of β-catenin activity in HCCs affects indirectly the differentiation of perichondrial-derived osteoblast precursors (Houben et al., 2016). HCCs also produce receptor activator of NF-κB ligand (RANKL) and its decoy receptor Osteoprotegerin, which positively and negatively, respectively, influence the differentiation of monocytes into osteoclasts at the chondro-osseous front (Usui et al., 2008; Silvestrini et al., 2005; Kishimoto et al., 2006). The expression of *Rankl* in HCCs is negatively controlled by β-catenin, leading to increased osteoclastogenesis and reduced trabecular bone formation in conditional *Ctnnb1* mice (Houben et al., 2016; Golovchenko et al., 2013; Wang et al., 2014a). As mentioned already, the matrix of the lower rows of HCCs mineralizes. HCCs utilize matrix vesicles to produce large amounts of microcrystalline, Ca^{2+}-deficient, acid-phosphate-rich apatite deposits in the collagen-rich matrix (Wuthier and Lipscomb, 2011). Matrix vesicle release occurs in a polarized fashion from the lateral edges of the growth plate HCCs, resulting in the mineralization of the longitudinal septae, while transverse septae remain unmineralized (Anderson et al., 2005a). The matrix vesicles then release the apatite crystals, which self-nucleate and grow to form spherical mineralized clusters in the calcified zone of the HCCs. Mitochondria may serve as storage containers for Ca^{2+}, with the mitochondria in HCCs reaching the highest Ca^{2+} concentrations and serving as the Ca^{2+} supply for matrix vesicles. The mitochondria loaded with Ca^{2+} can no longer produce sufficient amounts of ATP and the cells undergo a physiological energy crisis. As a consequence, the mitochondria produce increased amounts of reactive oxygen species (ROS) (Wuthier and Lipscomb, 2011). Increased ROS levels feed back on the chondrocytes, inducing them to hypertrophy (Morita et al., 2007).

Through knockout studies in mouse, numerous genes were identified that are involved in the regulation of the mineralization process, such as matrix Gla protein and tissue nonspecific alkaline phosphatase (encoded by the *Akp2* gene), ectonucleotide pyrophosphatase/phosphodiesterase type 1, progressive ankylosis gene, phosphoethanolamine/phosphocholine phosphatase, membrane-anchored metalloproteinase ADAM17, and, as already mentioned, OPN (Anderson et al., 2004, 2005b; Fedde et al., 1999; Hessle et al., 2002; Zaka and Williams, 2006; Harmey et al., 2004; Hall et al., 2013).

After the removal of HCCs, the mineralized longitudinal septae remain and are used by osteoblasts as a scaffold for the deposition of osteoid that calcifies into woven bone.

At the periphery, the growth plate is surrounded by a fibrous structure that consists of the wedge-shaped groove of Ranvier and the perichondrial ring of LaCroix (see Fig. 1.6G) (Brighton, 1978; Langenskiold, 1998). The groove of Ranvier serves as a reservoir for chondro-osteoprogenitor cells and fibroblasts, while the perichondrial ring of LaCroix may serve as a reservoir of precartilaginous cells (Fenichel et al., 2006; Shapiro et al., 1977). Interestingly, the two growth plates within a skeletal element have different activities leading to the differential growth of the distal and proximal parts (Pritchett, 1991, 1992; Farnum, 1994). Curiously, there seems to exist a temporal and local correlation between the appearance of the secondary ossification center and the activity of the nearby growth plate. For instance, in the humerus the secondary ossification center appears first in the proximal epiphysis and here, the proximal growth plate is more active than the distal one.

Mediators of skeleton formation

Accurate skeletogenesis, as well as postnatal growth and repair of the skeleton, depends on the precise orchestration of cellular processes such as coordinated proliferation and differentiation in time and space. Several signaling pathways impinge on the differentiation of the mesenchymal precursors as well as on the subsequent differentiation of chondrocytes and regulate the growth of the skeletal elements. Growth factor signaling is also partly controlled by the ECM and integrins (Munger and Sheppard, 2011; Ivaska and Heino, 2011). Cell-type-specific differentiation is under the control of distinct transcription factors with their activity being modulated by epigenetic factors and microRNAs. In addition to systemic and local factors, oxygen levels and metabolism also influence endochondral bone formation.

Systemic mediators

Longitudinal bone growth after birth is under the influence of various hormones, such as growth hormone (GH), insulin-like growth factors (IGFs), thyroid hormones, estrogen and androgens, glucocorticoids, vitamin D, and leptin. The importance of these hormones in skeletal growth has been demonstrated by genetic studies in animals and by "natural experiments" in humans (for reviews see Nilsson et al., 2005; Wit and Camacho-Hubner, 2011). Many of these systemic mediators interact with one another during linear growth of the juvenile skeleton and are differentially controlled by the nutritional status (Robson et al., 2002; Lui and Baron, 2011; Gat-Yablonski et al., 2008). Yet, only IGF signaling plays a role in endochondral ossification prior to birth.

Mice deficient for either *Igf1* or *Igf2* or the *Igf1r* gene display prenatal as well as postnatal growth defects, suggesting that IGFs act independent of GH on linear growth (Baker et al., 1993; Liu et al., 1993; Powell-Braxton et al., 1993). IGF1 was thought to affect chondrocyte proliferation, yet, a study on longitudinal bone growth in the *Igf1*-null mouse revealed no change in growth plate chondrocyte proliferation or cell numbers, despite the observed 35% reduction in the rate of long bone growth that was attributed to the 30% reduction in the linear dimension of HCCs (Wang et al., 1999). For more detailed information on the activities of GH and IGF signaling see reviews by Giustina et al. (2008), Kawai and Rosen (2012), Svensson et al. (2001), and Lindsey and Mohan (2016).

Local mediators

The various local mediators of endochondral and intramembranous ossification, which will be briefly discussed in the following, interact at multiple levels. Because of space constraints not all of these interactions can be mentioned.

Growth factor signaling pathways

Transforming growth factor β and bone morphogenetic proteins

The TGFβ superfamily is a large family of secreted polypeptides that can be divided into two subfamilies based on the utilization of the downstream signaling mediators, the regulatory SMADs (R-SMADs). The first one, encompassing TGFβ1−β3, activins, inhibins, nodal, and myostatin (GDF8), transduces the canonical signal through the R-SMADs 2 and 3. The second one consists of the BMPs 2 and 4−10 and most GDFs, transducing the canonical signal through R-SMADs 1, 5, and 8. The cofactor SMAD4 is utilized by both groups, forming a complex with the different activated R-SMADs. The receptor complexes are heterodimers consisting of serine/threonine kinase types I (ALKs 1−7) and II (TβRII, ActRII, ActRIIb, BMPRII, and MISRII) receptors. Ligand binding activates the type II receptor, leading to transphosphorylation

of the type I receptor. In addition to the SMAD-dependent canonical signaling, TGFβ/BMPs can signal through numerous SMAD-independent noncanonical signaling pathways (reviewed in Wang et al., 2014c; Wu et al., 2016).

Many of the TGFβ and BMP signaling molecules are involved in endochondral bone formation. In the mouse, all three *Tgfβ* isoforms are expressed in mesenchymal condensations, perichondrium/periosteum, and appendicular growth plates (Pelton et al., 1990, 1991; Schmid et al., 1991). Despite the numerous in vitro reports indicating a role for TGFβ molecules promoting mesenchymal condensation and the onset of chondrocyte differentiation, none of the individual *Tgfβ* knockouts supports such an early role in vivo. It has been proposed that transient activation of TGFβ and/or activin signaling primes mesenchymal cells to become chondroprogenitors (Karamboulas et al., 2010). Of the individual *Tgfβ* knockouts, only the $Tgf\beta2^{-/-}$ mutants displayed defects in intramembranous and endochondral bone formation (Sanford et al., 1997), some of which may be secondary due to defects in tendon formation (Pryce et al., 2009). The conditional ablation of the primary receptor for all three TGFβs and *Alk5*, in mesenchymal cells using the *Dermo1*-Cre line, resulted also in skeletal defects affecting intramembranous and endochondral bones (Matsunobu et al., 2009). The endochondral bone elements in the $Alk5^{-/-}$ animals were smaller and malformed, with ectopic cartilaginous protrusions present in the hindlimb. Conditional deletion of the *Tgfbr2* gene, encoding the TβRII receptor, in the limb mesenchyme with the *Prx1*-Cre line results in the absence of interphalangeal joints, probably due to a defect in downregulation of the chemokine MCP-5 in the joint interzone cells (Spagnoli et al., 2007; Longobardi et al., 2012). The appendicular skeletal elements of the *Tgfbr2*;*Prx1*-Cre embryos are also shorter, associated with altered chondrocyte proliferation and an enlarged HCC zone (Seo and Serra, 2007). This phenotype was also observed upon the expression of a dominant-negative form of the TβRII receptor or by expressing a dominant-negative TβRI (*Alk5*) construct in chondrocytes (Serra et al., 1997; Keller et al., 2011). Surprisingly, deletion of *Tgfbr2* in *Col2a1*-expressing cells resulted in defects only in the axial skeleton and not in the appendicular skeleton (Baffi et al., 2004). Nevertheless, the long bones of the *Tgfbr2;Col2a1*-Cre newborn mice were consistently shorter, but the difference was not significant. Sueyoshi and colleagues reported that deletion of *Tgfbr2* in HCCs results in a minor delay in chondrocyte differentiation around E14.5/15.5. Yet, at birth, no differences regarding the length of the long bones were observed, suggesting that this is a transient effect (Sueyoshi et al., 2012). Deletion of *Tgfbr2* in *Osx*-Cre-positive pre-HCCs and osteoblast precursors in the perichondrium led to postnatal alteration in the growth plate and affected osteoblastogenesis (Peters et al., 2017). This is probably associated with a loss of *TGFβ1* signaling (Tang et al., 2009). Nevertheless, inactivation of *Tgfbr2* may not be sufficient to eliminate all Tgfβ signaling, as TGFβ ligands were still capable of eliciting signals in the $Tgfbr2^{-/-}$ mice (Iwata et al., 2012). Furthermore, TGFβs can activate the canonical BMP/SMAD1/5/8 pathway through engagement of ALK1 (Goumans et al., 2002). TGFβ proproteins are sequestered by the ECM and can then be released and activated through, for instance, the activity of ECM degrading enzymes (Hildebrand et al., 1994; Pedrozo et al., 1998; Annes et al., 2003). For further information, in particular on the involvement of noncanonical TGFβ pathways in chondrogenesis and skeletogenesis and the implications of TGFβ signaling in osteoarthritis, see reviews by van der Kraan et al. (2009), Wang et al. (2014c), and Wu et al. (2016).

The cofactor SMAD4 is thought to mediate canonical signaling downstream of TGFβ and BMP signaling. Yet surprisingly, conditional mutants lacking *Smad4* in *Col2a1*-expressing cells are viable and display only mild phenotypic changes in the growth plate (Zhang et al., 2005; Whitaker et al., 2017). However, the prechondrogenic condensations do not form in mice lacking SMAD4 in the limb mesenchyme, supporting an essential role for TGFβ/BMP signaling in the early steps of chondrocyte differentiation, which appears to be independent of SOX9 (Lim et al., 2015; Benazet et al., 2012). Mice lacking either R-SMAD1/5 in *Col2a1*-expressing cells or all three R-SMADs (SMAD1, 5, and 8) acting downstream of BMP signaling are not viable and display a nearly identical severe chondrodysplasia phenotype (Retting et al., 2009). The axial skeleton is severely compromised, with vertebral bodies replaced by fibroblasts and loose mesenchymal tissue. This suggests that SMAD8 plays only a minor role in chondrogenesis. Furthermore, these results challenge the dogma that SMAD4 is required to mediate SMAD-dependent signaling downstream of BMPs and TGFβs.

Based on the analyses of gene knockout animals, the *Bmp/Gdf* family members *Bmp8*, *Bmp9/Gdf2*, *Bmp10*, and *Gdf10* appear to play no role in embryonic skeletogenesis (Zhao et al., 1996, 1999; Chen et al., 2004; Levet et al., 2013). The *short-ear* mouse is mutant for *Bmp5* and displays defects in skeletal morphogenesis and has weaker bones (Kingsley et al., 1992; Mikic et al., 1995). *Bmp6* mutants have sternal defects (Solloway et al., 1998). Mice mutant for *Bmp7* display skeletal patterning defects restricted to the rib cage, skull, and hindlimbs (Luo et al., 1995; Jena et al., 1997). In addition to *Bmp7*, *Bmp2* and *Bmp4* are expressed in the early limb bud. Conditional deletion of *Bmp2* and *Bmp4* in the limb mesenchyme results in an abnormal patterning of the appendicular skeleton with a loss of posterior elements in the zeugopod and autopod region probably due to a failure of chondrogenic differentiation of the mesenchymal cells caused by insufficient levels of BMP signaling (Bandyopadhyay et al., 2006). In addition, the skeletal elements that form are shorter and thinner. Chondrocyte differentiation within the skeletal elements is delayed but otherwise normal. Concomitantly, the endochondral ossification process is also delayed and bone formation is severely compromised in these

mice (Bandyopadhyay et al., 2006). Yet, of the two BMPs, BMP2 appears to be the crucial regulator of chondrocyte proliferation and maturation (Shu et al., 2011). GDF11/BMP11 is required for axial skeleton patterning and acts upstream of the *Hox* genes (McPherron et al., 1999; Oh et al., 2002). Postnatally, GDF11 acts on bone homeostasis by stimulating osteoclastogenesis and inhibiting osteoblast differentiation (Liu et al., 2016). Mutations in human *GDF5* (*BMP14, CDMP1*) or *BMPR1B* (*ALK6*) cause brachydactyly type C (OMIM 113100) and A2 (OMIM 112600), respectively (Lehmann et al., 2003; Polinkovsky et al., 1997). *Gdf5* and *Bmpr1b* mutant mice also display a brachydactyly phenotype (Storm et al., 1994; Baur et al., 2000; Yi et al., 2000). Closer examination of the *Gdf5* mutant brachypodism mouse revealed that the absence of the joint separating phalangeal elements 1 and 2 is due to the loss of the cartilaginous anlage and subsequent formation of the skeletal element by intramembranous instead of endochondral bone formation (Storm and Kingsley, 1999). The related family members *Gdf5*, *Gdf6*, and *Gdf7* are expressed in the interzone of different subsets of joints. *Gdf6* mutants display fusions of carpal and tarsal joints, and double mutants for *Gdf5/6* show additional skeletal defects (Settle et al., 2003). Interestingly, postnatally, GDF5 and GDF7 modulate the rate of endochondral tibial growth by altering the duration of the hypertrophic phase in the more active growth plate in opposite ways (Mikic et al., 2004, 2008). Bead-implant experiments in chicken and mouse embryos as well as various in vitro experiments revealed a prochondrogenic activity of BMP2, BMP4, or GDF5 protein, which can be antagonized by the secreted molecule Noggin (Zimmerman et al., 1996; Merino et al., 1999; Wijgerde et al., 2005). Consistent with this, Noggin-knockout mice display appendicular skeletal overgrowth and lack synovial joints (Brunet et al., 1998). Yet, surprisingly the caudal axial skeleton does not develop in the *Noggin* mutants. The vertebral phenotype can in part be reverted by the loss of one functional *Bmp4* allele, supporting the notion that too high levels of BMP4 signaling in the axial mesoderm may actually inhibit the differentiation of sclerotomal cells to chondrocytes. Instead, these cells take on a lateral mesodermal fate (Wijgerde et al., 2005; Murtaugh et al., 1999; Hirsinger et al., 1997). Double knockout of the BMP receptors *Bmpr1a* (*Alk3*) and *Bmpr1b* (*Alk6*) revealed a functional redundancy of these two receptors in endochondral ossification. Chondrocyte differentiation in the axial and appendicular skeleton is severely compromised in the mice lacking both receptors (Yoon et al., 2005). Conditional mutants for activin receptor type IA (*Alk2*) display only mild axial phenotypes. Double mutant analysis revealed a functional redundancy with *Bmpr1a* and *Bmpr1b* in endochondral skeletogenesis (Rigueur et al., 2015). Conditional postnatal deletion of *Bmpr1a* revealed a role for BMP signaling in the maintenance of the chondrogenic cell fate in the growth plate (Jing et al., 2013). Constitutively activating mutations in *ALK2* are found in patients with fibrodysplasia ossificans progressiva (OMIM 156400), a rare disorder in which the connective tissue progressively ossifies after traumatic injury (Shore et al., 2006). For further reading see reviews by Rosen (2006), Pogue and Lyons (2006), Wu et al. (2007, 2016), and Wang et al. (2014b).

Parathyroid hormone-related protein and Indian hedgehog

The paracrine hormone parathyroid hormone-related protein (PTHrP) and its receptor PTH1R are part of a crucial regulatory node, also referred to as the IHH/PTHrP feedback loop, coordinating chondrocyte proliferation with maturation in endochondral bone formation (Fig. 1.7). PTHrP is also required for normal intramembranous ossification (Suda et al., 2001). In the appendicular skeletal elements, PTHrP is expressed locally at high levels in the periarticular cells and at lower levels in the proliferating chondrocytes. Its receptor is expressed also at low levels in proliferating and at higher levels in pre-HCCs (Lee et al., 1996; Vortkamp et al., 1996; St-Jacques et al., 1999). *PTHrP* and *Pthr1* mutant mice display similar, but not identical phenotypes, with numerous skeletal abnormalities, including severely shortened long bones (Karaplis et al., 1994; Lanske et al., 1996). In both, the shortening of the long bones is associated with reduced chondrocyte proliferation and accelerated HCC maturation and bone formation (Amizuka et al., 1996; Lee et al., 1996; Lanske et al., 1998). Chimeric mice with $Pth1r^{-/-}$ clones in their growth plates revealed that the effects on chondrocyte maturation were direct but influenced by positional cues, as these clones expressed either *Ihh* or *Col10a1* ectopically dependent on their location within the proliferative zone (Chung et al., 1998). Concomitantly, mice overexpressing either PTHrP or a constitutively active form of PTH1R in chondrocytes show a delay in HCC maturation early and a prolonged persistence of HCCs associated with a delay in blood vessel invasion at later stages of development (Weir et al., 1996; Schipani et al., 1997b). PTH1R is a seven-transmembrane receptor coupled to heterotrimeric G proteins, consisting of α, β, and γ subunits. Its activation by PTHrP results in signaling via either the $G_s(\alpha)$/cAMP or the $G_q(\alpha)$/inositol-3-phosphate-dependent pathway. The two downstream pathways have opposing effects on chondrocyte hypertrophy with $G_q(\alpha)$/inositol-3-phosphate-dependent signaling cell-autonomously accelerating hypertrophic differentiation, while $G_s(\alpha)$/cAMP-signaling delays it (Guo et al., 2002; Bastepe et al., 2004). The intracellular mediator of the canonical WNT signaling pathway, β-catenin, interacts with the PTH1R and may modulate the switch from $G_s(\alpha)$ to the $G_q(\alpha)$ signaling (Yano et al., 2013; Yang and Wang, 2015). The $G_s(\alpha)$/cAMP signaling pathway is also involved in the maintenance of the pool of round proliferating

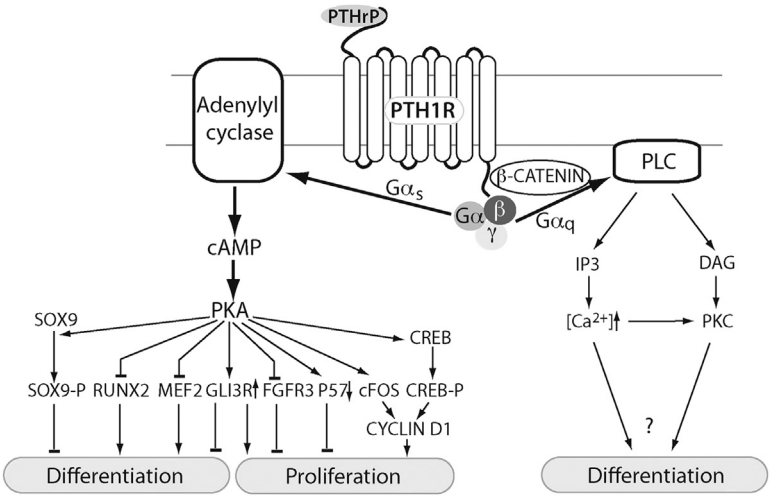

FIGURE 1.7 PTHrP/PTH1R signaling pathways and their functional consequences on chondrocyte differentiation and proliferation. The molecular mechanism underlying the differentiation-promoting effect of the PLC signaling branch is not yet understood. *PKA*, protein kinase A; *PLC*, phospholipase C; *PTHrP*, parathyroid hormone-related protein; *PTH1R*, PTHrP receptor.

chondrocytes (Chagin and Kronenberg, 2014). The inhibitory effect on chondrocyte hypertrophy is mediated through the activation of protein kinase A (PKA) downstream of $G_s(\alpha)$/cAMP signaling. This, in turn, promotes the following response: translocation of histone deacetylase 4 (HDAC4) into the nucleus where it binds to and inhibits the transcriptional activity of MEF2 transcription factors (Kozhemyakina et al., 2009). Furthermore, PTHrP signaling increases the expression of the transcription factor ZFP521, which negatively influences the transcriptional activity of RUNX2, again through recruitment of HDAC4 (Correa et al., 2010). In addition, PTHrP can decrease RUNX2 production and enhance its degradation specifically in chondrocytes (Guo et al., 2006; Zhang et al., 2009, 2010). MEF2 and RUNX2 are both positive regulators of chondrocyte hypertrophy (see later). PKA also phosphorylates SOX9, enhancing its DNA-binding activity, and stimulates GLI3 processing into its repressor fragment, thereby potentially interfering with chondrocyte maturation (Huang et al., 2000; Wang et al., 2000; Mau et al., 2007). PTH1R signal via PKA also inhibits the transcription of FGFR3 (McEwen et al., 1999). Furthermore, it leads to a downregulation of the cell-cycle-dependent inhibitor P57, a negative regulator of chondrocyte proliferation (Yan et al., 1997; MacLean et al., 2004). Last but not least, PTHrP signaling may stimulate proliferation through AP1/CREB dependent activation of cyclin D1 (Ionescu et al., 2001).

The findings in the different *Pthrp*/*Pth1r* mouse models can be correlated with activating mutations in the human PTH1R that lead to ligand-independent cAMP accumulation in patients with Jansen-type metaphyseal dysplasia (OMIM 156400) (Schipani et al., 1995, 1996, 1997a, 1999). On the other hand, the loss-of-function mutants correlate with the Blomstrand chondrodysplasia disorder (OMIM 215045) associated with the absence of a functional PTH1R (Karaplis et al., 1998; Zhang et al., 1998; Jobert et al., 1998). Interestingly, in the recessive Eiken skeletal dysplasia syndrome (OMIM 600002), a mutation leading to a C-terminal truncation of PTH1R has been identified that results in a phenotype opposite to that of Blomstrand chondrodysplasia and resembles a transgenic mouse model in which PTH1R signal transduction via the phospholipase C/inositol-3-phosphate-dependent pathway is compromised (Guo et al., 2002; Duchatelet et al., 2005).

Ihh, encoding a secreted molecule of the HH family, is expressed in pre-HCCs and has been shown to regulate the expression of *PTHrP* (Vortkamp et al., 1996; St-Jacques et al., 1999). This regulation is probably mediated by TGFβ2 signaling (Alvarez et al., 2002). *Ihh*-knockout mice display defects in endochondral and intramembranous bone formation (St-Jacques et al., 1999; Abzhanov et al., 2007; Lenton et al., 2011). In endochondral bone formation, IHH has multiple functions; it regulates proliferation and chondrocyte hypertrophy and is essential for osteoblastogenesis in the perichondrium. The last function of IHH apparently requires additional effectors other than RUNX2 (Tu et al., 2012). Conditional deletion of *Ihh* in *Col2a1*-CRE-expressing cells recapitulates the total knockout phenotype, including the multiple synostosis phenotype, a severe form of synchondrosis (Razzaque et al., 2005). In humans, *IHH* mutations are associated with brachydactyly type A1 (OMIM 112500), while copy number variations including the *IHH* locus are associated with syndactyly and craniosynostosis (Gao et al., 2009; Klopocki et al., 2011). The effects of *Ihh* on chondrocyte hypertrophy are PTHrP dependent as well as independent, while those on proliferation, osteoblastogenesis, and joint formation are PTHrP independent (Karp et al., 2000; Long et al., 2001, 2004; Kobayashi et al., 2005; Amano et al., 2016; Mak et al., 2008).

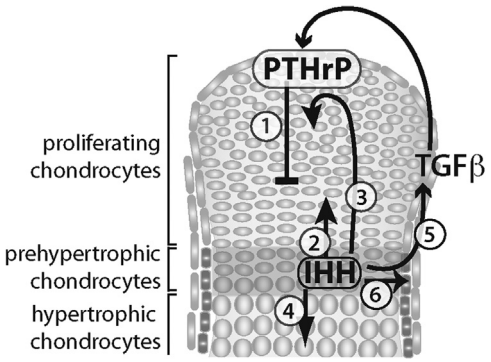

FIGURE 1.8 Parathyroid hormone-related protein (*PTHrP*) and Indian Hedgehog (*IHH*) interactions and functions in the growth plate. IHH and PTHrP participate in a negative feedback loop to regulate chondrocyte proliferation and differentiation. PTHrP is expressed from the perichondrial cells at the articular region and at low levels in round proliferative chondrocytes. It acts on proliferating chondrocytes, keeping them in a proliferative state and preventing their differentiation to prehypertrophic and hypertrophic chondrocytes (**1**). When the PTHrP concentration is sufficiently low enough, chondrocytes drop out of the cell cycle and differentiate into IHH-producing prehypertrophic chondrocytes. IHH, in turn, stimulates the proliferation of the adjacent flattened proliferating chondrocytes (**2**) and accelerates the progression of round to flattened proliferating chondrocytes (**3**) as well as the differentiation of prehypertrophic to hypertrophic chondrocytes (**4**). IHH also stimulates, probably mediated by transforming growth factor β (*TGFβ*) signaling, PTHrP production at the articular ends of the skeletal element (**5**) and acts on perichondrial cells, stimulating their differentiation into osteoblasts (**6**).

As mentioned earlier, the transcription factor GLI3 acts downstream of HH signaling, whereby HH signaling prevents the proteolytic conversion of GLI3 into the repressor form GLI3R. Mutations in *GLI3* are associated with Greig cephalopolysyndactyly (OMIM 175700) and Pallister−Hall syndrome (OMIM 146510) (Demurger et al., 2015). The mouse mutant extra-toes (Xt), a model for Greig cephalopolysyndactyly syndrome, has a deletion in the *Gli3* gene and displays numerous skeletal abnormalities, such as polydactyly, shortened long bones, split sternum, and craniofacial defects (Hui and Joyner, 1993; Vortkamp et al., 1992; Mo et al., 1997). Craniofacial abnormalities and shortened appendicular long bones are also reported in *Gli2* mutants (Mo et al., 1997). Interestingly, in double mutants for *Ihh* and *Gli3* the proliferation defect observed in the *Ihh* mutants is restored and the accelerated HCC differentiation, observed in $Ihh^{-/-}$ specimens, reverted (Hilton et al., 2005; Koziel et al., 2005). In contrast, the defects in osteoblastogenesis and cartilage vascularization are only partially rescued by the loss of *Gli3* (Hilton et al., 2005). Based on the observations in $Ihh^{-/-}$;$Gli3^{-/-}$ double mutants, Koziel and colleagues proposed a model whereby the IHH/GLI3 system regulates two distinct steps in chondrocyte differentiation: first, the transition from distal, round chondrocytes to the columnar chondrocytes, which appears to occur in a PTHrP-independent fashion, and second, the transition from proliferating to HCCs occurring in a PTHrP-dependent fashion (Koziel et al., 2005). Yet, Mak and colleagues proposed that Ihh also promotes chondrocyte hypertrophy in a PTHrP-independent way (Mak et al., 2008) (Fig. 1.8). In addition, Ihh activity is required for the maturation of the perichondrium and, in a cell-autonomous fashion, for the maintenance of endothelial cell fate (Colnot et al., 2005).

WNTs and β-catenin

As mentioned earlier β-catenin-mediated WNT signaling plays an important role as a permissive signal in the early steps of endochondral bone formation, enabling the differentiation of osteoblasts and cells contributing to the joint by repressing the chondrogenic potential within the respective precursor populations. The critical role of WNT/β-catenin signaling in osteoblastogenesis is first shown by the findings that human mutations in the WNT receptor LRP5 cause osteoporosis−pseudoglioma syndrome (OMIM 259770) (Gong et al., 2001; Lara-Castillo and Johnson, 2015). Mutations in the *WNT1* gene are causative for osteogenesis imperfecta, type XV, and an autosomal-dominant form of susceptibility to early onset of osteoporosis (OMIM 615220, 615221) (Keupp et al., 2013; Laine et al., 2013; Pyott et al., 2013). Numerous WNT-pathway molecules have been identified in genome-wide association studies related to skeletal phenotypes (Hsu and Kiel, 2012).

Stabilization of β-catenin in limb mesenchymal cells interferes with the initiation process of endochondral ossification (Hill et al., 2005). In contrast, expression of a constitutively active form of the downstream transcription factor LEF1 in *Col2a1*-expressing cells inhibits further maturation of chondrocytes and interferes with the formation of joints (Tamamura et al., 2005). Later during chondrocyte differentiation, WNT/β-catenin signaling regulates chondrocyte maturation in a positive manner (Hartmann and Tabin, 2001; Enomoto-Iwamoto et al., 2002; Akiyama et al., 2004; Day et al., 2005; Hill et al., 2005; Hu et al., 2005; Spater et al., 2006b; Joeng et al., 2011; Dao et al., 2012). This is mediated in multiple ways,

via direct regulation of *Ihh*, through interference with SOX9, and in a RUNX2-dependent fashion (Akiyama et al., 2004; Yano et al., 2005; Spater et al., 2006b; Dong et al., 2006; Dao et al., 2012; Mak et al., 2008). In HCCs, β-catenin signaling downregulates the expression of *Rankl*, thereby locally regulating the differentiation of osteoclasts at the chondro-osseous border (Golovchenko et al., 2013; Wang et al., 2014a; Houben et al., 2016). Based on overexpression of an intracellular inhibitor of β-catenin, ICAT, it has been proposed that β-catenin positively regulates VEGF and MMP13 (Chen et al., 2008). Yet, this has not been confirmed in conditional β-catenin mutants. Transient activation of β-catenin during early postnatal development leads to abnormal growth plate closure and promotes secondary ossification center formation (Yuasa et al., 2009; Dao et al., 2012). For further information see reviews by Baron and Kneissel (2013), Wang et al. (2014d), and Usami et al. (2016).

In addition to WNT/β-catenin-mediated signaling, a number of additional WNT signaling pathways are important within the growth plate. One that is highly relevant is the planar cell polarity pathway and its components, WNT5a and receptor tyrosine kinase orphan receptor (ROR2). Mutations in WNT5a and ROR2 are associated with Robinow syndrome (OMIM 268310; 164975) and brachydactyly type B1 (OMIM 113000) (Patton and Afzal, 2002; Person et al., 2010; Roifman et al., 2015). In mice, loss-of-function mutations in *Wnt5a*, *Ror2*, *Vangl2*, *Prickle1*, and *Ryk* result in skeletal dysplasias resembling those associated with Robinow syndrome (DeChiara et al., 2000; Takeuchi et al., 2000; Wang et al., 2011; Andre et al., 2012; Macheda et al., 2012; Gao et al., 2011; Yang et al., 2013b; Liu et al., 2014). In mice, *Wnt5a* and its related family member *Wnt5b* are both expressed in pre-HCCs (Yamaguchi et al., 1999; Yang et al., 2003; Witte et al., 2009). WNT5a promotes chondrocyte proliferation, as such mice lacking *Wnt5a* develop shorter skeletal elements due to a reduction in chondrocyte proliferation in zone II of the proliferating chondrocytes, encompassing the flattened proliferating chondrocytes (Yamaguchi et al., 1999; Yang et al., 2003). Furthermore, chondrocyte differentiation of HCCs is severely delayed in $Wnt5a^{-/-}$ mice as it is in *Ror2* mutants (DeChiara et al., 2000; Takeuchi et al., 2000; Oishi et al., 2003). Overexpression of either *Wnt5a* or *Wnt5b* primarily in chondrocytes also delays chondrocyte differentiation, yet, the two WNT ligands act on different chondrocyte subsets (Yang et al., 2003). The intracellular pathways underlying these effects are not known as of this writing. Compromised differentiation of HCCs may be associated with the capacity of WNT5a to induce the proteolytic cleavage of the transcription factor NKX3.2, which inhibits chondrocyte hypertrophy (Provot et al., 2006). In vitro, WNT5a and WNT5b can both activate calcium-dependent signaling leading to nuclear localization of nuclear factor of activated T cells (NFAT), as well as NF-κB signaling, and the kinase JNK (Oishi et al., 2003; Bradley and Drissi, 2010, 2011). The two pathways have differential effects on chondrogenesis (Bradley and Drissi, 2010). WNT5a signaling has also been shown to downregulate WNT/β-catenin signaling (Topol et al., 2003; Mikels and Nusse, 2006). However, experiments suggest that it can also enhance WNT/β-catenin signaling during osteoblastogenesis (Okamoto et al., 2014). Which pathway is preferentially activated may be decided at the level of the coreceptors (Grumolato et al., 2010).

Fibroblast growth factors and their receptors

FGF signaling also plays a critical role in the growth plate. Mutations in all three human *FGFRs* cause skeletal malformations, such as craniosynostosis syndromes (see also intramembranous ossification) and chondrodysplasia. Constitutively activating mutations in *FGFR3* are associated with hypochondrodysplasia (OMIM 146000), achondrodysplasia (OMIM 100800), and thanatophoric dysplasias type I (OMIM 187600) and type II (OMIM 187601). For reviews see Robin et al. (1993) and Ornitz and Marie (2015).

In the murine growth plate, *Fgfr2* is expressed at low levels in the round proliferating zone, also referred to as the resting zone. Proliferating and pre-HCCs express high levels of *Fgfr3*, and HCCs express high levels of *Fgfr1* (Ornitz and Marie, 2015). The growth retardation in conditionally deleted *Fgfr2* mice is attributed to alterations at the chondro-osseous junction (Yu et al., 2003). Yet, chondrocyte proliferation was unaffected in these mice. An increase in the zone of proliferating chondrocytes as well as HCCs was observed upon loss of *Fgfr3* (Colvin et al., 1996; Deng et al., 1996). In contrast, mice carrying an *Fgfr3* gene with human achondroplasia mutations display the opposite phenotype, a decrease in chondrocyte proliferation and a reduced zone of HCCs (Chen et al., 1999; Li et al., 1999). Of the different ligands, FGF9 and FGF18 have been identified based on their mutant phenotypes to be relevant in endochondral bone formation. Both are expressed in the perichondrium and periosteum. FGF9 and FGF18 are both required for chondrocyte maturation, as the onset of hypertrophy is delayed in $Fgf9^{-/-}$ and $Fgf18^{-/-}$ embryos (Hung et al., 2007; Liu et al., 2007). Yet, in the $Fgf9^{-/-}$ mutant only the stylopod elements are affected (Hung et al., 2007). Due to the delay in chondrocyte maturation vascular invasion is also delayed in both mutants. However, there is evidence that FGF18 may directly stimulate the expression of VEGF (Liu et al., 2007). In addition, FGF18 is required for chondrocyte proliferation. A 2016 allelic series study of *Fgf9*/*Fgf18* mutant embryos revealed unique and redundant roles of the two ligands in endochondral ossification (Hung et al., 2016).

C-type natriuretic peptide

In the growth plate, C-type natriuretic peptide (CNP) and its receptor GC-B are primarily expressed in proliferative and pre-HCCs (Chusho et al., 2001). In humans, homozygous loss-of-function mutations in the receptor cause acromesomelic dysplasia Maroteaux type (OMIM 602875), while heterozygous mutations are associated with short stature (Bartels et al., 2004; Olney et al., 2006; Vasques et al., 2013). Yet, the CNP/GC-B system is widely distributed in the body and as such, it was unclear whether it acts systemically or locally on endochondral ossification. Evidence for the latter is based on conditional knockouts of *Cnp* or *Gc-b* in *Col2a1*-Cre-expressing cells that recapitulate the dwarfism phenotypes of the respective full knockouts (Chusho et al., 2001; Tamura et al., 2004; Nakao et al., 2015). Dwarfism is associated with a decrease in the proliferative zone and in the number and size of HCCs. In contrast, loss of the clearance receptor NPR-C results in skeletal overgrowth similar to that in mice overexpressing the related molecule BNP (Suda et al., 1998; Jaubert et al., 1999). Skeletal growth can also be stimulated in a dose-dependent fashion by interfering with the clearance of CNP by overexpressing osteocrin, a natural NPR-C ligand (Kanai et al., 2017). In humans, overexpression of CNP is also associated with skeletal overgrowth (Bocciardi et al., 2007). Craniofacial studies in mice suggest that CNP/GC-B signaling primarily stimulates endochondral ossification (Nakao et al., 2013). Downstream signaling involves cyclic GMP-dependent kinase II but also interferes with the activation of the mitogen-activated protein kinase cascade downstream of FGF signaling (Miyazawa et al., 2002; Ozasa et al., 2005). For further reading see the review by Peake et al. (2014).

Notch signaling

Mutations in the Notch signaling components cause at least two human disorders with vertebral column defects, spondylocostal dysostosis (OMIM 277300, 608681, and 609813) and Alagille syndrome (OMIM 118450 and 610205) (Baldridge et al., 2010). Gain-of-function mutations in *NOTCH2* are found in Hajdu−Cheney syndrome, a rare skeletal disorder characterized by osteoporosis (OMIM 102500) (Majewski et al., 2011; Isidor et al., 2011). These diseases highlight, among others, the critical role of the segmentation clock in human axial skeletal development.

In chick and mouse, the Notch receptors 1−4 and the ligands, Delta1 and Jagged1/2, are expressed in a dynamic way within the developing limb skeleton, and inhibition of Notch signaling disrupts chondrocyte differentiation (Williams et al., 2009; Dong et al., 2010). Misexpression of the ligand Delta1 in chick inhibits the transition from pre-HCC to HCC (Crowe et al., 1999). A similar phenotype is observed upon conditional expression of the active Notch intracellular domain (NICD) in chondrocytes within the long bones, while a loss of skeletal elements due to impaired chondrogenesis is observed in the axial skeleton (Mead and Yutzey, 2009). The latter is associated with a downregulation of *Sox9* and, as shown in additional studies, with an enhanced proliferation of the mesenchymal progenitor cells, which is dependent on the activity of the transcriptional cofactor RBPjκ (recombination signal binding protein for immunoglobulin κ J region), which interacts with the NICD in the nucleus (Dong et al., 2010; Chen et al., 2013). Consistent with the osteoporosis phenotype in humans, the gain of Notch signaling in mice affects osteoblastogenesis of endochondral and membranous bones (Hilton et al., 2008; Mead and Yutzey, 2009; Dong et al., 2010). In contrast, interference with the Notch pathway by conditional deletion of *Presenilin 1/2*, encoding proteins required for the NICD release, or the *Notch1/2* receptors in the limb mesenchyme results initially in a delay of the onset of chondrocyte maturation and later in a delay of terminal differentiation leading to an elongated hypertrophic zone (Hilton et al., 2008). Conditional loss of the Notch effector RBPjκ results in a similar phenotype (Kohn et al., 2012). RBPjκ-independent Notch signaling, in contrast, affects the morphology of all growth plate chondrocytes and enhances osteoblast maturation (Kohn et al., 2012). In the articular chondrocytes, Notch signaling may be required for the maintenance of a chondroprogenitor population (Sassi et al., 2011).

Transcription factors

SOX9 and RUNX2 are master transcription factors that determine chondrocyte and osteoblast cell fates, respectively. It is not surprising that genetic defects in chondrocyte or osteoblast cell fate determination cause severe skeletal defects. Haploinsufficiency of SOX9 protein in humans causes campomelic dysplasia (OMIM 114290) with cartilage hypoplasia and a perinatal lethal osteochondrodysplasia (Meyer et al., 1997). Mutations in human *RUNX2* cause CCD (OMIM 119600), an autosomal-dominant condition characterized by hypoplasia/aplasia of clavicles, patent fontanelles, supernumerary teeth, short stature, and other changes in skeletal patterning and growth (Mundlos et al., 1997). The transcription factor OSX/SP7 acts downstream of RUNX2 within the osteoblast lineage (Nakashima et al., 2002; Nishio et al., 2006). Mutations in the human SP7 gene may be associated with osteogenesis imperfecta type XII (OMIM 613849) (Lapunzina et al., 2010).

Genetic studies in mice revealed that SOX9 plays numerous roles in skeletogenesis, from the initial differentiation of mesenchymal cells to chondrocytes to the maintenance of chondrogenic phenotype, survival, and the control of chondrocyte maturation (reviewed in Lefebvre and Dvir-Ginzberg, 2017). Its necessity for chondrocyte differentiation was first demonstrated by chimeric studies showing that *Sox9*-deficient cells are excluded from the cartilage (Bi et al., 1999). SOX9 activates the expression of two related family members, *Sox5* and *Sox6*, and cooperates with them, establishing and maintaining chondrocyte identity (Smits et al., 2001; Akiyama et al., 2002). SOX9 also interacts directly with and blocks the activity of the transcription factor RUNX2 at target promoters (Zhou et al., 2006). *Runx2* is expressed in pre-HCCs, HCCs, and osteoblast precursors and is important for HCC and osteoblast differentiation (Komori et al., 1997; Otto et al., 1997; Inada et al., 1999; Kim et al., 1999). Thus, this interaction maintains chondrocytes in a proliferative state and blocks their differentiation into HCCs and transdifferentiation into osteoblasts (Dy et al., 2012). RUNX2 acts partially redundantly with the related RUNX-family member RUNX3 on HCC maturation (Yoshida et al., 2004). *Mef2c* and *Mef2d*, members of the myocyte enhancer factor 2 family of transcription factors, are also expressed in pre-HCCs/HCCs. In contrast to *Mef2d*-knockout mice, which have no reported skeletal phenotype, *Mef2c*-deficient mice have shorter long bones associated with a delay in chondrocyte hypertrophy and downregulation of *Runx2* expression (Arnold et al., 2007; Kim et al., 2008). A constitutively active form of MEF2C upregulates *Runx2* and promotes chondrocyte hypertrophy, suggesting that MEF2C acts upstream of RUNX2 (Arnold et al., 2007). The activity of both transcription factors, MEF2C and RUNX2, is modulated by the histone deacetylase HDAC4 (see later). RUNX2 activity in HCCs is probably also modulated by interactions with other transcription factors such as *Dlx5/6*, which both physically interact with RUNX2 (Roca et al., 2005; Chin et al., 2007). Two members of the forkhead family of transcription factors, *Foxa2* and *Foxa3*, also play a role in HCCs. Both are expressed in HCCs and the loss of *Foxa2* results in decreased expression of hypertrophic markers, such as *Col10a1* and *Mmp13*, which is aggravated by the additional loss of *Foxa3* (Ionescu et al., 2012). The *SoxC* genes, *Sox4*, *Sox11*, and *Sox12*, are initially expressed in the mesenchymal progenitors of endochondral and intramembranous bone and become restricted to the perichondrium and joint as the chondrocytes differentiate (reviewed in Lefebvre and Bhattaram, 2016). In the progenitors, SOXC proteins are required for cell survival (Bhattaram et al., 2010). Later, during endochondral ossification, they are required for growth plate formation in part by promoting noncanonical WNT5a signaling (Kato et al., 2015). Other transcription factors, such as *Prrx1/Mhox* in combination with *Prrx2*, *Msx2*, and the *AP1* family member *Fra2*, also play roles in endochondral ossification (Martin et al., 1995; Lu et al., 1999; Karreth et al., 2004; Satokata et al., 2000). These can be acting locally restricted as is the case for *Prrx1/2* (Lu et al., 1999). For further information see reviews by Hartmann (2009), Karsenty (2008), and Nishimura et al. (2018).

The hypoxia-inducible transcription factor HIF consists of an α subunit that is regulated by oxygen and a β subunit that is constitutively expressed (Semenza, 2012; Ratcliffe, 2013). In growth plate chondrocytes, which are hypoxic, the subunit protein HIF-1α is stabilized and, on one hand, induces the expression of VEGF in HCCs and, on the other hand, regulates the oxygen consumption of chondrocytes through stimulation of anaerobic metabolism or glycolysis. Both downstream mechanisms are necessary for chondrocyte survival (Maes et al., 2012; Schipani et al., 2001, 2015; Cramer et al., 2004; Zelzer et al., 2004). The delayed differentiation observed in *Hif1a* mutants is probably a consequence of the initial delay in the initiation of chondrogenesis earlier in development (Provot et al., 2007; Amarilio et al., 2007). In contrast, mutation in the related α-subunit-encoding gene *Hif2a* results in only a transient and modest delay in endochondral ossification (Araldi et al., 2011). Yet, HIF2a appears to play a more prominent role postnatally in articular chondrocyte homeostasis (Pi et al., 2015; Yang et al., 2010).

Epigenetic factors and microRNAs

Since 2009, novel regulators of chondrogenesis and osteoblastogenesis have emerged, including epigenetic factors (reviewed in Furumatsu and Ozaki, 2010; Bradley et al., 2015). Among them is the histone deacetylase HDAC4, which plays a prominent role in HCC differentiation (Vega et al., 2004). HDAC4 binds to and inhibits the activity of two transcription factors that promote HCC differentiation, RUNX2 and MEF2C (Vega et al., 2004; Arnold et al., 2007). Histone-acetyl transferases such as P300 are important cofactors for BMP/SMAD1- and TGFβ/SMAD3-dependent signaling (Furumatsu et al., 2005; Pan et al., 2009; Sun et al., 2009). P300 also acts as a cofactor within the WNT/β-catenin pathway (Levy et al., 2004) and interacts with SOX9 (Furumatsu et al., 2005). SOX9 is also acetylated, which reduces its transcriptional activity, and this can be modulated by the NAD-dependent class III protein deacetylase Sirtuin (SIRT1) (Buhrmann et al., 2014; Bar Oz et al., 2016). SIRT1 and the histone methyltransferases SET7/SET9 also interact with P300 on the type II collagen promoter, promoting transcription (Oppenheimer et al., 2014). Conditional mouse mutants for the histone methyltransferase *Eset* have severely shortened limbs, a split sternum, and a widening of the sagittal suture of the skull (Yang et al., 2013a). The growth plates of *Eset* conditional knockout mice are disorganized, and HCC

differentiation appears to be accelerated. ESET interacts with HDAC4 to repress RUNX2 activity, thereby delaying hypertrophic differentiation (Yang et al., 2013a). Overall changes in the chromatin acetylation status in chondrocytes are induced through the interaction of the transcription factor TRPS1 with HDAC1 and HDAC4 (Wuelling et al., 2013).

Conditional deletion of *Dicer*, an enzyme that is required for the biogenesis of microRNAs, in chondrocytes revealed a functional role for microRNAs in chondrocyte proliferation and differentiation (Kobayashi et al., 2008). The latter is associated with a widened hypertrophic zone. Some of the specific microRNAs involved in these phenotypes are let-7 and miR-140 (Miyaki et al., 2010; Nakamura et al., 2011; Papaioannou et al., 2013). The noncoding RNA Dnm3os, a precursor for the microRNAs miR-199a, miR-199a*, and miR-214, is required for normal growth and skeletal development (Watanabe et al., 2008). In vitro, numerous microRNAs are differentially regulated during chondrogenesis and in osteoarthritis (Swingler et al., 2012; Crowe et al., 2016). For additional information on the role of microRNAs in skeletal development and homeostasis see Hong and Reddi (2012), Mirzamohammadi et al. (2014), and Fang et al. (2015).

Another class of RNA molecules with emerging functions in skeletal development are the long noncoding RNAs (lncRNAs). Mutations in the lncRNA *DA125942*, which interacts with PTHrP, result in brachydactyly type E (OMIM 613382) (Maass et al., 2012). The lncRNA *DANCR* promotes the chondrogenic differentiation of human synovial stem cell−like cells and is involved in osteoblastogenesis (reviewed in Huynh et al., 2017).

The functional roles of the vasculature in endochondral bone formation

Cartilage is an avascular and hypoxic tissue, yet, the ossification process and the remodeling of the cartilage template into cancellous bone require blood vessel invasion. Proliferating chondrocytes express numerous antiangiogenic factors, such as Chondromodulin I, Tenomodulin, Tissue-localized inhibitors of MMPs, and others (Maes, 2013). HCCs, in contrast, express VEGF, which is required to attract blood vessels to the perichondrium flanking the hypertrophic zone, as exemplified by mutant mice in which *Vegf* was deleted in cartilage or which lacked specifically the diffusible splice isoforms VEGF120 and VEGF164 (Zelzer et al., 2004; Maes et al., 2004, 2012). *Vegf* expression in HCCs is controlled by RUNX2 and, as mentioned earlier, by HIF1 (Zelzer et al., 2001). The invasion of blood vessels probably play an important role in the formation of the bone marrow cavity during endochondral ossification. Evidence for this is based on blocking VEGF signaling, which affects cartilage resorption, resulting in the elongation of the zone of HCCs (Gerber et al., 1999). Yet, as the monocytes, which are precursors for chondroclasts and osteoclasts, enter the bone marrow cavity via blood vessels, it is difficult to unambiguously distinguish between the functional requirements of the two components for the formation of the bone marrow cavity. Chondroclasts and osteoclasts produce matrix-degrading enzymes. Yet, the mineral dissolution function of osteoclasts is dispensable for the degradation of HCCs during long bone growth (Touaitahuata et al., 2014). Blood vessel endothelial cells also produce and secrete, among others, MMP9/Gelatinase B under proangiogenic conditions and may, therefore, be actively involved in the degradation of the cartilage matrix (Taraboletti et al., 2002). Blood vessels are, furthermore, important for trabecular bone formation during endochondral ossification. As mentioned previously, osteoblast precursors migrate into the forming bone marrow cavity along the blood vessels (Maes et al., 2010). In addition, it has been shown that the bone marrow cavity contains at least two types of blood vessels. In the embryo, an E and an L type can be distinguished, whereof the E type strongly supports osteoblast lineage cells (Langen et al., 2017). In the adult, the H-type vessels are the ones supporting osteoblast maturation (Kusumbe et al., 2014). Blood vessels also play a role as a structural component in trabecular bone formation. In addition to the mineralized cartilage matrix remnants, the vessels serve as structures for osteoid deposition (Ben Shoham et al., 2016).

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