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Part 1

Normal Fetal Skeletal Growth and Development

Skeletal Patterning, Bone Cell Differentiation and Bone Growth

The fetal skeleton comprises three embryonic origins: (1) the neural crest, (2) sclerotome and (3) lateral mesoderm, which give rise to (1) the craniofacial bones and clavicle, (2) axial skeleton – skull base (basiocciput), ribs, spine and sternum and (3) appendicular skeleton – shoulder girdle, pelvic girdle and tubular bones, respectively. Fetal bone development begins with condensation of undifferentiated mesenchymal cells. Formation of skeletal primordia is regulated by way of two distinctive, but synchronous, steps: patterning and differentiation. The former determines the position, number and shape of the primordial template. The latter refers to transition of the immature mesenchymal cells into bone cells (osteoblasts and osteocytes) and cartilage cells (chondroblasts and chondrocytes), which play a pivotal role in intramembranous ossification and endochondral ossification, respectively. These basic processes are almost completed during the early embryonic stage (8–12 weeks of gestation). At the end of the first trimester, most skeletal components are ossified and identifiable on postmortem radiograph. Afterward, the principal process is growth and maturation. Individual bones enlarge and mature according to the prenatal and postnatal timetables. Disruption of patterning leads to bone malformation (abnormally shaped bones) termed ‘dysostosis’. Impairment of differentiation results in generalised stunting of bone associated with disease-specific bone deformities, termed ‘bone dysplasia’.

Different Mechanisms of Ossification

Bone formation is governed by two different mechanisms, intramembranous ossification and endochondral ossification, which have different roles in bone formation. For embryonic development of primary ossification centres, the former contributes to ossification of the neural crest–derived bones (calvarium, facial bones, clavicles) and external surface (cortex) of the sclerotome/lateral mesoderm-derived bones (axial and appendicular skeleton), while the latter to the internal structure (spongiosa or cancellous bone) of the axial and appendicular skeleton. After primary ossifications centres are established, endochondral ossification occurs only at the epiphyseal cartilage (secondary ossification centre) and growth plate or growth plate equivalent of tubular bones, ribs, spine and flat bones. In the appendicular skeleton, and to a certain extent in the axial skeleton, endochondral bone formation contributes to longitudinal (directional) bone growth, while intramembranous bone formation contributes to radial (circumferential) bone growth.

Intramembranous Ossification

Intramembranous ossification is a relatively simple process. In neural crest–derived bones, undifferentiated mesenchymal cells condense as a primordium via cellular proliferation. Then, the mesenchymal cells evolve into osteoprogenitor cells and ultimately into osteoblasts that secrete the bone matrix proteins (e.g., type 1 collagen). The initial processes proceed in an avascular environment. Subsequently, vascular invasion into the bone anlagen occurs and induces matrix mineralisation. The initial small foci of mineralisation coalesce into a scaffold of immature trabeculae, into which osteoblasts are entrapped and differentiated into mature osteocytes. The immature bone (woven bone) is gradually remodelled into mature bone (lamellar bone) via repeated cycles of bone resorption and new bone formation. Defective ossification of neural crest–derived bones is clinically observed in cleidocranial dysplasia due to loss-of-function mutations in the *RUNX2* gene (**Figure 1.1a–c**). Intramembranous ossification is also essential in external or cortical bone formation of the axial and appendicular skeleton, which is closely linked to internal or cancellous bone formation through endochondral ossification.

Endochondral Ossification

Internal structures of the axial and appendicular skeleton are created by a complex process termed endochondral ossification, in which a cartilage intermediate plays an essential role. As with intramembranous ossification, the first step of endochondral ossification is condensation of undifferentiated mesenchymal cells to a primordium. The primitive condensation predetermines the position, size and shape of bone (patterning). Then, the mesenchymal cells differentiate into chondroblasts in an avascular environment. Simultaneously, the periphery of the condensation evolves into the perichondrium composed of immature perichondral cells. Perichondral cells retain chondrogenic potential, adding chondrocytes into the periphery of cartilage templates and contributing to their radial growth.

Chondroblasts further differentiate into chondrocytes, which undergo centrifugal maturation, developing into proliferating chondrocytes and differentiating into hypertrophic chondrocytes with their columnar formation. Chondrocytes secret cartilage matrix proteins (i.e., type 2 collagen and aggrecan). Hypertrophic

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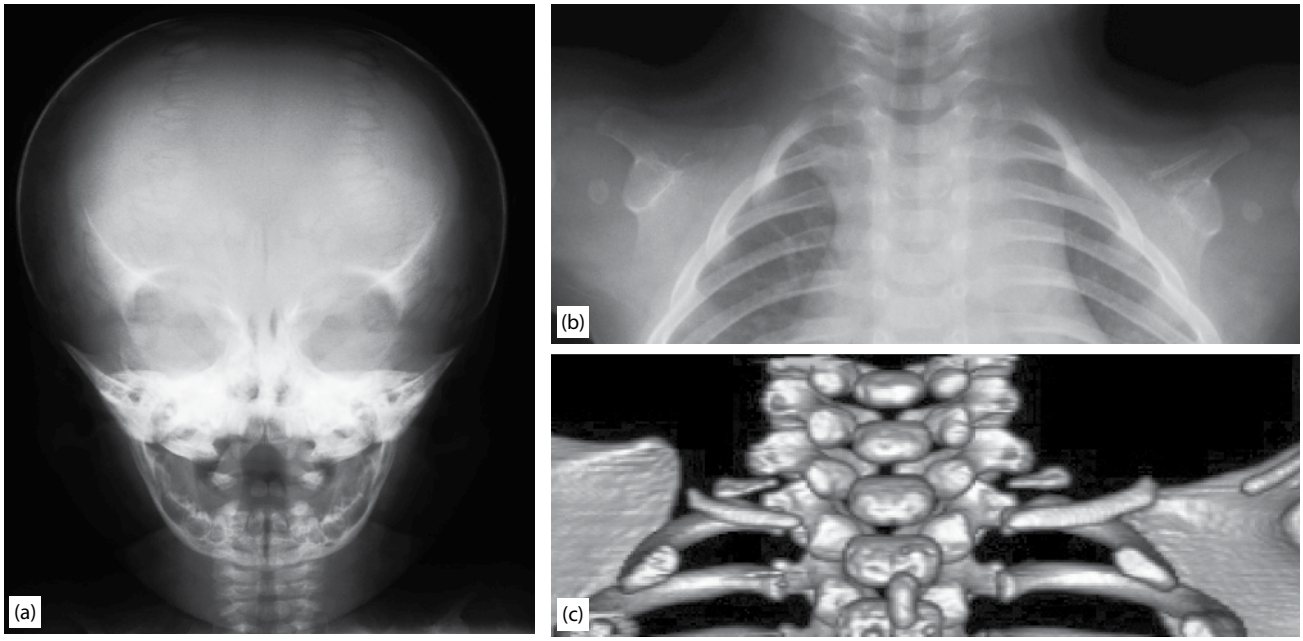

FIGURE 1.1 (a–c): Cleidocranial dysplasia. Note wide fontanelles with multiple Wormian bones and hypoplastic clavicles. The medial segment of both clavicles is formed. However, the right distal segment is missing, and the left is rudimentary along with pseudoarthrosis.

chondrocytes progress to the stage of terminal differentiation and undergo apoptosis. The subsequent processes are matrix mineralisation, vascular invasion and matrix absorption. Then, endochondral bone formation begins with deposition of immature osteoid. The woven bones are subsequently remodelled into mature bones via consecutive bone resorption and new bone apposition. While chondrocyte maturation proceeds, the perichondrium begins with intramembranous ossification for radial or circumferential growth of bone collars. External intramembranous ossification and internal endochondral ossification are linked to each other. External perichondral intramembranous ossification begins at the hypertrophic zone of the inner template.

During fetal growth, centrifugal endochondral ossification continues toward bone ends and ultimately forms growth plates (physes) and secondary ossification centres (epiphyses). The periphery of growth plates remains outlined by the perichondrium (perichondral ring), which probably adds chondrocytes to the growth plate. Endochondral bone growth at the growth plates follows the development of primary ossification centres. From the third trimester, epiphyseal ossification starts and continues to proceed postnatally.

The timing of growth plates formation is assumed to be programmed in advance. In fact, the growth plates are distant from bone ends, along with vertically long epiphyses in disorders with delayed terminal differentiation of chondrocytes (e.g., metaphyseal dysplasia Jansen type due to gain-in-function mutations of the *PTHLH* gene and cleidocranial dysplasia due to loss-of-function mutations in the *RUNX2* gene) (**Figure 1.2a**). Growth plates are scooped or cupped in shape in disorders with premature cessation of the terminal differentiation (e.g., achondroplasia due to gain-in-function mutations in the *FGFR3* gene). The finding is attributable to a relatively normal growth potential in chondrocytes that are newly added from the perichondrium to the growth plate (**Figure 1.2b**).

Embryonal Stage

The human skeleton originates from cells derived partially from paraxial mesenchyme and partially from the neural crest (part of the cranial vault and the viscerocranium). Bone tissue forms through a process involving ossification of a primordial cartilage model, which arises from mesenchyme. This process is defined as *indirect* or *endochondral* ossification and occurs in most bones. The bones of the cranial vault, part of the viscerocranium and the clavicle, form through a process known as *direct* or *intramembranous* ossification, in which bone develops from specialised regions of mesoderm, parts of which differentiate into osteoblasts and osteoclasts.

Morphogenesis is determined by a web of molecular programmes that overlap and regulate processes concerning position, differentiation, modelling, proliferation, apoptosis and cell renewal. Bone tissue forms and develops through a modelling process involving production of bone matrix by osteoblasts and resorption by osteoclasts. Osteoblasts derive from multipotential mesenchymal cells and further differentiate into bone-lining cells and osteocytes. Osteoclasts originate from haematopoietic precursor cells in common with monocytes and macrophages. Ossification starts in the eighth gestational week and continues postnatally until the mid-20s are reached. The human skeletal system is divided into two principal groups: the axial skeleton and the appendicular skeleton. The axial skeleton consists of the vertebral column, the thoracic cage and the skull. The appendicular skeleton consists of the pectoral girdles, the upper limbs, the pelvic girdles and the lower limbs.

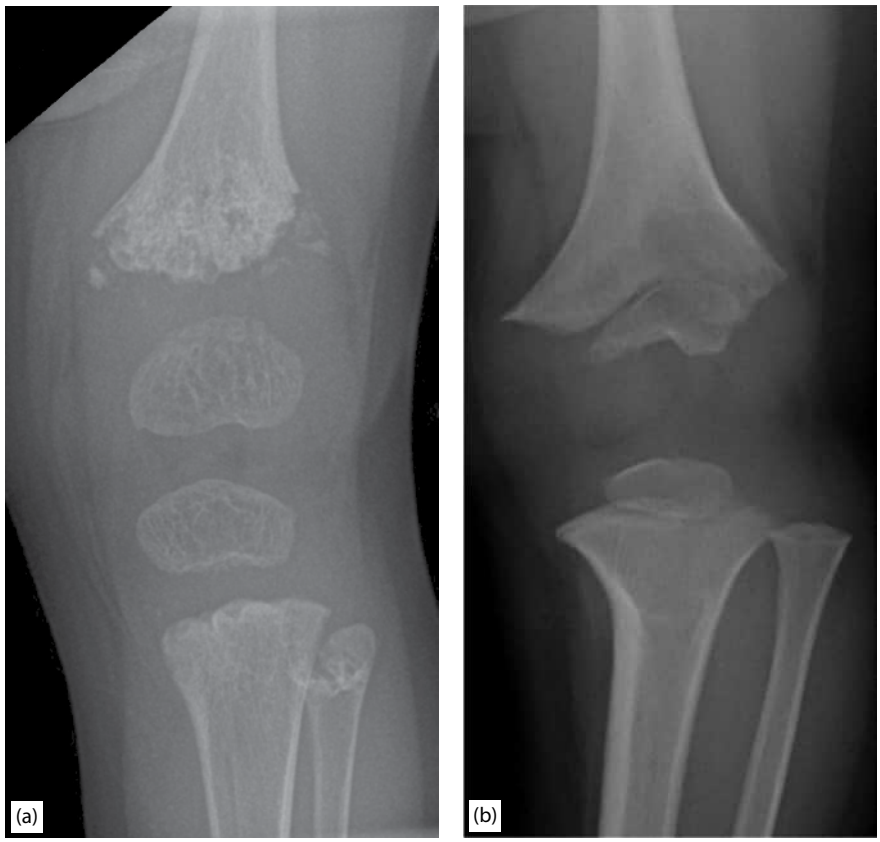

FIGURE 1.2 (a) A 3-year-old child with metaphyseal dysplasia Jansen type. Note wide growth plates, metaphyseal irregularities and mega-epiphyses. (b) A 3-year-old child with achondroplasia. Note metaphyseal cupping, metaphyseal flaring and small epiphyses.

Vertebral Column

During the third gestational week, the paraxial mesoderm condenses into spherical structures called somites, which are paired on either side of the neural groove and form in a craniocaudal direction. Within each somite are three distinct and temporally transient structures forming in a dorsoventral (DV) pattern: (1) the dermomyotome, (2) the myotome and (3) the sclerotome. The sclerotome forms bone tissue (i.e., the vertebral column). The myotome is a precursor for muscular structures, while the dermomyotome produces the progenitor cells of the dermis. The sclerotome undergoes a process known as 'epithelio-mesenchymal transition'. This differentiation is regulated by a number of surrounding tissues including the notochord, neural tube, lateral plate mesoderm and myotome. Later differentiation of the vertebrae and intervertebral discs involves remodelling of the initial segmentation in which a half-somite shifts caudally. Within the somites, it is possible to identify alternating loose and dense tissues that do not mix; the loose, cephalic tissue is the precursor of the centrum, while the dense, caudal tissue is the precursor of the intervertebral disc. The centrum encloses the notochord and gives rise to most of the vertebral body. The neural processes extend dorsally on each side of the neural tube, and later right and left processes unite to complete the neural arch. Therefore, cells derived from two adjacent somites form each vertebra.

The segmentation process is genetically controlled by an oscillating 'segmentation clock', which is determined by pulses of signalling of *Notch*, *Wnt* and fibroblast growth factor (*FGF*). The Notch pathway is particularly important for proper patterning of the developmental axes and vertebral modelling, and many components of this pathway have been identified (*DLL1*, *DLL3*, *LFNG*, *MIB1*, *POFUT1*, *PSEN1*, *CSL/RBPJ*), as well as some of the target genes (*HES7*, *MESP2*, *LFNG*).

HOX genes play a fundamental role both in axial skeletal patterning and in the proper development and modelling of the vertebrae, but the molecular net they rule is still largely unknown.

The vertebral column forms as a cartilaginous template that is later converted into bone by endochondral ossification. Ossification is incomplete, with an articular cartilage remnant adjacent to the intervertebral disc. The intervertebral disc then differentiates into a fibrocartilaginous disc. The vertebral body ossifies before the vertebral arch, which partially surrounds the spinal cord (a dorsal opening is closed by the dorsal ligament) to allow further growth of the spinal cord.

The ossification process starts at the ninth gestational week. At birth, the majority of the vertebrae show three ossification centres, one for the centrum and one for each lateral process.

Ribs

At the side of the vertebrae of the neck and trunk, close to the vertebral arches, small condensations of mesenchyme develop to form the costal processes. The ribs arise solely from the costal processes of the thoracic vertebrae. They start to develop on day 35, and by day 45 the first seven ribs fuse ventrally to the sternum. The other five ribs do not connect directly with the sternum and therefore are defined as false ribs. At the end of the embryonal stage, the cartilage model of the thoracic cage is complete. Around the sixth week an ossification centre appears at the angle of each rib. Other ossification centres will appear around puberty in the tubercles and head of each rib. The costal processes of the cervical vertebrae will form the anterior part of the transverse foramina, through which the vertebral arteries pass, while the costal processes of the lumbar vertebrae will form the transverse processes. At the sacral level, the costal processes take part in the formation of the alae.

Sternum

The sternum develops from two condensed structures of mesenchymal cells in the ventral body wall known as sternal bars. It fuses with the cartilaginous tissue of the ribs in a craniocaudal direction. By the ninth week the cartilaginous model of the sternum is complete. Ossification proceeds in a craniocaudal direction until the fifth postnatal month.

Skull

The skull has distinctive features that make it a unique structure within the skeletal system: an embryonal origin from neural crest cells in addition to formation via an intramembranous ossification process.

The bones of the skull are divided into two groups: (1) the viscerocranium, which includes the bones of the face and originates from neural crest cells, and (2) the cranial vault or neurocranium, which originates from the occipital somites. The bones of the cranial vault develop through intramembranous ossification, while the bones that form the base of the skull are formed by endochondral ossification.

The viscerocranium arises from the first two branchial arches. The first arch gives rise to the maxilla, zygomatic bone, temporal bone (dorsal portion), mandible and sphenomandibular ligament (ventral portion or Meckel cartilage). The second arch gives rise to the incus, malleus and stapes, which are the first bones to be fully ossified (4 months). The face develops between the fourth and tenth gestational weeks by fusion of five structures: the frontonasal process, a pair of maxillary swellings and a pair of mandibular swellings. Two nasal placodes, formed by condensed ectodermal tissue, form and develop over the frontonasal process and contribute to the nasal structures, the philtrum and the primary palate. The external auditory meatus and the tympanic cavity derive from the first pharyngeal cleft and pouch, respectively.

The cranial vault starts its development around the membranous labyrinth. The first structure to appear is the otic capsule followed by the basioccipital region. The process moves from the base of the cranium to the parietal region, and by the end of the embryonal stage, formation of the cartilaginous templates of the skull is complete. The bones of the cranial vault have large, flexible, fibrous joints (sutures: coronal, lambdoid, metopic, sagittal, squamous temporal), which allow the skull remarkable flexibility, enabling the head to pass through the birth canal and allowing postnatal growth of the brain.

The sutures gradually fuse at different postnatal times – first the metopic suture in infancy, then followed by the others much later. Ossification continues through puberty until the mid-20s. In old age, the sutures separating the vault plates are often completely ossified. Recent studies have shown that *noggin* (a bone morphogenetic protein [BMP] antagonist) is involved in closure of the sutures. *Ephrin*, *FGF2*, *FGFR2* and Twist-related protein 1 (*TWIST1*) also regulate the timing of sutural closure. Abnormal premature fusion (synostosis) of any of the sutures will lead to a number of different skull shape deformities.

Limbs

The first sign of the limbs is a small protrusion at the flank of the embryonal body. These limb buds arise from mesenchymal cells derived from the lateral plate mesoderm and are covered by an ectodermal layer that forms the apical ectodermal ridge (AER). The posterior half of the bud encompasses a region known as the zone of polarising activity (ZPA). The ZPA has a central and unique function in normal limb bud development, controlling survival and differentiation of the mesenchyme along the anteroposterior (AP) axis. The upper limb buds appear around day 24 at the cervicothoracic transition point, and the lower limb buds appear around day 28 at the lumbosacral transition point. The precise point in which the bud shows up along the AP axis is probably determined by the expression of *HOX* genes.

The limb bud grows and elongates in a proximal-to-distal direction and develops through stages that include mesenchymal condensation, differentiation into cartilage, creation of a cartilage anlage and endochondral ossification. The condensation of the mesenchyme produces three different elements in chronological sequence: (1) stylopod (proximal), (2) zeugopod (intermediate) and (3) autopod (distal). The patterning of the developing limb is also established in relation to the anteroposterior (AP) and dorsoventral (DV) axes.

In the upper limb, the first primordial bone to appear is the humerus, followed by the radius, ulna, metacarpals and phalanges. The tip of the developing buds acquires a flattened shape and is defined as the hand plate (day 32) or the foot plate (day 36). The mesenchyme then condenses to form a radial chondrogenic structure that represents the blueprint of the fingers. The digital rays are linked by a mesenchymal membrane, which progressively disappears by apoptosis to enable shaping of individual digits (days 46–49).

The cartilaginous template of the limb bones is completed around the eighth week, following which the ossification process

slowly begins. Cartilage, bones, tendons and skin derive from the mesenchyme within the limb buds. Muscles, nerves and vessels originate from the myotome and migrate progressively within the limb buds. The formation of the limb – from the first sign to the completion of the cartilaginous limb – takes 4 weeks. At the sixth week the limbs rotate anteriorly, so that the elbows and knees point laterally, and the palms and soles point medially toward the trunk. At the seventh week the limbs undergo another torsion, 90° along the longitudinal axis, so that the elbows are directed caudally and the knees cranially. At the eighth week a third counter clockwise rotation completes the positioning.

Limb morphogenesis and three-dimensional patterning are coordinated by interactions between the centres of different limb regions through the molecules that they produce: (1) AER – *FGFs*, (2) ZPA – sonic hedgehog (*SHH*) and (3) dorsal ectoderm (*WNT7A*). Abnormal coordination between these centres is responsible for abnormalities of the three-dimensional development of the limb – namely, reduction defects and duplications.

Proximal-Distal Axis: Fibroblast Growth Factors

Growth along the proximal-distal axis is dependent on the AER, a specialised epithelial layer set on the tip of the limb bud. The *FGFs*, four of which are specifically expressed within the AER, are the key molecular players of this specialised region. Removal of the AER in the chick embryo results in the loss of formation of adjacent skeletal structures: early in the limb bud phase, it results in the complete loss of the limb, while in later stages it results in the normal formation of proximal structures with loss of the more distal elements.

Anteroposterior Axis: Sonic Hedgehog

The posterior mesenchyme of the limb bud, the ZPA, has been linked to limb morphogenesis along the AP axis. *SHH* is the key signalling molecule of the ZPA: the addition of *SHH* proteins to the distal portions of the limb bud of the chick embryo triggers the development of mirror-image digital duplication, while the removal of *SHH* results in loss of skeletal elements in the AP axis.

SHH regulates the expression of *GLI3*, which is an important regulator of the AP patterning of the distal portion of the limb bud. *GLI3* and *dHAND* are expressed early in the limb bud in the anterior and posterior portions, respectively, and their expression appears to be mutually exclusive. Although they are interconnected, the expression of *dHAND* seems to be independent of the *SHH* pathway. *dHAND* is essential for establishing the normal AP patterning of the proximal structures (stylopod and zeugopod).

Dorsoventral Axis: WNT7A

DV patterning is dependent on signals derived from the ectoderm overlying the limb bud. At a very early stage of limb budding, *WNT7A* is expressed in the dorsal ectoderm and induces the transcription factor *LMX1B*, which is necessary for dorsal morphology of the target cells, particularly in the autopod. *WNT7A* expression is repressed in the ventral ectoderm by the transcription factor Engrailed (*EN1*), which is induced by *BMP* through activation of the receptor *BMPR1A*. The absence of *WNT7A* expression determines a biventral pattern of the autopod, while the absence of *ENL* or *BMP* expression causes a bidorsal pattern.

Fetal Stage

Up until the 14th week, development of the skeleton is relatively fast. Beyond 14 weeks, development is much slower and involves growth and modelling of the segments. There is good correlation between overall fetal development and femoral growth and modelling.

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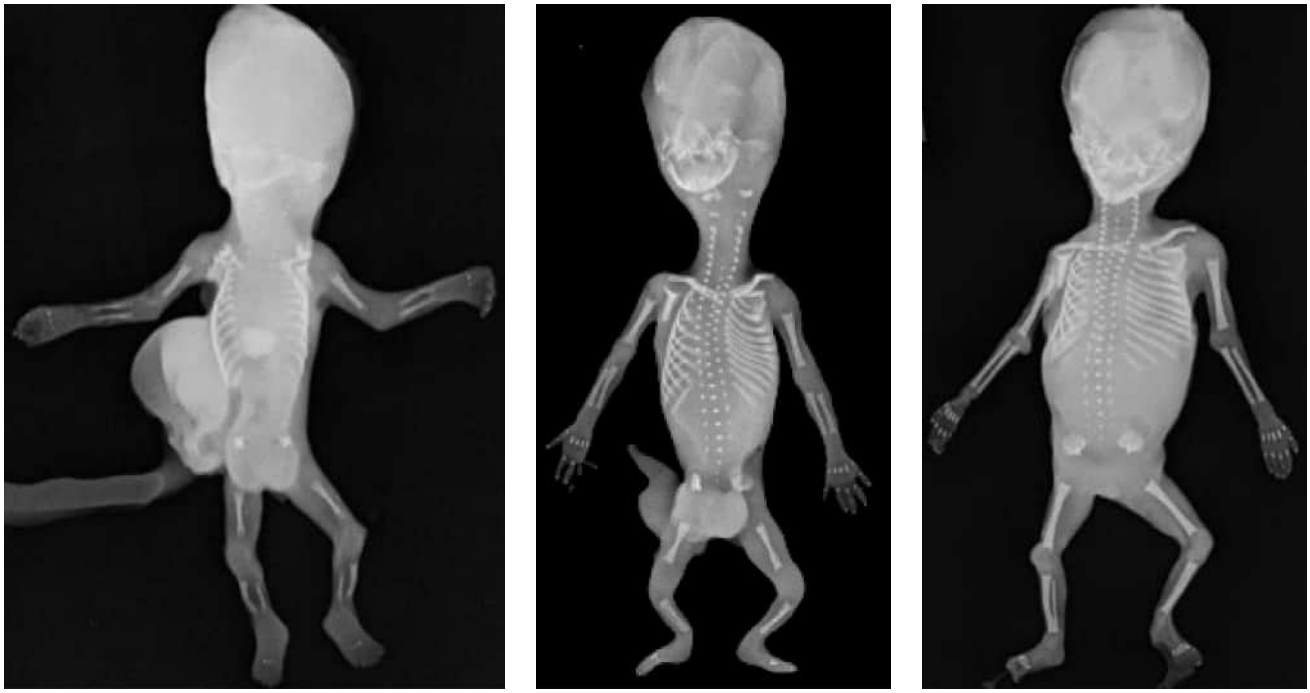
NORMAL FETAL RADIOGRAPHS

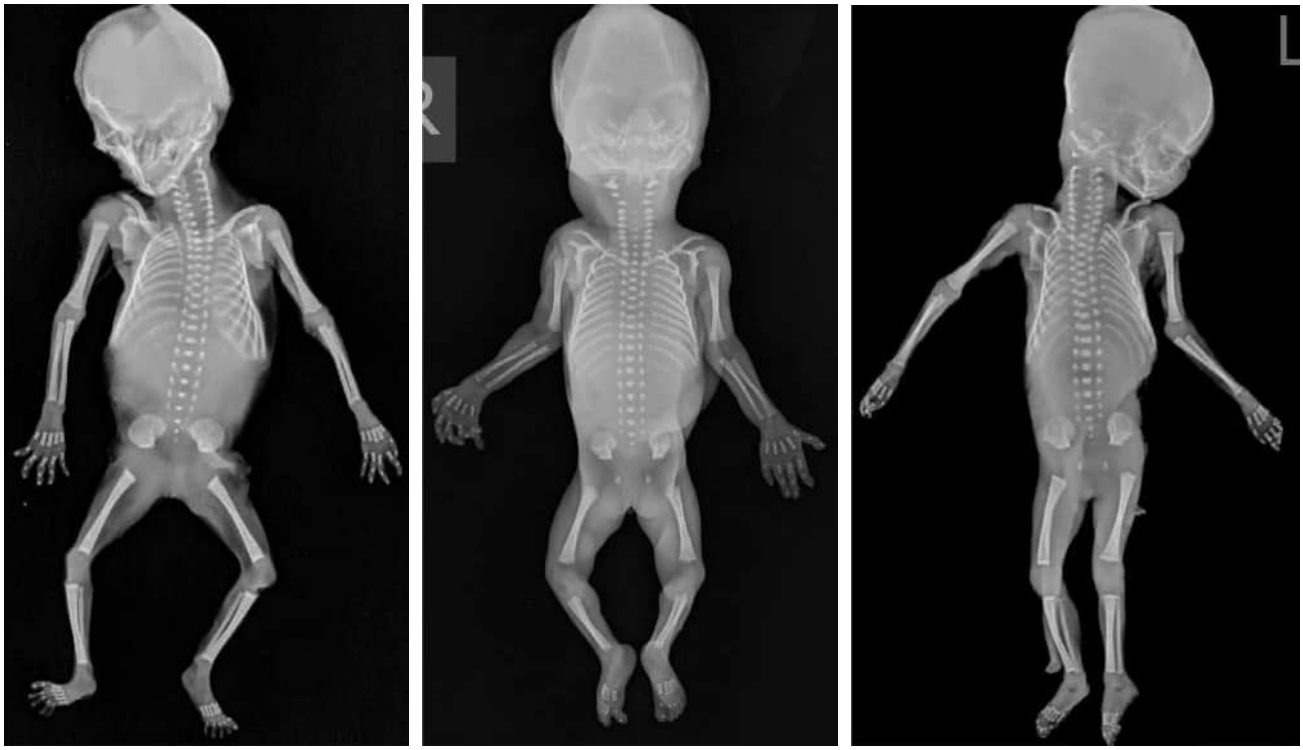
These images next should be consulted in conjunction with the synopsis of fetal development in Part 1.

11–14 weeks (11, 12 and 14 weeks):



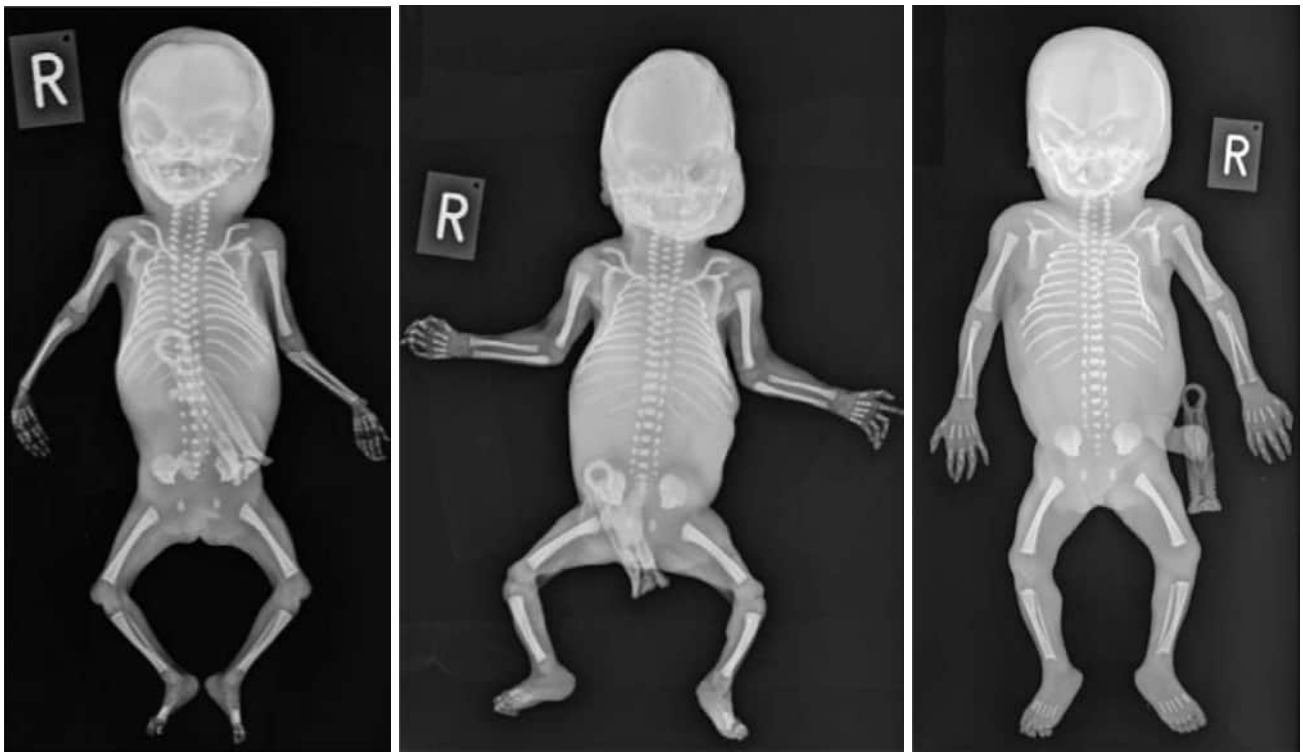
At 11 weeks of gestation, only the clavicles, scapulae, ilia and tubular bones are ossified. The short tubular bones are partly ossified. At 12 weeks, the thoracic and lumbar spine are ossified. At 14 weeks, the cervical spine and sacrum are partly ossified. All short tubular bones are ossified, but ossification of the middle phalanges is still incomplete.

15–17 weeks (15, 16 and 17 weeks):



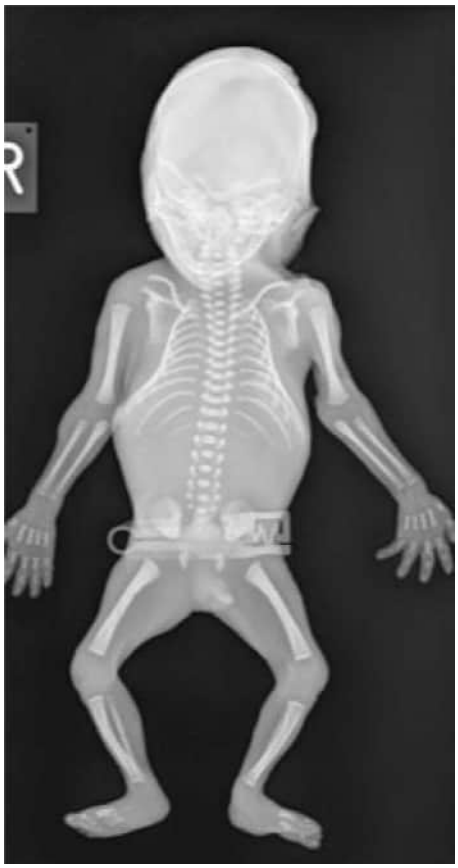
At 16 weeks, the ischia are ossified.

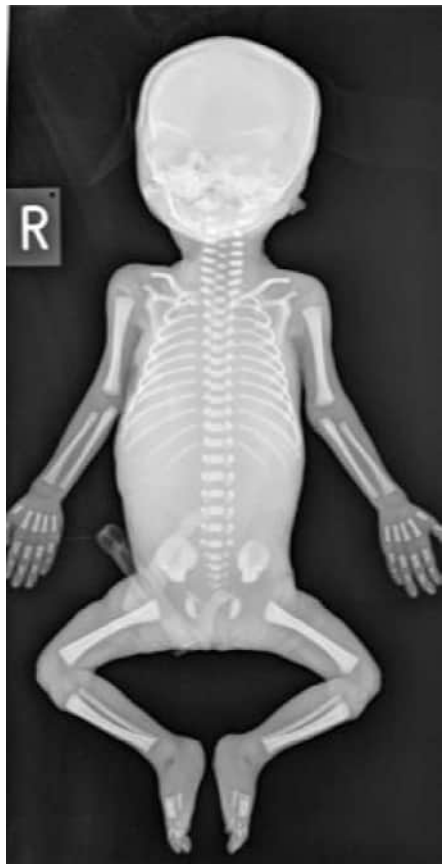
19–21 weeks (19 weeks, 20 weeks and 1 days and 21 weeks):

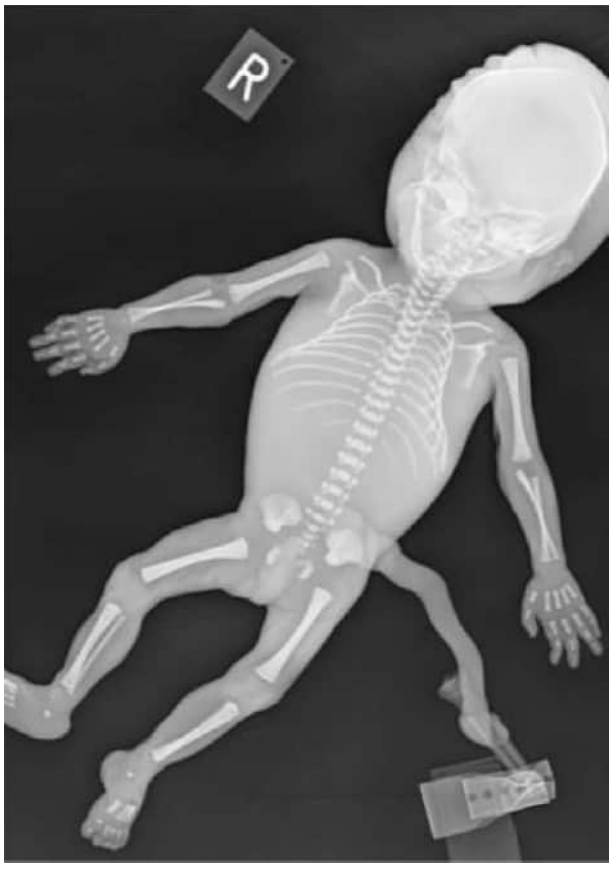


Ossification of all primary ossification centres, other than the pubic bones, is complete.

22–26 weeks (22 weeks, 24 weeks and 6 days and 26 weeks):

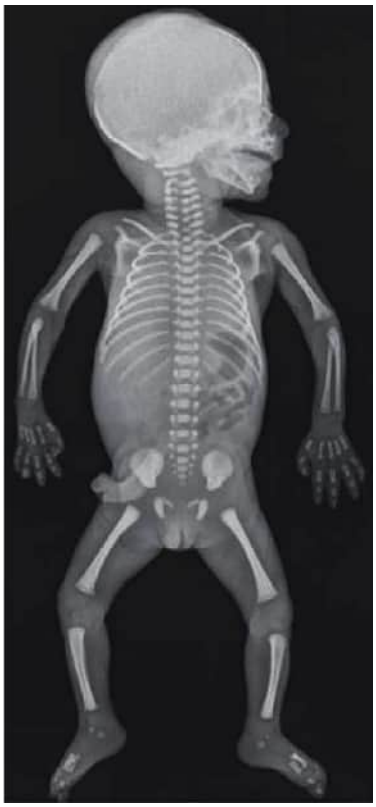


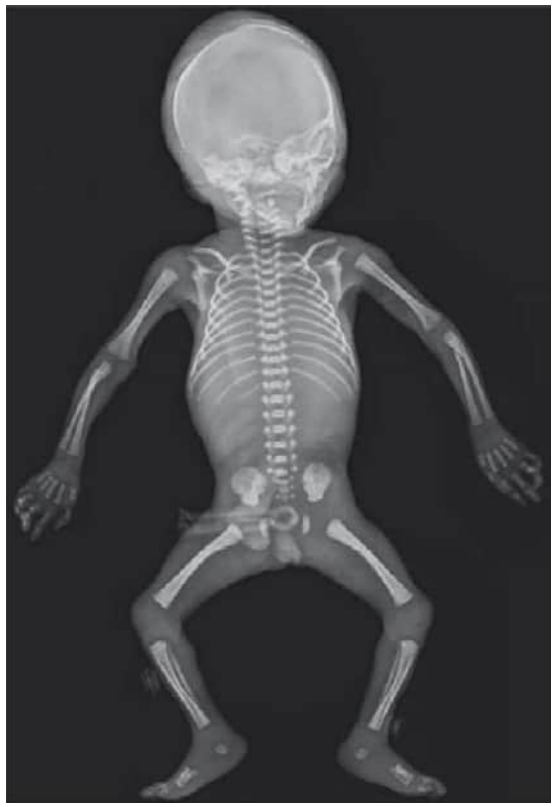




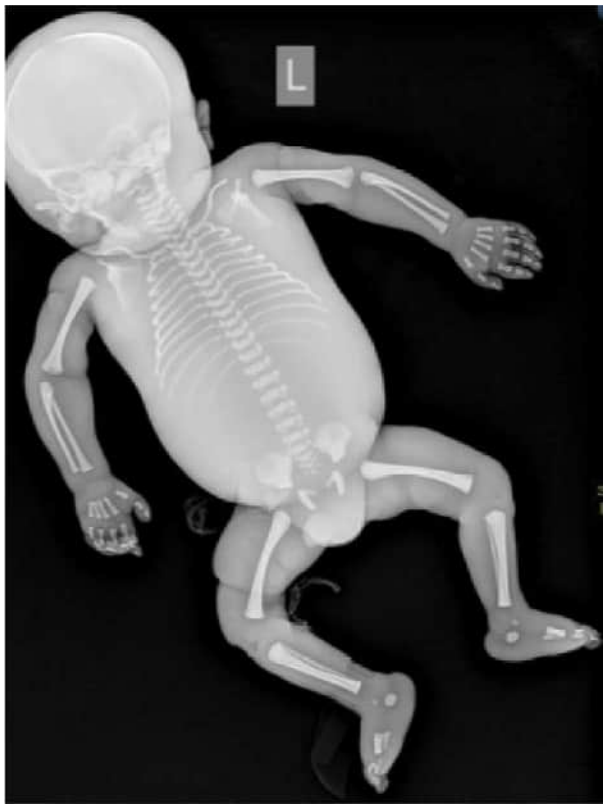
At 25 weeks (24 weeks and 6 days), pubic ossification is seen. At 26 weeks, the calcanei are ossified.

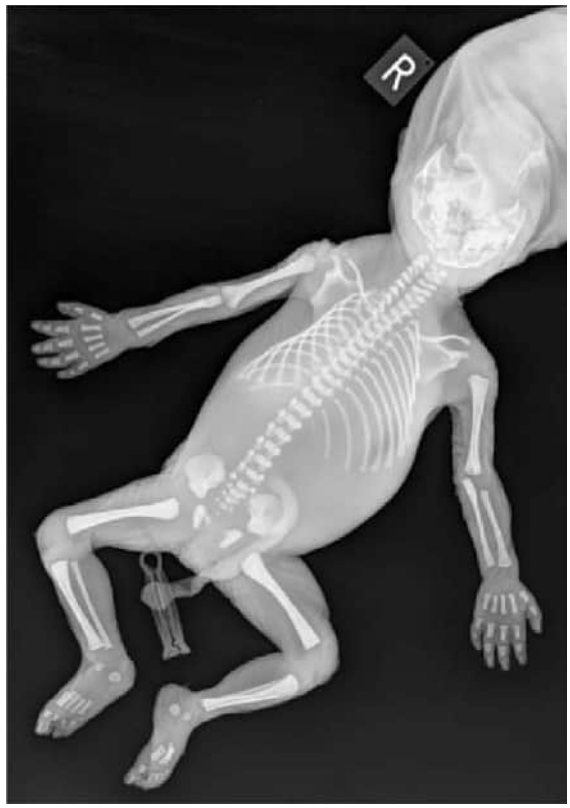
26 and 27 weeks:





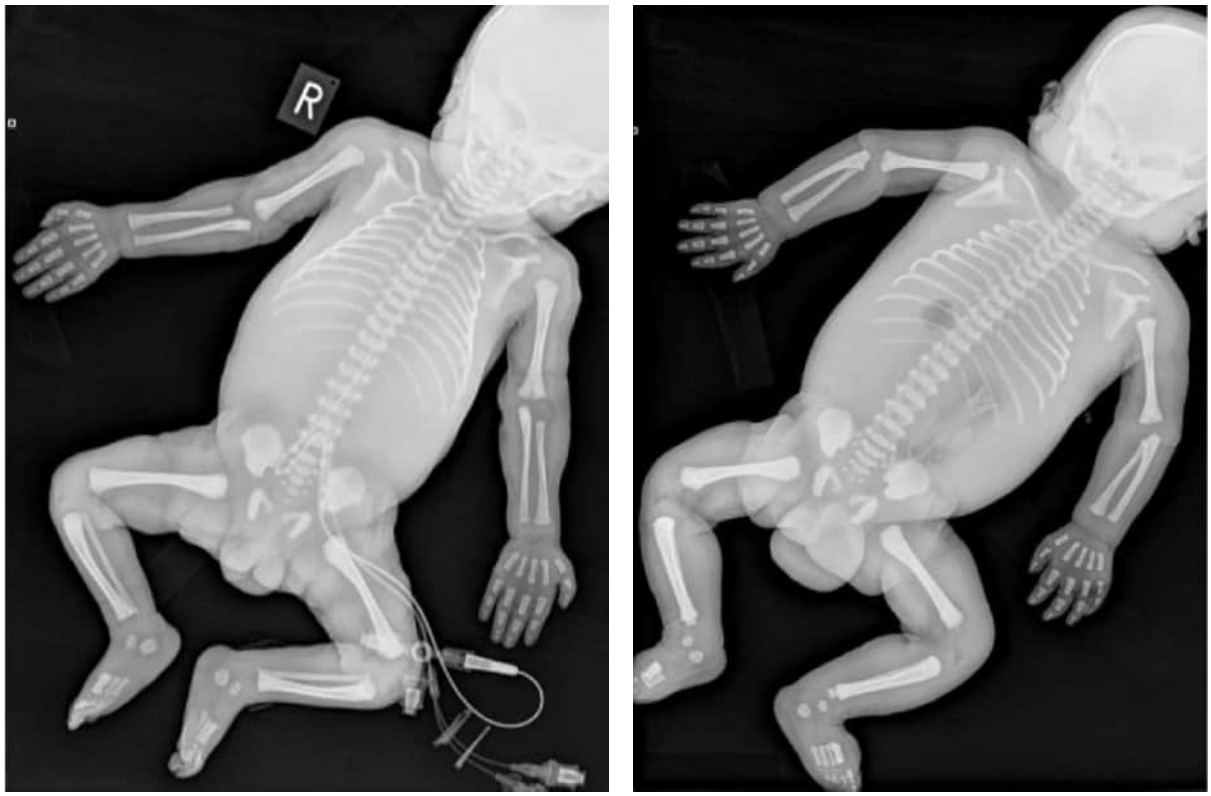
27–30 weeks (27 weeks and 30 weeks):





At 27 weeks, ossification of the calcanei and tali is seen.

32–35 weeks (32 weeks and 2 days and 35 weeks):



The calcanei and tali are well ossified at these gestational ages.

38–40 weeks (38 weeks and 2 days and 39 weeks and 3 days):