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Bone development in laboratory mammals used in developmental toxicity studies

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Evaluation of the skeleton in laboratory animals is a standard component of developmental toxicology testing. Standard methods of performing the evaluation have been established, and modification of the evaluation using imaging technologies is under development. The embryology of the rodent, rabbit, and primate skeleton has been characterized in detail and summarized herein. The rich literature on variations and malformations in skeletal development that can occur in the offspring of normal animals and animals exposed to test articles in toxicology studies is reviewed. These perturbations of skeletal development include ossification delays, alterations in number, shape, and size of ossification centers, and alterations in numbers of ribs and vertebrae. Because the skeleton is undergoing developmental changes at the time fetuses are evaluated in most study designs, transient delays in development can produce apparent findings of abnormal skeletal structure. The determination of whether a finding represents a permanent change in embryo development with adverse consequences for the organism is important in study interpretation. Knowledge of embryological processes and schedules can assist in interpretation of skeletal findings.

KEYWORDS
embryology, developmental toxicity testing, embryofetal toxicology testing, ossification, skeletal development, supernumerary ribs, wavy ribs

1 | DEFINITION OF THE PROBLEM

Developmental toxicity testing includes assessment of the near-term fetal skeleton in laboratory animals, particularly rats, mice, and rabbits. Alterations associated with treatment usually are categorized as malformations or variations, defined in section 2, below. The distinction between variations and malformations is not always clear, and different laboratories may produce different assessments. The biological importance of variations may also be unclear. We will review the published data on these skeletal findings with emphasis on the biological significance of the skeletal changes that have been the most variably interpreted.

We are not the first authors to undertake an assessment of developmental changes in the skeleton. Among the helpful reviews in the literature are those by Kimmel and Wilson (1973), Chernoff and Rogers (2004), Tyl, Chernoff, and Rogers (2007), Daston and Seed (2007), Carney and Kimmel (2007), Beyer et al. (2011), Kimmel, Garry, and DeSesso (2014), and Hofmann, Buesen, Schneider, and van Ravenzwaay (2016).

2 | REGULATORY REQUIREMENTS

2.1 | Techniques

Most pharmaceutical and many non-pharmaceutical chemicals are evaluated for developmental toxicity in experimental animals prior to marketing approval. The requirements for pharmaceutical chemicals have been harmonized for use in Europe, the US, and Japan (ICH, 2005, 2017). The draft revised ICH guidelines (R3) indicate that skeletal

examination of preferably all fetuses is part of the developmental toxicity evaluation; however, the method of skeletal examination is not specified. The newer techniques of microcomputerized tomography and magnetic resonance imaging are mentioned as acceptable methods, although traditionally, skeletal evaluation involves clearing of soft tissue with glycerin after maceration in potassium hydroxide, staining of bone with alizarin red, and, if desired, staining of cartilage with alcian blue (Redfern & Wise, 2007; Redfern, Wise, & Spence, 2007). One advantage of imaging studies for the evaluation of the skeleton is the ability to perform serial evaluations on the same individuals, permitting a determination of the permanence of findings noted near the time of birth (De Schaepdrijver, Delille, Geys, Boehringer-Shahidi, & Vanhove, 2014; Solomon et al., 2016).

For chemicals subject to regulation by the U.S. Environmental Protection Agency (EPA), the requirements for testing include examination for skeletal variations and malformations in approximately one-half of each litter in rodents and the entire litter in rabbits, with a preference for double staining of bone and cartilage (U.S. EPA, 1998). The Organisation of Economic Cooperation and Development test guideline 414 (OECD, 2001) is similar to the EPA guideline with respect to developmental toxicity testing.

2.2 | Definitions

The definitions of skeletal alterations in the draft ICH guidelines (ICH, 2017) are:

Malformation: Permanent structural deviation that generally is incompatible with or severely detrimental to normal postnatal development or survival.

Variation: Structural change that does not impact viability, development, or function (e.g., delays in ossification) which can be reversible, and is found in the normal population under investigation.

The ICH R3 draft goes on to say

> [R]eversible or minor manifestations of developmental toxicity (e.g., changes in fetal weight, skeletal variations) by themselves are of minimal concern from a risk assessment perspective. However, an increased incidence of variations can influence the interpretation of an equivocal increase in related malformations. The extent of concern will be influenced by other factors (e.g., exposure multiple at which the findings occurred, cross-species concordance).

The U.S. EPA (1998) guideline does not define malformation or variation, but the Developmental Risk Assessment Guideline (U.S. EPA, 1991) uses these definitions:

> A malformation is usually defined as a permanent structural change that may adversely affect survival, development, or function. . .The term variation is used to indicate a divergence beyond the usual range of structural constitution that may not adversely affect survival or health. Distinguishing between variations and malformations is difficult since there exists a continuum of responses from the normal to the extremely deviant. There is no generally accepted classification of malformations and variations. Other terms that are often used, but no better defined, include anomalies, deformations, and aberrations.

The OECD Test Guideline 414 includes these definitions:

Malformation/Major Abnormality: Structural change considered detrimental to the animal (may also be lethal) and is usually rare.

Variation/Minor Abnormality: Structural change considered to have little or no detrimental effect on the animal; may be transient and may occur relatively frequently in the control population.

These definitions include two criteria of importance for our discussion: Malformations are permanent and malformations are detrimental to the health of the organism. Both of these criteria are satisfied for unequivocal malformations.

A series of workshops on terminology in developmental toxicology were organized in Berlin, most recently in 2011 and 2014 (Solecki et al., 2013, 2015). Surveys were used to assess levels of agreement on nomenclature, including the distinction between malformations and variations. There was general agreement that variations might be distinguished from malformations based on their transience and lack of impact on health or viability of the animal. It also was agreed, however, that for some alterations, there were insufficient data to evaluate transience or health impact. Wavy ribs, which are transient in rodents and very infrequent in rabbits and primates, were accepted as variations. Ossification delays, supernumerary ribs, and vertebral centrum abnormalities were generally considered variations, although there was uncertainty about cervical ribs based on the association in humans with symptoms in some patients.

3 | EMBRYOLOGY OF THE SKELETON

3.1 | Introduction to skeletal development and timing

The development of all mammalian organisms extends from the time of fertilization through the postnatal period. While

birth demarcates the end of gestation, it does not represent the end of development (Morford, Henck, Breslin, & DeSesso, 2003). The gestation period of rats, mice, and rabbits is brief compared to that of humans. Consequently, much development in rodents and rabbits occurs after birth.

One aspect of development that occurs late in gestation is the mineralization of osseous tissue. Ossification proceeds by two mechanisms described as intramembranous or endochondral (described in textbooks of histology [e.g., Bloom & Fawcett, 1968; Mescher, 2016] and in reviews [e.g., Rivas & Shapiro, 2002]). Intramembranous ossification occurs within condensed mesenchyme as cells differentiate into osteoprogenitor cells, which proliferate, differentiate into osteoblasts, and begin to secrete extracellular osteoid matrix, within which calcification takes place. The flat bones of the skull, the bones of the face, and portions of the clavicle and scapula undergo intramembranous ossification. In contrast, nearly all of the extracranial bones of the body, including the long bones (e.g., bones of the arm, forearm, and digits), the sternum, ribs, and vertebral bodies undergo endochondral bone formation. During endochondral ossification, a hyaline cartilage model of the long bone appears first and is gradually replaced by ossified tissue as the organism matures.

Development and ossification of the digits, sternum, and other bones in rodents and rabbits occurs in the perinatal period (i.e., near the time of birth). As a result, the extent of prenatal ossification in fetuses has been used as an indicator of fetal maturity in developmental toxicity studies (Aliverti, Bonanomi, Giavini, Leone, & Mariani, 1979; Fritz, 1975). If a particular fetus has fewer ossified bones or bones with less ossification (judged by radiography or intensity of staining with bone-specific dyes) than is typically seen in control animals at the time of euthanasia, that fetus is considered to have experienced a reduced rate of development (Carney & Kimmel, 2007; Khera, 1981). Observations of reduced ossification are not malformations, because they are transient and typically catch up during the lactation period, which is a period of rapid growth during which rodent/rabbit offspring increase their body mass by 9–10 fold (Ellis-Hutchings et al., 2010). This increase in body mass is accompanied by a proportional increase in the size of the bones.

In order to understand the genesis of the various skeletal elements in the near-term fetus, it is important to review early embryogenesis, with emphasis on the establishment of the mesodermal germ layer and mesenchymal tissues in the embryo. While the length of gestation varies greatly among mammals, the order of occurrence of gestational milestones during mammalian development is virtually identical across species. Table 1 presents a list of major gestational milestones and the time in gestation at which they occur for important rodent and primate species, including humans. Because the literature places greater emphasis on human development, the following discussion typically describes events relative to human development. By using the data in

TABLE 1 Selected comparative gestational milestones in skeletal development (gestational days)

Gestational milestone	Rat	Mouse	Rabbit	Monkey	Human
Gestation length	22	20	32	165	266
Implantation	5.5–6	4.5–5	7–7.5	9	6–7
Primitive streak	8.5	7	7.25	15	13.5
Start of somite phase	9.5	7.75	7.75	20–21	19–21
10 Somites	10.5	8.5	8.5	23	25
Second pharyngeal arch and pouch	11	8.75	9.5	25	25
Forelimb buds appear	11	9.5	10.5	25	26
Third pharyngeal arch and pouch	11.5	9.5	–	27	28
Hindlimb buds appear	12	10.3	12	28	28
Meckel's cartilage	13.5	13	–	35–38	33–36
Forelimb digital rays	14	12.3	14.5	34	36
Hindlimb digital rays	15.5	13	16	36	38
Ossification centers appear in ribs	15–16	13–14	21	–	44
Hard palate closes	17	15	19	45	56

Note. Data from DeSesso (2012).

Table 1, one can readily translate the timing to the appropriate gestational age for the species of interest. The three major territories of the body that are discussed are the trunk, head and limbs.

3.2 | Trunk

3.2.1 | Somites

Appreciation of the complexity involved in development of the fetal/neonatal skeleton requires a brief introduction to the embryology of the skeleton.

The skeleton develops from mesenchymal tissues, most of which are formed as a result of gastrulation. Gastrulation occurs during the second week of gestation and is the process whereby the cells of the epiblast ingress through the primitive streak to form the mesodermal germ layer, which lies between the outer ectoderm and the inner endoderm (Figure 1). With continued development, the mesoderm near the midline develops into a pair of thickened columns of cells (paraxial mesoderm) that run parallel to the developing neural tube. More peripherally, the mesoderm remains flat and is termed the lateral plate. Within the paraxial mesoderm, starting at the cranial end of the embryo, cells organize into

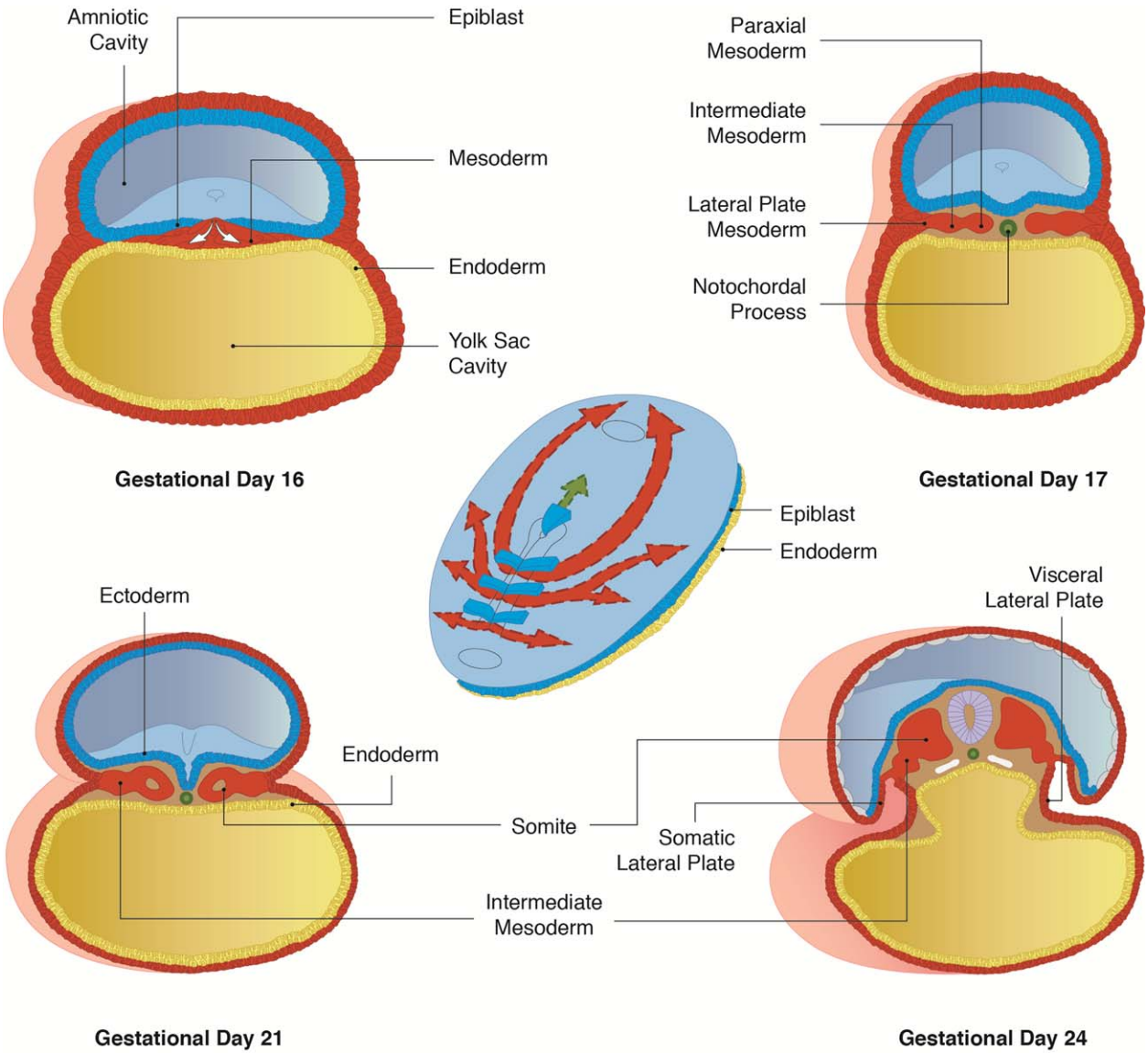

FIGURE 1 Formation of germ layers. The central image is a dorsal view of the embryo during ingression. The red arrows show the movement of epiblast cells toward and through the primitive streak and the migration of newly formed mesoderm. The green arrow denotes the notochord. The four surrounding cross sections illustrate the sequential organization of the mesoderm. At gestational Day 16, the epiblast actively migrates through the primitive streak to form mesoderm. At gestational Day 17, the mesoderm organizes into paraxial mesoderm (adjacent to the notochord), lateral plate mesoderm at the periphery, and intermediate between the two. By gestational Day 21, the paraxial mesoderm organizes into a somite. By gestational Day 24 the somite enlarges and the lateral plate splits into a somatic layer (associated with the ectoderm) and a visceral layer associated with the endoderm) Reproduced from DeSesso JM. Vascular ontogeny within selected thoracoabdominal organs and the limbs. Reproductive Toxicology, 2017; **70**: 3–20, copyright Elsevier, Inc

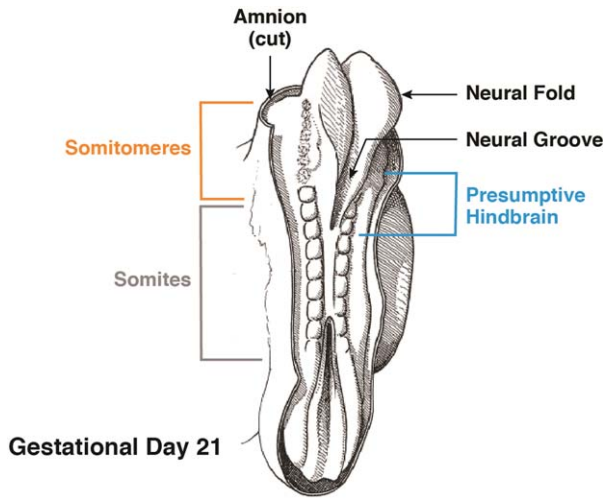

FIGURE 2 A human embryo at the end of the third week of gestation. The neural tube is closing. Note the pairs of block-like somites on either side of the closed neural tube. The whorl-like structures extending toward the cranial region are somitomeres

bilateral, loose whorls called somitomeres. The cells of the first seven pairs of somitomeres dissociate and migrate into the head region to form striated muscles in the jaws, throat, and ocular region. The cells of the eighth pair of somitomeres (and caudal somitomeres in sequence) undergo compaction to form somites. The elapsed time for formation of a pair of somites in rodents is approximately 1.5–2 hr; in humans, the timing is approximately 5–7 hr (Webb & Oates, 2016).

The pairs of somites give the appearance of a series of paired blocks along the back of the embryo, and each pair of blocks is considered a "segment" (Figure 2). While these segments presage the vertebral column, the vertebrae are actually intersegmental structures as detailed below. Shortly after formation, somites exhibit a roughly triangular shape when cut in cross-section (Figure 3a). The somites become polymorphous and gradually break apart allowing the cells to migrate throughout the embryo. Initially somite cells are pluripotential; however, as development proceeds different regions of the somites become committed to various fates due to interactions with the surrounding tissues. The first region to emigrate from the dissolving somite is the sclerotome (Figure 3b), which will give rise to the vertebral column as described below. The remaining regions (Figure 3d) are the dermatome (forerunner of the dermis of the back) and the myotome (gives rise to voluntary muscles of the trunk and limbs).

3.2.2 | Vertebral column

The bodies of the vertebrae arise from the sclerotome portions of adjacent somites. The sclerotome cells dissociate and emigrate from the somite (Figure 3c) migrating medially and ventrally to surround the notochord. A small portion of them pushes dorsally to surround the neural tube to form the neural arch (Figure 3d). These sclerotome cells differentiate into fibroblasts (connective tissue) and chondroblasts (cartilage). Ultimately, they form the vertebral column; however, a complex migration must occur to align the segmental nerves exiting from the spinal cord with the foramina between the vertebral body segments (Figure 4). When viewed in longitudinal section, after the sclerotomes from either side have surrounded the notochord (Figure 4a), each sclerotome is composed of three unequally sized regions (Figure 4b). The caudal (inferior) region of each sclerotome is populated with numerous cells (termed the dense region) in contrast to the loosely arranged (less dense) cranial region. Between the two regions is a very thin, sparsely populated region. The second stage of sclerotome migration involves the caudal migration of the caudal (dense) regions of one sclerotome to join with the superior, less dense region of the subjacent sclerotome (Figure 4b). The newly formed, combined structure (the nascent vertebral body) is composed of portions of two adjacent sclerotomes (Figure 4c). For example, the caudal (dense) region of the fourth cervical sclerotome descends to combine with the cranial (less dense) portion of the fifth cervical sclerotome to form the fourth cervical vertebral body. The thin territory between the dense and less dense regions of the original sclerotome is enlarged by the descendant migration of the caudal half of the sclerotome. This region is the site of the intervertebral disc. The notochord disappears from all areas except in the region of the intervertebral disc, where it gives rise to the nucleus pulposus in the center of the intervertebral disc.

Mineralization of vertebral bodies occurs by means of bilateral ossification centers (one in each of the population of migrating sclerotome cells). Thus, at first, the vertebral body has two separate ossification centers (Figure 5a); as ossification continues, the two centers begin to join (forming a dumbbell-shaped structure), and eventually fusion results in a single ossification center. It is possible that one of the ossification centers will begin functioning in advance of the other one, resulting in an asymmetric appearance (Figure 5b). As long as both sclerotomes have migrated appropriately, this asymmetry is merely a difference in developmental timing. In the event one of the sclerotomes is deficient in size or is absent, the resulting structure is a hemivertebra (Figure 5c), which is a cause of scoliosis. If caudal migration occurs incorrectly, there may be no separation between adjacent vertebrae. This condition (termed "block vertebra"; Figure 5d) will limit the range of motion in the back.

3.2.3 | Ribs

The anlagen for most of the ribs (Figure 6) are derived from sclerotome cells that migrate into the body wall after

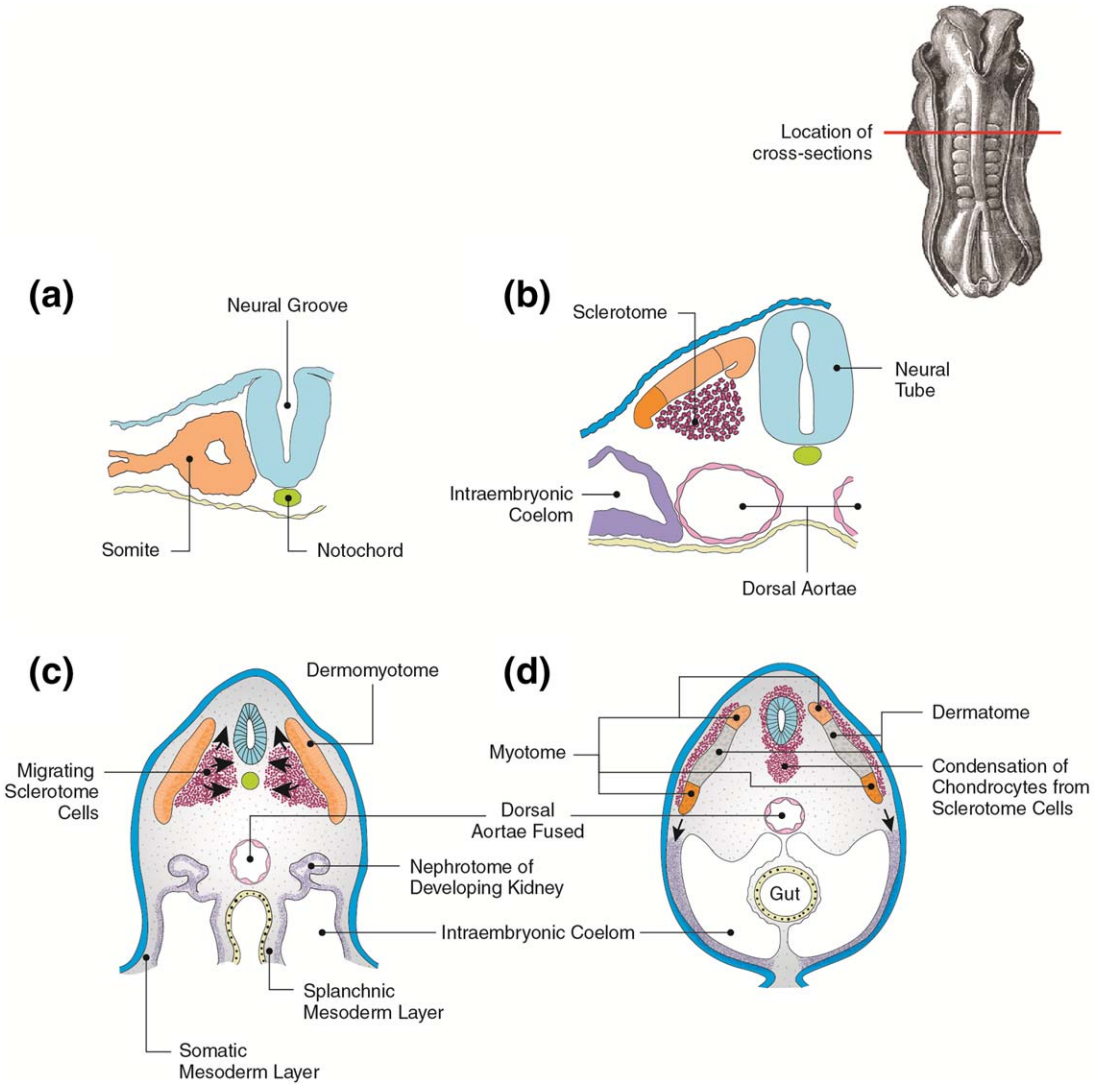

FIGURE 3 Somite development: Migration of sclerotome. Image (a) is a mature somite in its epithelial stage. In image (b), the sclerotome cells dissociate from the somite and the sclerotome cells migrate toward the notochord and neural tube (image c). In image (d), sclerotome migration has surrounded the notochord and neural tube, presaging the structure of a vertebra

resegmentation (caudal migration of sclerotomes) has occurred (Huang et al., 2000). Consequently, like the vertebral bodies, ribs are intersegmental structures. The sclerotome cells develop into the head, neck, tubercle, and most of the body (shaft) at least as far distally as the midaxillary line (Aoyama, Mizutani-Koseki, & Koseki, 2005; Huang et al., 2000). The portions of the ribs that are adjacent to the sternum are likely derived from lateral plate mesoderm (Aoyama et al., 2005), as is the sternum (discussed below). The territory between adjacent ribs is occupied by cells of the dermomyotome that give rise to the intercostal muscles (Aoyama et al., 2005).

The ribs mineralize by endochondral ossification of the cartilaginous rudiment that was laid down by condensations of mesenchymal cells regardless of whether they are of sclerotome [somitic] or lateral plate origin. In rodents this process begins at mid-gestation and continues until parturition.

3.2.4 | Sternum

The sternum is the bone that lies in the midline of the anterior chest wall where it overlies the heart and portions of the lungs. The sternum serves as the attachment of true and false ribs, that is, those attaching directly to the sternum and those attaching through a costal cartilage. The sternum is composed of 3 bony elements: the broad, superiorly placed manubrium, the narrow, elongated body, and the small xiphoid process (Figure 7e). These bony elements are joined together by symphyses (closely bound, fibrocartilaginous joints; Drake, Vogl, & Mitchell, 2010; Hebel & Stromberg, 1986), which fuse as the individual ages (McCormick & Nichols, 1981).

In order to understand the development of the sternum, it is important to first briefly discuss the development of the thoracic (chest) body wall. The bulk of the bony thoracic

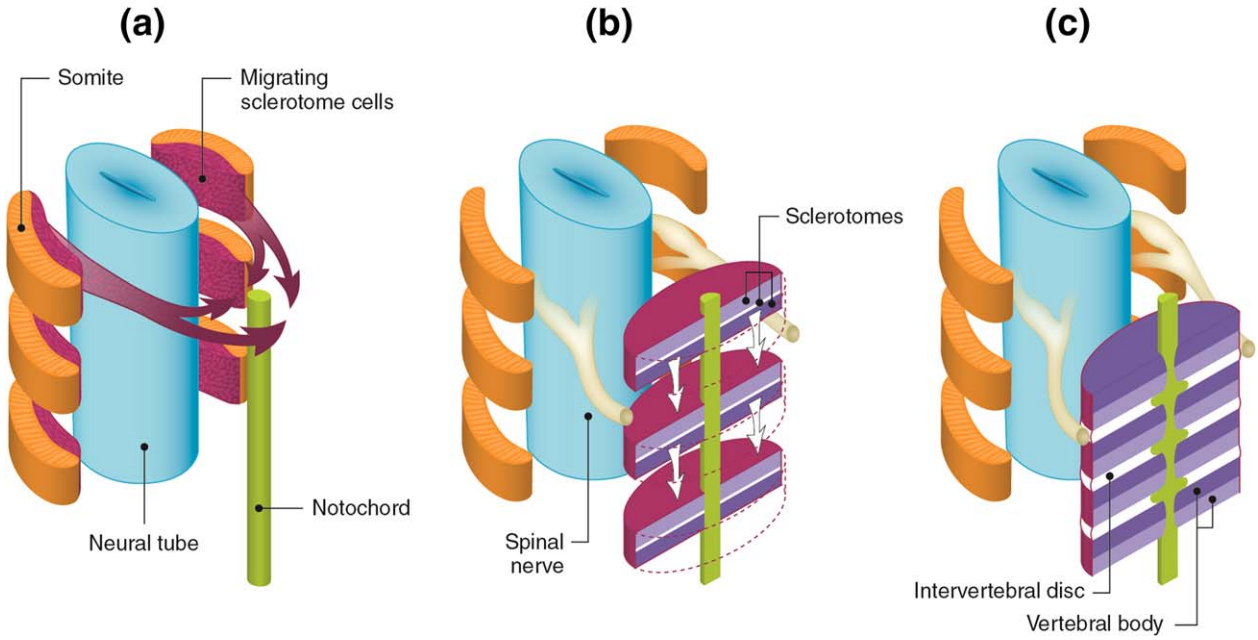

FIGURE 4 Development of vertebral bodies. Image (a) depicts migration of the sclerotome to surround the notochord. Image (b) reveals the organization of the migrated sclerotome with a densely populated lower region (dark colored area) and a less dense upper region separated by a small sparsely populated area (white). The arrows indicate resegmentation of the sclerotomes. In image (c), the intersegmental vertebral bodies are composed of cells from the densely populated region of the upper sclerotome and cells of the less densely populated region of the sclerotome below. Note that the spinal nerve is aligned with the intervertebral disc (between adjacent vertebrae) allowing for escape from the spinal canal

body wall is made up of the thoracic vertebrae (12 in humans and monkeys; 13 in rodents; 12 or 13 in rabbits), pairs of ribs consistent with the number of thoracic vertebrae, and the sternum. These structures are derived from mesoderm from different sources. Based on the origin of the mesoderm, the developing thoracic wall can be divided into three compartments (Figure 6; Aoyama et al., 2005). The first two compartments are derived from the sclerotome. The first site contributes to the developing vertebrae and proximal ribs. The second site arises from migration of cells out of the first site and contributes to the distal ossified portion of the ribs. The mesoderm of the third compartment comes from the lateral plate and participates in the non-ossified portions of the ribs (adjacent to the sternum) and the sternum itself.

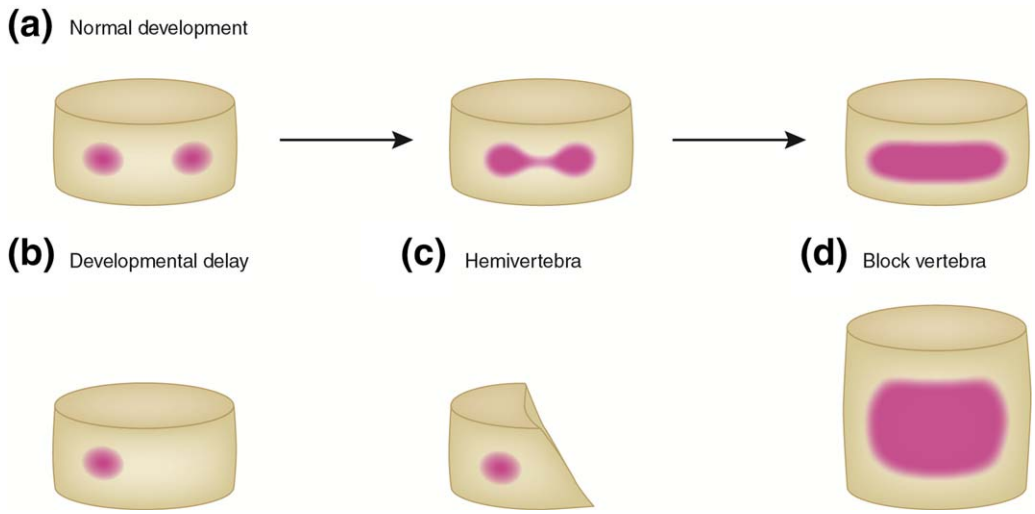

FIGURE 5 Ossification of vertebral bodies. The sequence of images in (a) begins with ossification centers on each side of the vertebral body. With continued growth, the ossification centers touch each other (forming a dumbbell-shaped profile). The ossification centers continue to expand throughout the vertebral body. Asynchronous appearance of ossification centers in a segment is illustrated in image (b). If the overall shape of the vertebral body is appropriate, the condition is a developmental delay that will rectify after birth. If, however, a sclerotome fails to migrate on one side (image c), the result is a hemivertebra, which contributes to scoliosis. If the caudal migration of sclerotomes is faulty and no intervertebral region appears, two adjacent vertebrae fuse into a "block vertebra" (image d)

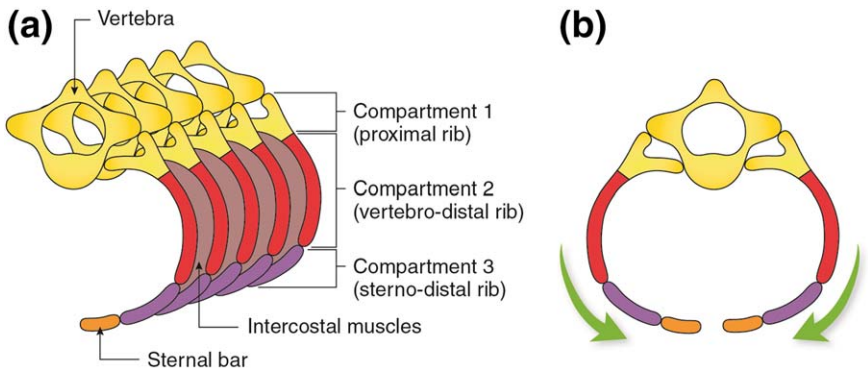

FIGURE 6 Development of ribs. The vertebrae and three rib compartments are illustrated. Compartment 1 (yellow) is formed by the extension of the sclerotome cells that migrated to form the neural arch and gives rise to the head and articular facets of the rib. Compartment 2 (red) forms by extension of cells migrating from compartment 1and gives rise to the angle of the rib and extends to (and beyond) the midaxillary line. Compartment 3 (purple) is closely associated with the sternal bars and forms from cells of the somatic layer of the lateral plate

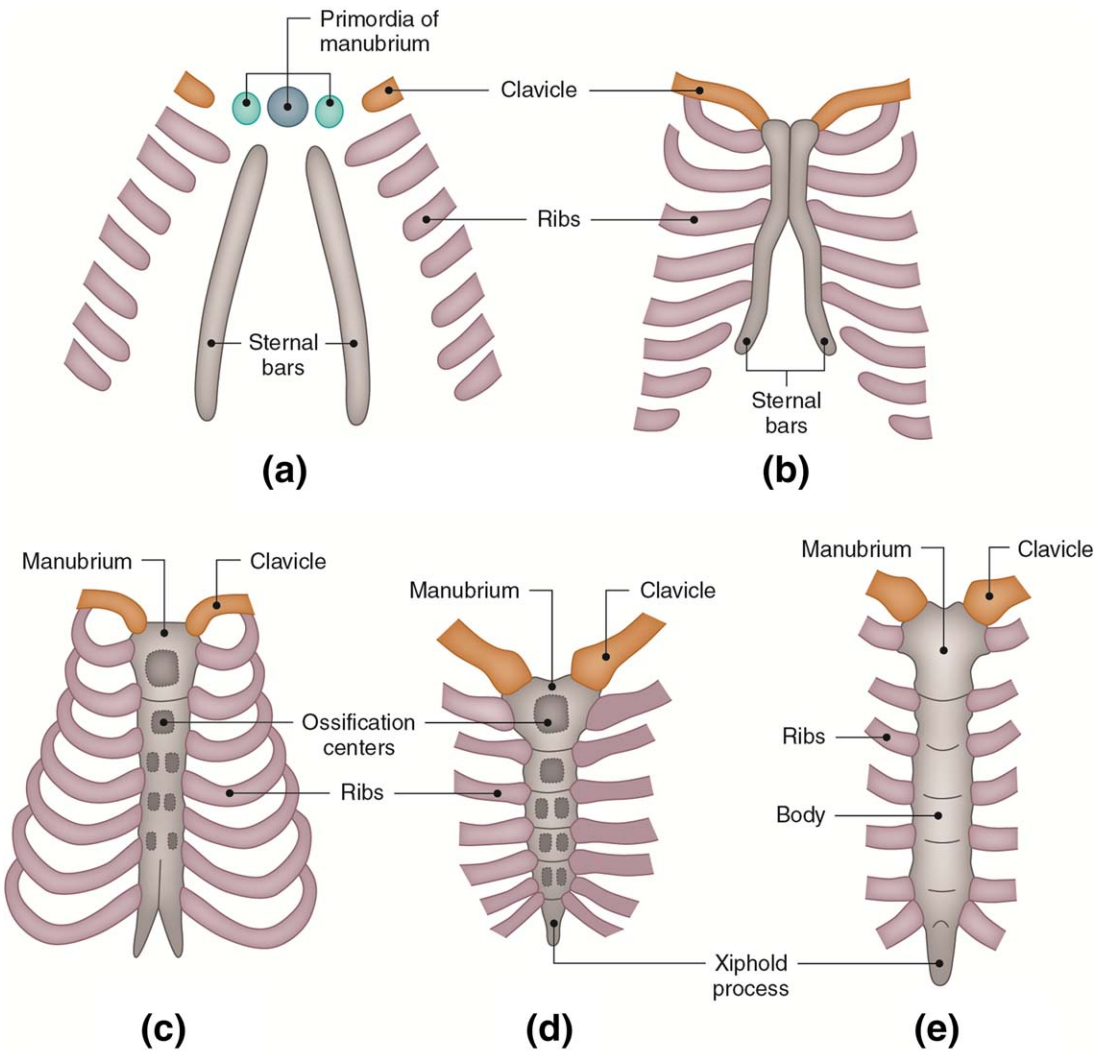

FIGURE 7 Development of sternum. Views of the anterior chest wall at successive stages (a–e) of development illustrating the movement toward the midline of the sternal bars and their fusion, which begins at the cranial region and moves caudally. Complete ossification (e) occurs in postnatal life
Modified from Carlson (2014)

The development of the sternum is complex and has been described as comprising various pathways by numerous authors (Carlson, 2014; Hamilton & Mossman, 1972; Sadler, 2000; Schoenwolf, Bleyl, Brauer, & Francis-West, 2015; O'Neal et al., 1998). The following is a distillation of that information.

The manubrium of the sternum is located in the most cephalic position (Figure 7a). The manubrium has a complicated embryology that involves migration of cells from the somitic sclerotome, the mesenchymal lateral plate, and the influx of neural crest cells (Klíma, 1968; Rodriguez-Vazquez, Verdugo-Lopez, Garrido, Murakami, & Kim, 2013). Despite its complex origin, there are relatively few findings of anomalies in the manubrium.

The body of the sternum arises as a pair of condensed mesenchymal bars that are derived from lateral plate mesoderm and are positioned on either side of ventral midline of the anterior chest wall (Aoyama et al., 2005; Carlson, 2014; Chen, 1952; Engum, 2008; Rodriguez-Vazquez et al., 2013). The bars are discrete entities that are parallel to but separate both from each other and from ventromedial ends of the costal rudiments (developing ribs) (Figure 7a). The bars reside in and participate with the tissue in the third mesodermal compartment that gives rise to the ventral body wall (Mekonen, Hikspoors, Mommen, Köhler, & Lamers, 2015). The mesenchymal bars fuse, beginning at the cephalic region (Figure 7b); fusion progresses caudally, but with decreasing speed such that the lowest segment (xiphoid process) may remain bifid (Engum, 2008).

During the same period that the sternal bars are undergoing fusion, the tissue within them begins to form cartilage, the first step in endochondral ossification (Figure 7c). As discrete portions of the cartilage begin to undergo endochondral ossification, the sternal anlagen organize into a series of segments (Figures 7c and 7d). The ossification centers form the central portion of each segment where they remain surrounded by cartilage. The first sternebra forms the manubrium and is actually not part of the sternal bars. As the sternal bars fuse, the segments from each side unite. The second through the fifth sternebrae give rise to the body of the sternum. The sixth sternebra will become the xiphoid process. The cartilaginous intersections between adjacent sternebrae mark the sites at which the ribs join the sternum (discussed further below).

Typically, one or two ossification centers can be seen in each sternebra depending on timing and whether ossification began before or after the right and left segments united as well as how quickly the ossification centers merged. The most common pattern of ossification centers is one in each of sternebrae 1 and 2 and two ossification centers in each of sternebrae 3–6 (Ashley, 1956). Ashley was clear to point out that when two ossification centers are present in a given sternebra, they can be either located directly across from each other or obliquely relative to each other (Ashley, 1956). In rodents, ossification centers first begin to appear near the end of organogenesis; appearance of ossification centers continues into the peri-parturition time period. If ossification is slightly delayed, the right and left ossification centers may appear as two sites (sometimes called bipartite sternebrae) or they may be partially fused (dumbbell-shaped sternebrae). These findings are normal stages in the development of a sternebra, transient findings that will resolve shortly.

Regardless of the number of ossification centers within a given sternebra, the ribs attach to the sternum at the cartilaginous areas of the sternum between adjacent sternebrae. It is notable that the right and left ribs of a given rib pair attach directly across from each other. The arrangement between the non-ossified ends of the ribs and the intersternebral regions of the body of the sternum has been studied and evidence has been developed that supports a role for signaling emanating from the ends of the ribs that guides segmentation of the sternum (Chen, 1953). More recent investigations suggested that the signals involved may be due to mechanical stress (Wong & Carter, 1988) or may be involved with the *Wnt* signaling that is part of the larger issue of ventral body wall closure (Snowball et al., 2015). Regardless of the signaling, ossification of the body of the sternum is completed postnatally during adulthood (McCormick & Nichols, 1981).

3.3 | Head and neck

The skull is a complex osseous structure that serves to support and protect the cephalic portion of the nervous system (brain and brainstem) and the anterior end of the gastrointestinal and respiratory systems (pharynx). The deep interior portion of the skull is formed by endochondral ossification; its cartilaginous precursor serves as the floor that supports the brain; it is also the site of attachment of the superior end of the pharynx, which is located below the brain. The bones of the cranial vault and those of the face are formed by intramembranous ossification. Figure 8 illustrates the organization of the bones in the human fetal skull.

3.3.1 | Neural crest cells and head mesenchyme

The embryological sources of the cells that give rise to the various skull bones differ according to location (Carlson, 2014; Ishii, Sun, Ting, & Maxson, 2015; Schoenwolf et al., 2015; Tubbs, Bosmia, & Cohen-Gadol, 2012; Yoshida, Vivatbutsiri, Morriss-Kay, Saga, & Iseki, 2008). The cranial vault (calvaria) has contributions from neural crest cells, which migrate superiorly along the tissue that surrounds the neural tube (presumptive dura mater; Smith & Tondury, 1978) and give rise to the squamous portions of the frontal and temporal bones, as well as the superior squamous portion

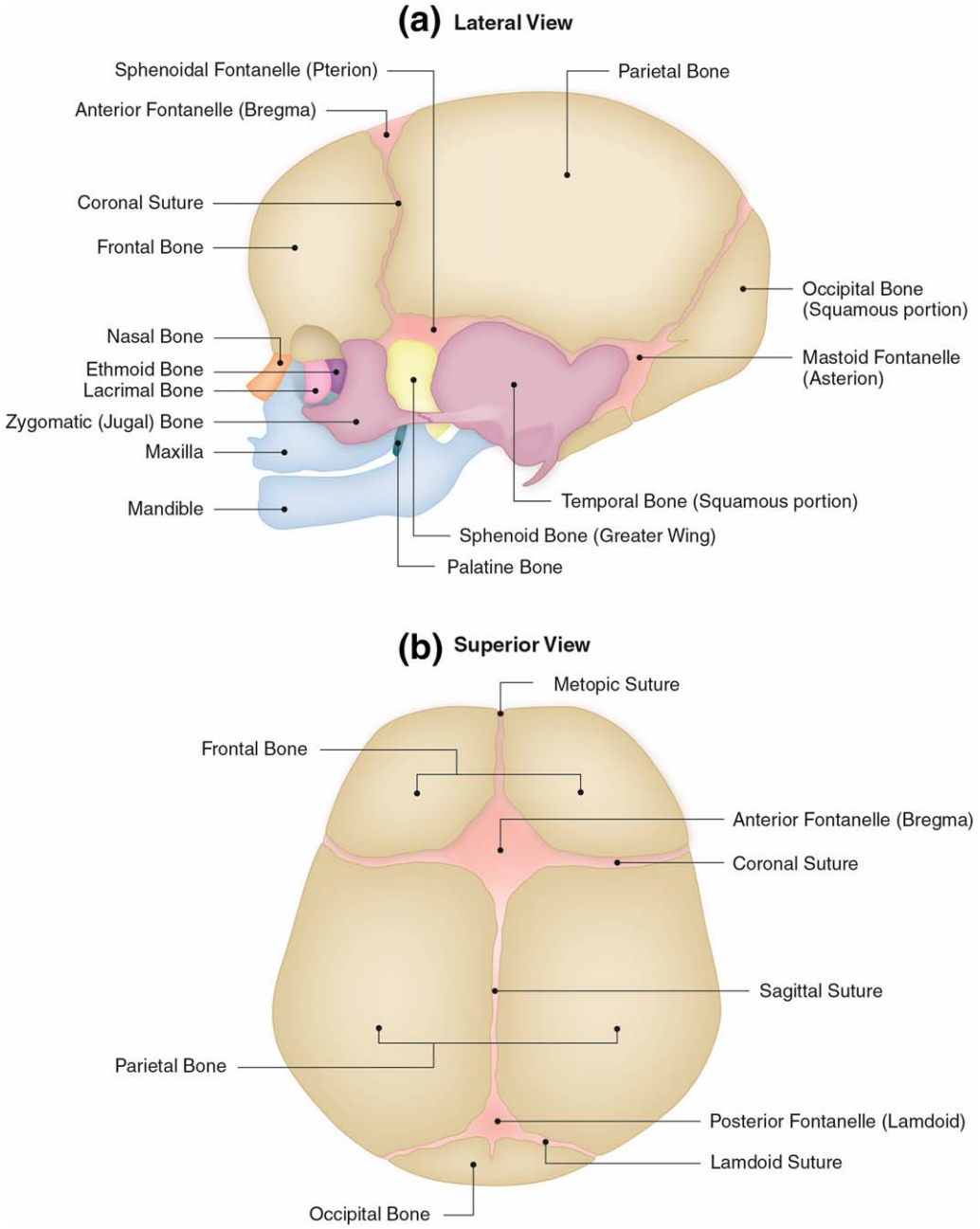

FIGURE 8 Fetal human skull. Image (a) is a lateral view of the fetal skull. Note the bones forming the calvaria (dome of the skull), which include the frontal, parietal, greater wing of the sphenoid, as well as the squamous portions of the temporal and occipital bones. All of the aforementioned bones form within the outer layer of the dura mater by intramembranous ossification. The membranous connections between these bones are sutures; the membranous areas where several of these flat bones approach each other are fontanelles. Note the sphenoidal fontanelle (pterion) and mastoid fontanelle (asterion). Image (b) is a superior view of the fetal skull. The anterior fontanelle (bregma) and posterior fontanelle (lambdoid or occipital fontanelle) are easily discerned in the midline. The midline suture between the frontal bones (metopic suture) is transient and typically closes within 3–9 months after birth

of the occipital bone. In the face, neural crest cells migrate inferiorly into the pharyngeal arches and ultimately give rise to the maxilla (including the palatal shelves), palatine, nasal, and lacrimal bones, the mandible, and the zygomatic (jugal) bone. There is also a neural crest contribution to the portion of the sphenoid that participates in the forming the lower portion of the cranial vault. All of these bones undergo intramembranous ossification. Failure/reduced migration of neural crest cells into the locations of the primordia of the aforementioned bones can lead to malformations including absence of all or parts of the bones.

Head mesenchyme arises from the migration of mesodermal cells that have invaginated through the primitive streak and migrated anteriorly toward the cephalic end of the embryo. Some of these cells migrate to surround the inferior portion of the developing brain to give rise to the parietal

bones and the orbital plate of the frontal bones, both of which undergo intramembranous ossification, and the portion of the occipital bone that surrounds the foramen magnum, which undergoes endochondral ossification.

3.3.2 | Base of the skull

The base of the skull is formed from a series of paired cartilages that surround the rostral end of the notochord. These cartilages give rise to the ethmoid bone (trabeculae cranii), the body (hypophyseal cartilage) and lesser wing (ala orbitalis) of the sphenoid, the petrous portion (ala temporalis), and otic capsule of the temporal bone, and the basal portion of the occipital bone (parachordal cartilage). Both head mesenchyme and neural crest cells contribute to these bones, which undergo endochondral ossification. Malformations in this area are rare (Di Ieva et al., 2014) and, when they do occur, they typically involve unclosed spaces between bones, for example, persistent/large foramen cecum between the nasal cavity and meninges or large spaces between adjacent bones making up the floor of the brain case, allowing herniation of meninges that may contain brain tissue (meningoencephaloceles).

3.3.3 | Occipital bone

The occipital bone is made up of two portions: an upper, squamous portion that is in contact with the parietal bones and contributes to the calvaria and the basal portion that arises from the parachordal cartilage and surrounds the foramen magnum. The squamous portion is formed by neural crest cells; the basal portion is formed by head mesenchyme. The two regions fuse to form a single bone. This fusion typically occurs before birth in humans and nonhuman primates, but the bones are typically distinct in rodent fetuses. When distinct, the presumptive squamous portion is termed the interparietal bone.

In embryo-fetal development studies (developmental toxicity studies) in rodents and rabbits, findings that are frequently encountered include reduced ossification and large fontanelles. Because ossification of the calvaria begins shortly before parturition and because the fetuses are typically taken 24–26 hr before natural parturition, these findings are most likely due to fetuses being collected early in the process of ossification. They are not indications of a perturbed developmental event. To determine if there is an adverse finding, it is important to observe the shape of the calvaria; it should be a smooth, oval dome with no protrusions of tissue between ossified areas.

3.3.4 | Mandible

During development of the lower portion of the face, neural crest cells migrate inferiorly along the course of the aortic arches to populate and expand areas beneath the brain on either side of the developing pharynx. These bilateral structures, known as pharyngeal (branchial) arches, each contain an aortic arch, a cranial nerve, a muscular component, and a skeletal element (typically a cartilaginous bar) (Moore, Persaud, & Torchia, 2013; Schoenwolf et al., 2015). The first pharyngeal arch is unique in that it has a smaller upper portion and a larger and lengthier lower portion. The mandible develops within the lower portion of the first pharyngeal arch. Within the lower first pharyngeal arches, rod-like cartilaginous bars (Meckel's cartilage) develop, unite distally at the presumptive chin, and support the tissues that will form the floor of the mouth and upper throat. As development proceeds, additional neural crest cells invade the first pharyngeal arch and take up residence exterior to Meckel's cartilage. The new cell population undergoes intramembranous ossification to form the mandible (Lee et al., 2001; Moore et al., 2013). Concomitantly, most of Meckel's cartilage degenerates. The only derivatives of Meckel's cartilage include the mandibular symphysis (the region where the two cartilages meet) and small bilateral portions of the proximal Meckel's cartilages that give rise to two ear ossicles (malleus and incus) (Carlson, 2014; Parada & Chai, 2015). The mandible increases its length considerably near the end of organogenesis, just prior to rotation of the palatal shelves (Moore et al., 2013).

3.3.5 | Palate

The bony and soft palates separate the oral and nasal cavities in mammals, forming the roof of the mouth and the floor of the nasal cavity. The palate is one of the last major structures to form during organogenesis. The embryology of this structure has been dealt with in numerous texts and articles (e.g., Bush & Jiang, 2012; Diewert, 1983, 1985, 1986; Greene & Pisano, 2010; Moore et al., 2013; Sadler, 2012; Schoenwolf et al., 2015) and is summarized here.

Most of the face (including the nasal and oral cavities) is derived from neural crest cells that migrate via different routes to populate the frontonasal process and the first pharyngeal (branchial) arches. Pharyngeal arches are bilateral structures that join in the ventral midline so that they form a U-shaped structure that surrounds the developing pharynx. The first pharyngeal arch on each side is divided into two processes. The upper process (maxillary process) gives rise to the upper jaw and eventually forms the roof of the mouth; the lower process (mandibular process) is the forerunner of the mandible.

Prior to the closure of the secondary palate, there is no roof to the mouth, and the oral and nasal cavities are continuous (Figure 9a). At this time in development the mandible is small and, consequently, the tongue is compressed into a hillock that occupies the mid-portion of the oronasal cavity. On

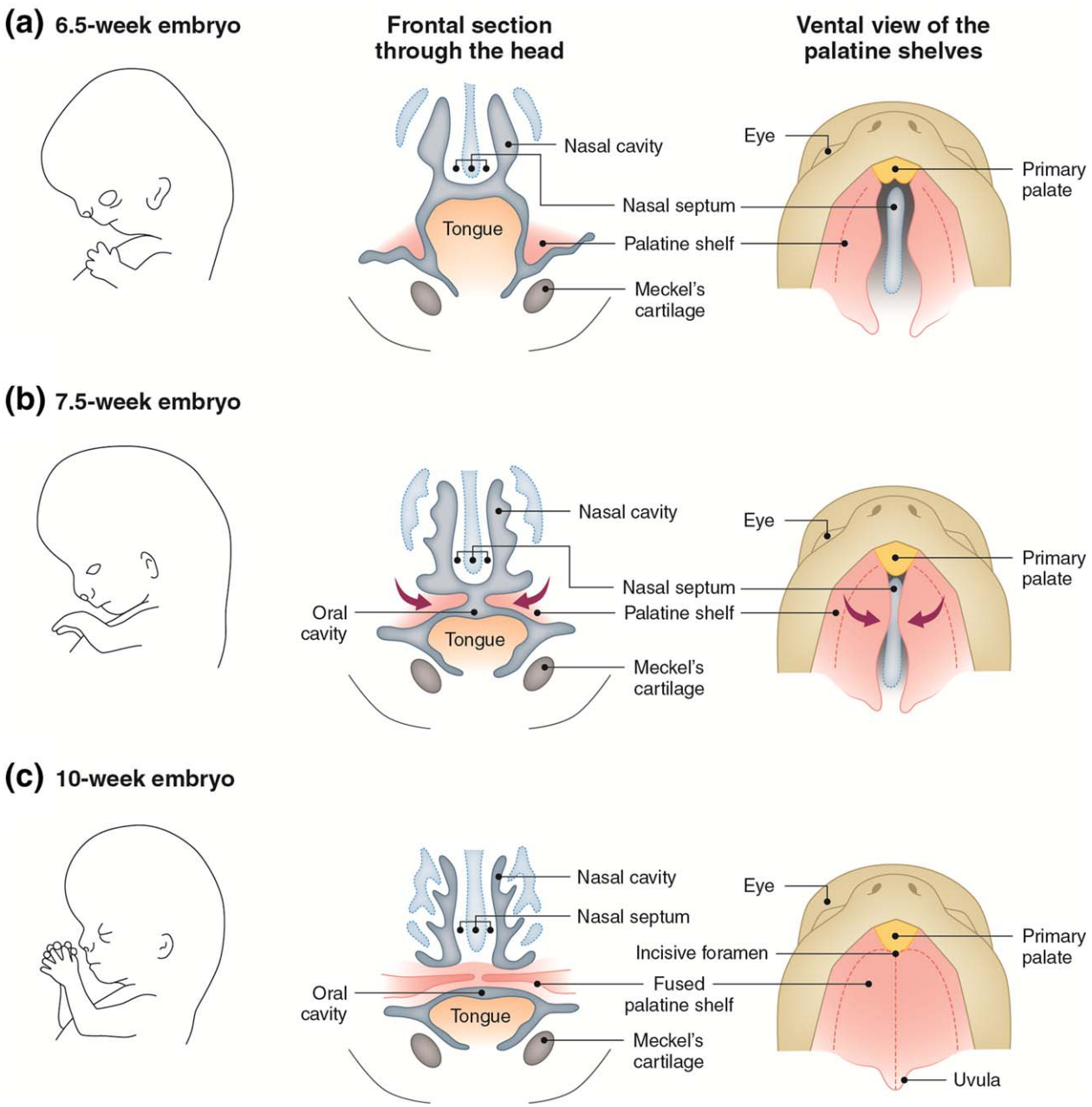

FIGURE 9 Development of palate. Each series shows (1) a line drawing of the embryo, (2) a coronal section through the head illustrating the relationship among the nasal and oral cavities and the tongue, and (3) the condition of the roof of the mouth. In series (a), the oral and nasal cavities are continuous; upper and lower jaws are short, leaving no room for the tongue, which is pushed up into the nasal cavity. The palatine shelves are parallel to the sides of the tongue. In series (b), the mandible has grown extensively, allowing the tongue to flatten and make room for the palatine shelves to rotate 90° thereby separating the oral and nasal cavities. In series (c), the palatine shelves have merged to form the roof of the mouth and the nasal septum has fused with them

the lateral sides of the oronasal cavity, two vertical palatine shelves grow downward from the presumptive maxilla (in the region of the upper jaw that is just medial to where the teeth appear). As the period of major organogenesis ends, the mandible elongates allowing the tongue to flatten out. This flattening vacates the space between the two palatine shelves (Figure 9b), which allows the palatine shelves to rotate 90° to the horizontal position where the distal ends of the shelves meet and fuse, thereby forming the secondary palate. The secondary palate separates the nasal cavity from the oral cavity (Figure 9c). The palate is joined by the perpendicularly positioned nasal septum. The bony portions of the palate and nasal septum undergo intramembranous ossification, typically commencing near the time of palatal shelf rotation.

An explanation of the propensity of mice to exhibit cleft palate may be helpful. Mice have a very short gestation (~19 to 20 days) and have short windows during which certain developmental events must occur (DeSesso, 2012). In the case of all mammals, but especially important for mice because of their short gestational period, the skull is rapidly

expanding at the same time due to the growth of the brain. This means that the skull is expanding in a lateral direction, effectively widening the gap that must be closed by the palatine shelves (Diewert, 1983, 1985, 1986). The rate of lateral expansion is greater than the rate of growth of the palatine shelves (i.e., the lateral spread of the oral cavity is faster than the medial growth of the palatine shelves if they have not fused). Consequently, if the rotation of the shelves is delayed or impaired for any reason (such as delayed growth of the embryo), and the shelves do not meet and fuse immediately at the end of rotation, a cleft palate will result. Catch up growth of the palatine shelves is not sufficient to close the gap. In species with longer gestations, the available window of time for uniting the palatal shelves is proportionally longer, and developmental delays will not have the same impact on palate closure. Consequently, cleft palate occurs more often in mice than in other species (including rats and rabbits) and is not a good predictor of cleft palate in humans.

3.3.6 | Hyoid bone

The pharynx is the region of the throat that lies behind the nasal passages and the oral cavity. It is a common conduit shared by the respiratory and alimentary canals and ending at the larynx. The hyoid bone is a U-shaped bone that serves to anchor the superiorly placed tongue and the floor of the mouth and to stabilize the inferiorly placed larynx (Bass, 1971; Warwick & Williams, 1973). The central portion of the U is the body, the two arms of the U are the cornua, termed alae in rodents and sometimes referenced as horns. Muscles involved in swallowing and vocalization attach to the cornua (alae).

The hyoid bone is the only non-sesamoid bone in the body that does not articulate directly with another bone. It is derived embryologically from the cartilages of the second and third pharyngeal arches. The cartilages of the second and third arches undergo endochondral ossification and eventually fuse to form the definitive hyoid bone. The second arch gives rise to the upper body and the lesser horns; the third arch cartilages develop into the lower body and greater horns (see Figure 10). The fusion of the cartilaginous rudiments occurs late in gestation of short-gestation species. Prior to fusion, the rudiments are tethered by dense mesenchymal connective tissue. During normal development of bones, developing musculature applies progressively increased mechanical loads that help to shape the bones (Sharir, Stern, Rot, Shahar, & Zelzer, 2011). If there is a developmental delay in the maturation in ossification of the hyoid bone and the nascent musculature attached to the horns begin to contract forcefully prior to fusion of the second and third arch cartilages, the orientation of the superior horn may tip toward the midline, giving the appearance of an "angulated" or "bent" hyoid. Typically, with continued growth, the portions of the hyoid bone unite, the overall structure of the bone remodels, and the angulation resolves.

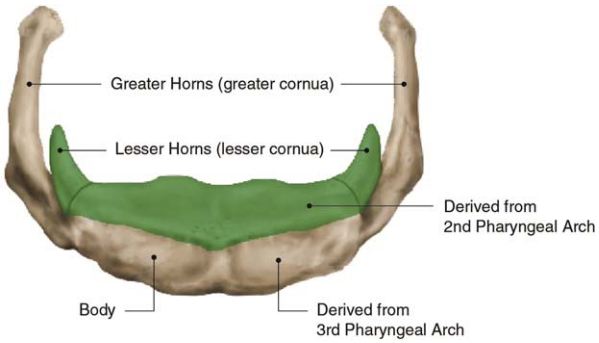

FIGURE 10 Hyoid bone. The central area (body) is located in the anterior portion of the throat between the inferior edge of the mandible and the superior edge of the thyroid cartilage (top of the larynx). The long rami on either side are the greater cornua (horns); the smaller more medially located rami are the lesser cornua. The portion colored in green derives from the cartilages of the second pharyngeal arch. The beige colored region forms from the cartilages of the third pharyngeal arch

3.4 | Limbs

3.4.1 | Early development

The limbs arise on the flanks of the embryo as paired, bilateral hillocks termed limb buds. Early limb buds are composed of a core of mesenchymal cells that are formed by the proliferation of cells from the somatic lateral plate mesoderm (Figure 11). The mesodermal hillock is overlain by ectoderm, which acquires a unique, thickened organization at the tip of the limb bud (apical ectodermal ridge; AER). The AER extends along the margin of the developing limb at the border between dorsal and ventral ectoderm. As the limb bud grows, it acquires additional cells by migration of myoblasts from the myotome of the adjacent somites. The myotome cells develop into the musculature of the limbs, while cells derived from the lateral plate give rise to the skeletal elements of the limb. Limb bud outgrowth depends upon reciprocal interactions between the AER and limb bud mesenchyme, the specifics of which can be found in textbooks of embryology (e.g., Carlson, 2014; Schoenwolf et al., 2015) and review articles (e.g., Amprino, 1984; Hopyan, Sharpe, & Yang, 2011; Ribatti & Santoiemma, 2014; Wolpert, 1999).

As the limbs grow out from the flank, they organize into territories of specification that are laid down in a proximal-to-distal order (Figure 12). The proximal region is the stylopod (humerus, femur), the middle region is the zeugopod (radius-ulna, tibia-fibula), and the distal region is the autopod (carpus, tarsus). Within each of these regions, the bones of the limbs mineralize by endochondral ossification.

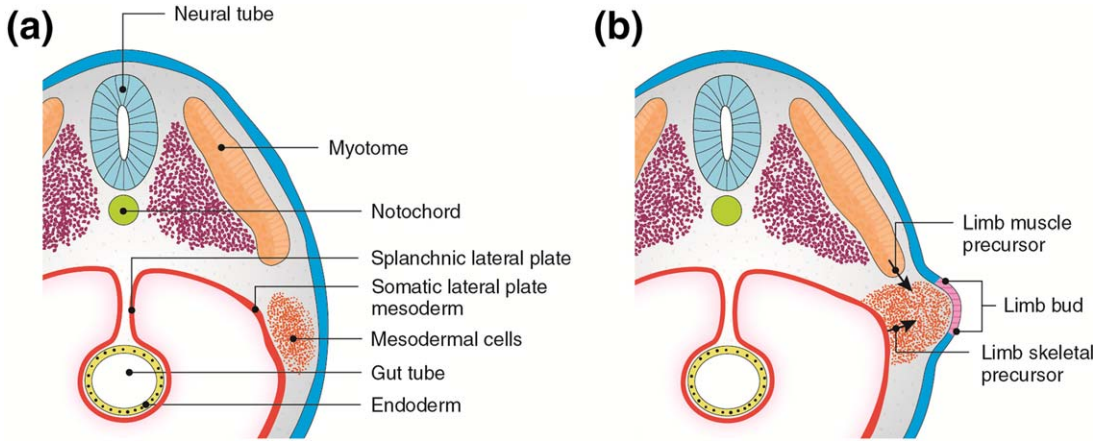

FIGURE 11 Early events in limb bud development. In image (a), mesodermal cells dissociate from the somatic lateral plate and proliferate on the flank of the embryo. As the mass of the lateral plate-derived cells increase (presumptive skeletal elements of the limb), the shape of the early limb bud is established (image b). Myoblasts from the somitic mesoderm (presumptive muscular elements of the limb) migrate into the limb bud. Note the specialized apical ectodermal ridge (AER), which directs limb outgrowth

In the upper limb, the arm forms first, followed by forearm, wrist and hand. Ossification of the bones of the limbs occurs in a similar sequence, except that the bones of the wrist ossify after birth (Aliverti et al., 1979; Gardner, Gray, & O'rahilly, 1969). In the hand, the metacarpals (bones of the palm of the hand) ossify before the phalanges (bones of the fingers). Because the phalanges undergo ossification near the time of birth in rodents and rabbits, small delays in the onset of ossification or brief (early) changes in the time of harvesting of fetuses can result in reduced numbers of ossification centers in the forepaws. In small or growth-retarded fetuses, poor (reduced or absent) phalangeal ossification can be a manifestation of a reduced rate of development rather than a direct disruption of fetal development by the test agent.

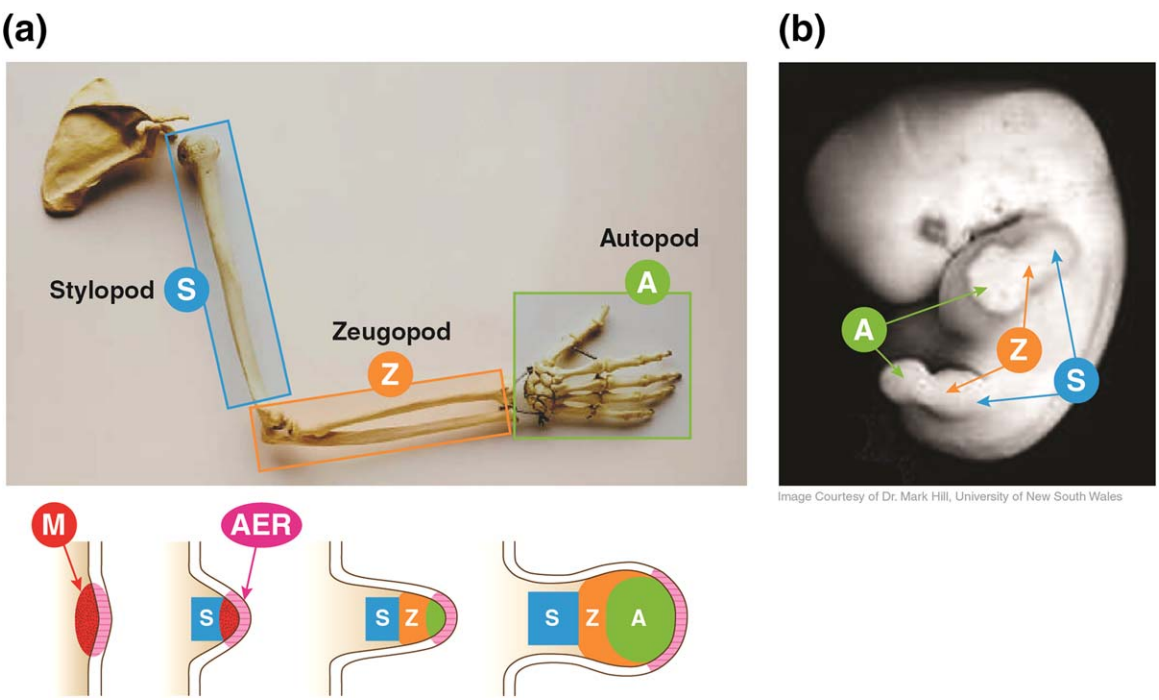

FIGURE 12 Regions of limb specification and derivative structures. Image (a) depicts the skeleton of and adult human forelimb. The bony structures derived from each of the regions of specification (stylopod, zeugopod, and autopod) are outlined. The inset shows the proximal-to-distal sequence of development of the limbs. The stippled red area (M) is an area of high mitotic activity (often called the progress zone), which is the source for the cells that will differentiate into limb bones. The magenta colored AER maintains orderly, sequential outgrowth of the limb bud. Note that the stylopod is laid down first and the limb bud grows out from that point adding the zeugopod and finally the autopod. Image (b) illustrates the location of the regions of specification in both the fore- and hindlimbs

3.4.2 | Ossification of digits

The digits of the forepaw are numbered from 1 to 5, with the pollex (thumb) designated as digit 1 and the small digit (*digiti minimi*) designated as digit 5. Among the laboratory animals typically used for developmental toxicity studies, mice have the shortest gestation (19–20 days; DeSesso, 2012). In mice, ossification of the thumb is typically not apparent until the postnatal period (Fritz & Hess, 1970; Patton & Kaufman, 1995). In rats, which have a slightly longer gestation period of 21–22 days (DeSesso, 2012), ossification of the thumb has often been described as occurring in the postnatal period (Aliverti et al., 1979; Fritz & Hess, 1970; Strong, 1926).

Rabbits have a longer gestation period than rats or mice (31–32 days), but fetuses are harvested on either gestational Day 28 or 29 (Tyl & Marr, 2012). This difference in timing must be recognized and accounted for when comparing studies. In much of the classical literature on skeletal development of rabbits, ossification status of the first digit is ignored/not mentioned (e.g., Crary & Sawin, 1949; Fritz, 1975; Sawin & Crary 1964). There are a few papers that describe ossification of the thumb beginning during the week before study termination (e.g., Danielson & Kihlström, 1986; Kamata et al., 2007). This literature provides an indication of the variability in the schedule of ossification in control rabbits.

Understanding the schedule of development for the bones of the digits is important because evaluation of near-term rodent and rabbit fetuses for potential developmental toxicity involves inspection of the skeletal system, including the bones of the paws. For many years, this evaluation entailed staining whole fetuses with alizarin red S, followed by clearing of the remaining tissue so that the bony skeleton could be evaluated. This technique identifies ossified bone, but is unable to demonstrate the presence of the cartilage that makes the initial "model" of the long bones and is subsequently replaced by ossified bone during development and maturation. Alternative methods involve double staining using Alcian blue to also stain cartilaginous tissues. The cartilaginous models are not the size of the entire bone; rather they are the point of origination for the primary ossification centers of the bones. With continued development, the ossified portion of the bone grows thereby extending the length of the nascent bone. In preparations that stain only with alizarin red S, any cartilage that is present remains clear and, thus, is invisible to examiners. The absence of alizarin-stained tissue would have led to the reporting of "agenesis" of the tissue, even when a cartilaginous rudiment was present and the development of that bone was actually only slower than that of controls. Beginning in the mid-1980s, it became more common to double stain the fetuses with alizarin red and alcian blue in order to identify the presence of both bone and cartilage. In double-stained paws, cartilage rudiments are apparent, and what might appear as missing bones can be reported as developmental delay or reduced ossification rather than agenesis.

3.4.3 | Evaluation of digit 1 adactyly

In rabbits, digit 1 (the pollex) develops near the time of birth. If development of the pollex is slowed, ossification might not begin until after GD 29, the typical date of cesarean section in the rabbit. Although the pollex is evaluated on external examination, the absence of alizarin staining would give the appearance that the thumb bones were absent, leading to an incorrect call of adactyly. However, the cartilaginous model upon which the thumb bone develops would likely have been present. It is paramount to recognize that during fetal evaluations, the observer captures only a snapshot of a continuing developmental process. Development of the organs does not stop at birth, but continues throughout the postnatal period, at least until the time of sexual maturity.

3.5 | Scapula

The scapulae undergo a complicated development that likely involves both intramembranous and endochondral ossification as described by Kimmel et al. (2014). The body of the scapula (the flat, thin triangular portion of the bone) likely develops by accretion of endochondral ossification sites and intramembranous ossification, similar to the majority of the flat bones in the body (Junquiera & Carneiro, 2005). In intramembranous ossification, calcification of bony matrix occurs directly within connective tissue without the formation of a cartilage model. The ossification center for the body of the scapula is located near the glenoid cavity (where the head of the humerus attaches to the shoulder). Because muscles develop in association with the bones they move, the muscles begin to contract as they differentiate. Any delay in ossification or in the timing of appearance of the ossification centers will result in an area of the scapula that is only connective tissue and will be susceptible to bending when the subscapularis and infraspinatus muscles begin to contract. The scapula continues to develop and ossify after birth, typically resulting in a normal shape by the end of the lactation period (Kimmel et al., 2014).

4 | OSSIFICATION

4.1 | Normal ossification schedule

The extent of ossification of the skeleton has been evaluated routinely for decades, but the ossification status of the fetus continues to elicit controversy. As discussed in the embryology section, above, the ossification process is highly dependent on the maturity of the fetus, and small mammal fetuses

actively ossify their skeletons in the last three days of pregnancy and the first days of postnatal life. A schedule of ossification in Sprague-Dawley rats and NMRI mice was published by Fritz and Hess (1970). They examined alizarin-stained skeletons from cleared fetuses and pups from 1 or 2 days before term and up to Day 2 after birth. Two days before term in rats, more than 90% of fetuses had ossification centers in all sternebrae, but these ossification centers were often bipartite, particularly in the fifth sternebra. Ossification of the sternebrae was complete by 12 days after birth, although nearly all sternebrae were ossified by 2 days after birth. Only 22% of fetuses had visible cervical vertebral ossification centers 2 days before term, and thoracic vertebral ossification centers were dumbbell shaped, representing incomplete ossification, in 14% of fetuses. Thoracic vertebral ossification was complete 2 days after birth. The supraoccipital bone of the skull had a dumbbell-shaped ossification center in about 2% of rat fetuses 2 days before term. Phalanges developed ossification centers by 2 days before term except for the proximal phalanx of the fifth digit of the fore- and hindlimb and the middle phalanx of digits 2–4 in the forelimb, in which ossification centers developed somewhat later. Aliverti et al. (1979) provided a detailed description of ossification over the last three days of gestation in the Wistar rat and proposed that ossification was a more reliable indicator of developmental delay than fetal weight.

In the mouse, more than 90% of sternebrae had ossification centers 2 days before term. Cervical and caudal vertebral ossification centers were present in a minority of specimens 2 days before term and in about 90% of specimens 1 day before term. The majority of phalanges had ossification centers by 1 day before term, but there were far fewer fetuses with phalangeal ossification centers 2 days before term. Only about half the fetuses had an ossification center of the calcaneus 1 day before term, with the remainder showing this ossification center at term. Two days before term, fewer than half of mouse fetuses had ossification of the parietal bone, giving the appearance of wide sutures. The supraoccipital bone ossification center was dumbbell-shaped 2 days before term. Many sternebrae were dumbbell-shaped until 2 days after term.

In rabbits, patterns of ossification are strain-dependent, suggesting genetic influence (Sawin & Crary, 1956, 1964). A timetable for ossification in the rabbit fetus was described by Fritz (1975), who removed fetuses from Silver Fawn rabbits daily from gestation day (GD) 21 to 30 (insemination = GD 0). Fritz noted that there was a clear order in which bones ossified, and that fetal maturity was reflected in the degree of ossification, particularly in distal limbs and sternum. Sternebrae 1–5 were minimally ossified on GD 21, and only about 90% of sternebrae 5 were ossified by GD 30. The calvaria did not completely ossify until term.

4.2 | Delayed ossification and decreased fetal weight

Fritz (1975) noted that, "A marked inhibition of skeletal development is. . .in most cases associated with a decrease in weight." A test compound may cause a reduction in fetal body weight through effects on maternal food intake, maternal physiology, or perhaps direct effects on the fetus. Recovery of offspring body weight to control levels after delivery is common, and achievement of normal ossification under these circumstances is expected. For example, Marr, Price, Myers, and Morrissey (1992) administered ethylene glycol 2,500 mg/kg bw/day to pregnant rats on GD 6–15 (sperm = GD 0). Fetuses and pups were examined at intervals before and after natural parturition, designated postnatal day (PND) 0, which occurred on approximately GD 21. Maternal body weight gain was decreased 13% by ethylene glycol treatment compared to vehicle-treated controls. Fetal weight was reduced an average of about 25% by ethylene glycol treatment during the last three days of gestation and recovered nearly to control levels within 2 weeks after birth. Ossification of sternebrae, vertebral centra, forelimb phalanges, and hindlimb metatarsals was decreased on GD 20; however, when statistically adjusted for fetal body weight, ossification was not significantly altered by treatment. Ossification of sternebrae and vertebral centra was decreased on PND 1, and the difference from control persisted after adjustment for pup body weight. Ossification improved after delivery and reached control levels by PND 14 in the limbs and PND 63 elsewhere. Bipartite thoracic vertebral centra and cartilage peaked in incidence on PND 1 (23 of 50 pups) and PND 21 (21 of 32 pups) but by PND 63 had decreased to 1 of 39 pups. Rib and sternebral abnormalities, some of which were also seen in control animals, decreased in incidence or disappeared by PND 63. The authors noted that assessment on PND 21 did not show the same degree of resolution of skeletal findings as assessment on PND 63, which might explain why some studies (e.g., Thiel et al., 1989) reported persistence of skeletal alterations in control pups at the time of weaning. These data are consistent with delays in the developmental schedule as discussed above in the Embryology section.

An evaluation of 1,484 historical control Wistar rat fetuses on GD 21 (sperm = GD 0) showed almost complete ossification of sternebrae, metatarsals, and distal phalanges of fore- and hindlimb (Chahoud & Paumgartten, 2005). Almost all fetuses demonstrated ossification of 8 of 10 metacarpals, and there was no association of metacarpal ossification with fetal body weight. Fetal body weight was associated with the number of ossification sites of proximal and medial phalanges (fore- and hindlimb). Ossification of cervical and sacrococcygeal vertebrae was also incomplete on GD 21 and was associated with fetal body weight.

In CD-1 mice evaluated on GD 18 (copulation = GD 0), delayed ossification of the skull was observed in 1.57% of untreated fetuses, and delayed ossification of vertebrae was noted in 2.25% of untreated fetuses (Perraud, 1976). In Sprague-Dawley derived rats evaluated on GD 20 (plug = GD 0), delayed ossification of thoracic vertebrae was noted in 2.36% of control fetuses (Perraud, 1976). The prevalence of delayed ossification of the skull and hyoid were 0.43% and 0.18%, respectively. In NZW rabbits, decreased skull ossification was a temporary observation, occurring in 0.24% of about 8,000 controls (Palmer, 1968).

4.3 | Maternal toxicity

In regulation-compliant whole-animal studies, maternal toxicity is manifest by clinical signs, reduced food or water consumption, reduced body weight gain, reduced absolute or uterus-corrected body weight, histopathological lesions associated with altered organ function (e.g., liver, kidney, heart, lungs), or maternal death (Tyl & Marr, 2012). Although a decrease in maternal weight or weight gain might be secondary to a compound-induced reduction in fetal growth or number, an evaluation of 125 National Toxicology Program developmental studies in mice, rats, or rabbits showed that fetal weight reduction was usually the endpoint determining the lowest observed adverse effect level for development and that there was a strong relationship between reduction in fetal weight and reduction in maternal carcass weight, suggesting that maternal toxicity usually played a role in fetal weight reduction (Chernoff, Rogers, Gage, & Francis, 2008).

Assessments of maternal toxicity using body weight and clinical signs are crude and perhaps insensitive. Additional endpoints that might increase the sensitivity of maternal toxicity detection have been proposed including serum concentrations of acute phase proteins, serum zinc, hepatic metallothionein, hematology, clinical chemistry, organ weights, and histopathology of selected organs (European Centre for Ecotoxicology and Toxicology of Chemicals, 2004). These endpoints might be derived from subchronic studies in nonpregnant animals, although some of the biochemical endpoints might differ between pregnant and nonpregnant animals.

4.4 | Feed restriction studies

Feed restriction during pregnancy is a way to produce a variety of clinical signs often seen in test agent-induced maternal toxicity and body weight reduction independent of treatment with a xenobiotic and has been used to dissociate effects of maternal toxicity from possible direct effects of a treatment on the fetus.

4.4.1 | Rats

A review of 12 papers that investigated the impact of feed restriction in pregnant rats found decreased ossification in most of the studies in which it was assessed (Nitzsche, 2017). Some of the papers will be discussed here in more detail.

Shrader and Zeman (1973) mated Sprague-Dawley rats over a 2-hr period (mating = GD 0) and fed them a control diet (24% casein as the protein source) or a protein-deficient diet (6% casein). Fetuses evaluated periodically from GD 17–22 for the number of ossification sites in cleared, alizarin-stained skeletons showed delayed appearance of ossification sites (Figure 13) and fainter staining associated with the protein-deficient diet. The number of ossification sites remained lower through PND 14 in pups from the protein-deficient group fostered to dams on an adequate diet. The number of ossification centers per pup was correlated with pup body weight.

Pregnant Sprague-Dawley rats were feed-restricted on GD 6–17 (sperm = GD 0) and evaluated on GD 21 (Fleeman, Cappon, Chapin, & Hurtt, 2005). Prior to and after the restriction period, dams had ad libitum access to feed and consumed 21–26 g/day. During the restriction period, a control group was maintained on ad libitum feeding, and restriction groups were given feed at 20, 15, 10, or 7.5 g/day. There was a decrease in mean dam body weight during part or all of the feed restriction period, and there was a decrease in body weight gain at 20 and 15 g/day. The dams fed 10 or 7.5 g/day lost body weight during the feed-restriction period. Maternal carcass weight on GD 21 was reduced in all feed-restriction groups with a net loss of corrected body weight in the 10- and 7.5-g/day groups. There were no effects of

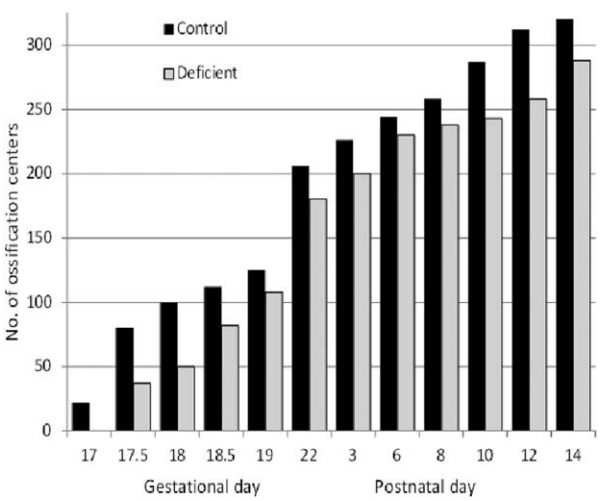

FIGURE 13 Number of ossification sites in fetuses/pups from Sprague-Dawley rat dams fed a control diet or a protein-deficient diet. Postnatal data from pups fostered to dams on a control diet with 4 pups/litter. Control and deficient columns are statistically different at each time point. Figure drawn from data presented in Shrader and Zeman (1973)

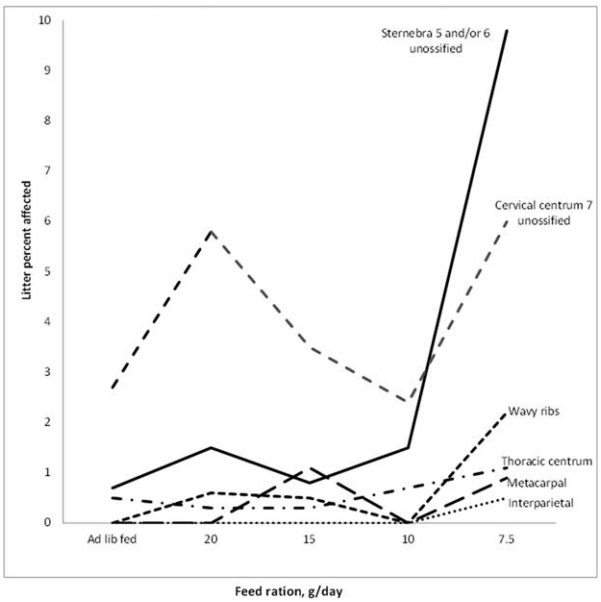

FIGURE 14 Effects of feed restriction of pregnant Sprague-Dawley rats on fetal ossification. The labels thoracic centrum, metacarpal, and interparietal refer to reduced ossification at those sites Drawn from data in Fleeman et al. (2005)

maternal feed restriction on the numbers of viable fetuses or resorptions. Mean fetal weight was progressively reduced as the level of feed-restriction increased. There was no statistical effect of feed-restriction on malformations. There was an increase in unossified sternebrae 5 and/or 6 in the most severely feed-restricted group. Other skeletal variations were not statistically increased, although a feed-restriction effect was suspected (Figure 14).

The dissociation of malformations from delayed ossification was demonstrated in rats in a food-restriction study by De-Carvalho, Delgado, Souza, Chahoud, and Paumgartten (1994). Pregnant rats were randomized on GD 0 to be fed ad libitum or to be given half the food ration of the well-nourished control animals. Food deprivation caused a 27% decrease in maternal carcass weight at term, a 22% decrease in mean fetal weight, and a two-fold increase in the proportion of fetuses with ossification delay. There was no increase in skeletal malformations.

4.4.2 | Rabbits

A review article listed six studies in which pregnant rabbits were feed restricted (Nitzsche, 2017). Reduced ossification was noted in two of the three studies that included ossification as an endpoint. These studies will be discussed in more detail here.

New Zealand white rabbits were given feed rations of 150, 50, or 15 g/day on GD 6–18 (insemination = GD 0) and 150 g/day on other days of gestation (Clark et al., 1986). Fetuses were evaluated on GD 28. Feed restriction was associated with altered maternal biochemical endpoints, including decreased urea nitrogen, protein, albumin, and alkaline phosphatase and increased creatinine, potassium, and chloride. Decreased live fetal body weights occurred in both feed-restricted groups. Litters in the 15-g/day group experienced an increase in resorptions as well as increased incidences of incompletely ossified sites in surviving fetuses. An increase in malformations was described in this group, including sternebral malformations, which were not otherwise defined.

Another feed restriction study in New Zealand white rabbits provided a 150-g/day ration to control animals (Cappon, Fleeman, Chapin, & Hurtt, 2005). Restricted groups were given rations of 110, 75, 55, 35, or 15 g/day on GD 7–19. From GD 20 until termination on GD 29, all groups were given 150 g/day. A decrease in body weight gain during the restriction period occurred in all groups, and the dams given 15 g/day lost body weight. Maternal body weight increased with resumption of the control feeding level in all groups, although not all groups attained control body weight levels by termination. At a feed ration of 75 g/day or less, mean fetal weights were decreased compared to control. There were no malformations in fetuses of any group, but at a feed ration of 55 g/day or less, ossification decreases were seen in sternebrae 5 and 6. At a feed ration of 35 g/day, the authors described a decrease in ossification of metatarsals, metacarpals, and caudal vertebrae, although the findings were not statistically significant. Abortion was increased in the group given a ration of 15 g/day.

By contrast, Petrere, Rohn, Grantham, and Anderson (1993) did not identify adverse effects of feed restriction on fetal development in New Zealand white rabbits. Pregnant animals were given ad libitum access to feed except on GD 6–18 when animals were given 150, 75, or 15 g/day with a control group continuing to have ad libitum access to food. Fetuses were evaluated on GD 30. There were no effects of feed restriction on resorption, and there were no dead fetuses. Fetal weight was decreased in males by 9% in the 15 g/day group. An apparent 12% decrease in female fetal body weight was not significantly different from the ad libitum fed control. There was no increase in malformations in any group and ossification of sternebrae, olecranon, humeral tuberosities, and epiphyses of fore- and hindlimb were similar across groups. Differences between this study and that of Clark et al. included use of an ad libitum fed control group, a different source of rabbits, and a different rabbit chow. In addition, this study included an additional two days of ad libitum feeding after restriction, perhaps permitting more time for ossification to catch up. Ossification was assessed only at five sites, among which only the sternebrae are typically involved in delayed ossification in fetuses evaluated near term.

4.5 | The interpretation of reduced ossification

Because ossification delay can be associated with fetal weight reduction, the interpretation of reduced ossification incorporates information about fetal weight and maternal toxicity. Maternal toxicity is generally defined as a reduction in weight gain during pregnancy of at least 10%, the presence of clinical signs (e.g., sedation), or mortality. A French Teratology Association working group, which included a representative from the French Medicines Agency, suggested that the importance of delayed ossification is minimized by the presence of fetal weight reduction and maternal toxicity (Guittin, Eléfant, & Saint-Salvi, 2000).

Carney and Kimmel (2007) wrote, "...delayed ossification is generally a finding that denotes generalized growth delays with subsequent catch-up postnatally. It also does not seem to have general predictive value for teratogenicity." These authors suggested that the pattern of bones that are involved can be helpful; if the reductions in ossification are restricted to bones that normally ossify late in gestation, the interpretation of a transient delay is tenable. These bones include the phalanges, sternebrae 5 and 6, the centra of the thoracic, sacral, and caudal vertebrae, and the calvaria. Carney and Kimmel suggested that the presence of maternal toxicity would be consistent with delayed ossification of no consequence. Makris et al. (2009) in a description of harmonized terminology from the Berlin Workshops emphasized the difference between delayed ossification and structural abnormality throughout their table of skeletal findings.

Delayed ossification of frontal and/or parietal bones can result in a report of enlarged fontanelles. An examination of the shape of the fontanelles can provide a clue about which bone is involved. Because these bones arise from intramembranous ossification, staining for cartilage will not be useful in evaluating fontanelles. An interpretation of apparently enlarged fontanelles can include an evaluation of the size of the brain and lateral venticles; in the face of a normally shaped brain and intact dura mater, apparent enlargement of fontanelles can be considered to be due to a delay in ossification.

5 | MATERNAL TOXICITY AND OTHER SKELETAL FINDINGS

Some investigators have evaluated possible associations between maternal toxicity and what were considered malformations of the skeleton and other organs. These studies did not include an assessment of the permanence of the findings or their effects on offspring health and, as will be discussed, some of the skeletal findings are more consistent with variations than malformations.

Khera (1984) reviewed 85 published mouse teratology studies and found 39 that included dose levels that were demonstrated to produce maternal toxicity, defined as reduced body weight gain, clinical signs, or death. Among these studies, 11 did not show fetal malformations even in the presence of maternal toxicity. Among the 28 studies in which malformations accompanied maternal toxicity, 19 studies included a characteristic pattern of malformations with exencephaly, open eye, fusion of the atlas with the occipital or axis, hemivertebrae, fused vertebral arches or centra, missing, forked, fused, or supernumerary ribs, and missing, fused, divided, or scrambled sternebrae.

In a similar review that included studies in hamsters, rats, or rabbits, there were 192 studies with sufficient information on fetal response at a maternally toxic dose level of test compound among which 106 were reported to show consistent types of fetal alterations (Khera, 1985). Frequently reported findings in hamsters included fused ribs, exencephaly, encephalocele, and micro/anophthalmia. In rats and rabbits, frequently reported findings involved ribs (fused, extra, missing, wavy), vertebrae (fused, retarded, missing, split), and sternebrae (missing, fused, nonaligned). In some instances, alterations suggestive of maternal toxicity occurred in the absence of manifest maternal toxicity, leading the author to propose that these fetal effects might occur through direct action of the test chemical on the fetus. Khera subsequently summarized and reviewed these studies (Khera, 1987).

Kavlock, Chernoff, and Rogers (1985) treated pregnant CD-1 mice on GD 8 (plug = GD 1) with one of eight chemicals at dose levels predicted to be around an LD_{10} and an LD_{40}, based on previous studies in nonpregnant animals. Fetuses were evaluated on GD 18. A dose-related decrease in maternal weight gain occurred for three of the compounds, and a decrease in fetal weight occurred for two of the compounds. Skeletal alterations were increased for two of the compounds. The most consistent fetal finding associated with maternal toxicity was an increase in supernumerary lumbar ribs, which correlated inversely with maternal weight gain ($r^2 = .45$, $p < .001$). Delayed ossification was noted in dose groups with decreases in fetal weight.

In a study evaluating Khera's theory that maternal toxicity is associated with a defined syndrome of developmental defects, Chernoff, Setzer, Miller, Rosen, and Rogers (1990) administered eight different compounds to Sprague-Dawley rats at dose levels associated with maternal toxicity in preliminary studies. Decrements in maternal weight gain or maternal death occurred with most of the compounds but a decrease in fetal weight was noted for only two of the compounds. An increase in supernumerary ribs occurred with two other compounds than those associated with decreased fetal weight. No syndrome of developmental effects at maternally toxic dose levels was identified with these compounds.

In a study of the effects of maternal stress on supernumerary ribs, Beyer and Chernoff (1986) (reported again in Chernoff, Kavlock, Beyer, & Miller [1987]) restrained pregnant CD-1 mice on GD 9 and Sprague-Dawley rats on GD 10 (sperm or plug positive = GD 1), previously determined as sensitive periods. Groups of animals were restrained supine from 9 a.m. to 9 p.m. and other groups from 9 p.m. to 9 a.m. with lights on during the restraint period and food and water withheld. Control groups were deprived of food and water during the same periods. An additional control group for each species had ad libitum access to food and water. Mouse fetuses were evaluated on GD 18, and rat fetuses were evaluated on GD 21. There was no effect of restraint stress in the rat on maternal weight gain, fetal weight, mortality, or supernumerary ribs. In mice restrained during either time period, there was no effect on maternal weight gain, fetal weight, or mortality, but there was an increase in supernumerary ribs and fused ribs. In the group restrained 9 a.m. to 9 p.m., there was an increase in exencephaly and in extra rib ossification sites defined as structures less than half the length of the last thoracic rib. There was a statistically significant linear relationship between the maternal weight loss during the immobilization period and the prevalence of lumbar ribs and ossification sites ($r^2 = .49$, $p < .01$). This relationship extended to the food- and water-deprived control animals.

Treatment of pregnant Sprague-Dawley rats with the loop diuretics indacrinone or furosemide produced maternal toxicity characterized by reduced body weight and hypokalemia with wavy ribs, shortened humeri, and misshapen scapulae in fetuses (Robertson, Minsker, Bokelman, Durand, & Conquet, 1981). The skeletal changes were reduced or prevented by provision of 1% potassium chloride in drinking water. Coadministration of amiloride, a potassium-sparing diuretic, with the loop diuretics also reduced the appearance of skeletal alterations.

Diflunisal, a nonsteroidal anti-inflammatory drug, produced fused, malaligned, and split vertebrae and fused, branched, and hypoplastic (or absent) ribs after administration to pregnant rabbits, an effect due to maternal hemolytic anemia and not to access of the drug to the conceptus (Clark et al., 1984). These authors cited older reports in the German literature showing similar skeletal effects in rabbits exposed to hypobaric oxygen or depletion of blood volume, leading them to conclude that maternal hypoxia mediated the abnormalities of fetal skeletal development.

Treatment of pregnant rats from GD 6 with lovastatin resulted in an increase in fetal skeletal findings including malformations of vertebrae, missing vertebrae, as well as rib fusions, agenesis, hypoplasia, and absence of ribs (Lankas, Cukierski, & Wise, 2004). The findings were associated with maternal toxicity attributable to a forestomach lesion. When dosing was begun 2 weeks prior to pregnancy, permitting the forestomach lesions to resolve, maternal toxicity and the fetal skeletal findings were prevented in spite of similar fetal exposure to lovastatin, demonstrating that maternal toxicity was more likely than drug exposure to have caused the developmental toxicity.

Although there are exceptions (Chernoff et al., 1990), most studies have shown some impact of maternal toxicity on the developing skeletal system. Rational dose levels can be based on toxicokinetic data, avoiding the maternal toxicity that can occur at the maximum tolerated dose or with a mandated limit dose (Saghir et al., 2012).

6 | FINDINGS IN SPECIFIC BONES

6.1 | Wavy ribs and bent bones

Wavy ribs, defined as "undulation(s) along the length of a rib" (Makris et al., 2009), and bent bones are often considered together, because they may be manifestations of similar ossification delays. Wavy ribs can be present in untreated animals; in CD-1 mouse dams treated with a control substance, wavy ribs occurred in 0.05% of fetuses (Perraud, 1976).

Khera (1970) in an abstract reported that wavy ribs were usually bilateral in the fourth to twelfth ribs in Wistar rats. These alterations were not noted before gestation Day 18, and the author proposed that areas of reduced replacement of cartilage with osteoid tissue were subject to bending from the activity of muscles with costal attachments. He identified the relevant muscles as the posterior and medial scalenes and the anterior and posterior serratus, which exert force 180° opposed to the external oblique muscles.

Baier, Cordts, and Stei (2016) used historical control data from the Wistar rat to characterize the phenotype of fetuses with spontaneous wavy ribs. Sex distribution favored males and clustering within litters was common. Median number of affected ribs per fetus was 11 (range 2–20). Ribs were affected bilaterally, but when single, favored the right. Wavy ribs were associated with delayed ossification of frontal, parietal, interparietal, squamosal, orbitosphenoidal, supraoccipital, zygomatic arch, and forelimb proximal phalanx. Fetal weight was not related to the presence of wavy ribs.

Pregnant Sprague-Dawley-derived rats treated with a beta-adrenergic blocking drug (Nishimura, Iizuka, Iwaki, & Kast, 1982; Saegusa, Kaneko, Sato, Narama, & Segima, 1980) or with a loop-diuretic (Hayasaka, Tamaki, Uchiyama, Kato, & Murakami, 1985) had fetuses with an increase in wavy ribs. The curved portions of the ribs showed delays in ossification prior to term (specifically, on GD 17 or 18), suggesting bending by muscle forces of these rib segments. Over the lactation period, the curvature in the ribs decreased, and by weaning, there was little detectable curving. Dietary boric acid during pregnancy at dose levels associated with

decreased fetal weight caused an increase in wavy ribs in fetuses examined on GD 20 (Price, Strong, Marr, Myers, & Murray, 1996). There were no wavy ribs in offspring examined on PND 21.

Kast (1994) reviewed 74 publications with information on wavy ribs. He emphasized the transient nature of the finding and its association with delays in ossification with consequent bending due to muscular forces. He analogized wavy ribs to bent scapulae and long bones, which arise from the action of muscles on under-mineralized bone. Bending of fetal bones has been described in mice, rats, and rabbits after exposures associated with embryofetal death or growth impairment, with or without maternal toxicity (e.g., Clark et al., 2004; J. E. Gibson & Becker, 1968; Treinen, Gray, & Blazak, 1995; Viertel & Güttner, 2000). The importance of muscle force in the developing embryo in the shaping of long bones was demonstrated in mice using serial micro-computerized tomography both in normal animals and in mutants genetically lacking muscle activity (Sharir et al., 2011).

A review of wavy ribs and bent or short long bones and scapulae summarized 41 informative papers on developmental toxicity in rodents and rabbits from a variety of exposures (Kimmel et al., 2014). The bony alterations were seen at dose levels associated with maternal and/or fetal toxicity. In general, wavy ribs occurred at a higher incidence than bent bones, and both kinds of alterations were infrequent in rabbits, attributable perhaps to the longer time period after dosing in rabbit studies during which bone remodeling could occur prior to fetal assessment near term. In rats, wavy ribs and bent/short bones were transient and reversible (De Schaepdrijver et al., 2014; Hayasaka et al., 1985; Kast, 1994; Mitchard & Stewart, 2014), leading to the conclusion that these changes, as well as bent scapulae (Kimmel et al., 2014), were variations associated with maternal or fetal toxicity and should not be used in determining effect levels.

6.2 | Supernumerary ribs

Supernumerary ribs are cervical or lumbar, occurring most typically on the seventh cervical or first lumbar vertebra. A distinction was made between rudimentary and extra lumbar ribs based on whether the supernumerary rib was less than or greater than half the length of the thirteenth rib in rats (Kimmel & Wilson, 1973). A subsequent paper suggested that the bimodal distribution of supernumerary lumbar ribs was better defined by considering rudimentary ribs to be 35% or less than the length of the thirteenth rib and extra ribs to be more than 35% of the length of the thirteenth rib (Chernoff & Rogers, 2004). A project to harmonize terminology adopted the latter criterion (Makris et al., 2009), writing:

> supernumerary cervical and supernumerary thoracolumbar ribs fall into two categories; these were named as "supernumerary ribs" and "ossification sites." "Supernumerary ribs" are larger (longer) structures with distal cartilage present and are likely to be permanent, ultimately remaining as distinct ribs; "ossification sites" are smaller (shorter) structures without distal cartilage and are likely to be transient. . .

6.2.1 | Mice

Among CD-1 mouse dams that were untreated or treated with a control substance, fused ribs occurred in 0.07% and cervical ribs in 2.4% of fetuses (Perraud, 1976). In another series of untreated adult mice, 14 ribs were present in one-quarter of individuals (Beck, 1983). In this series, administration of acetazolamide on GD 8 increased the number of animals with 14 ribs, and this increase persisted to adulthood. After treatment of pregnant Swiss Webster mice by gavage with corn oil vehicle on GD 6–15, 11.6% of fetuses had supernumerary ribs, about two-thirds of which were rudimentary (less than half the length of the thirteenth rib) and all of which had resolved by PND 40 (Chernoff, Rogers, Turner, & Francis, 1991). The fetal prevalence of supernumerary ribs was increased by gavage treatment on GD 6–15 (plug = GD 0) with bromoxynil 96.4 mg/kg/day, a dose level associated with maternal mortality and decreased fetal weight. The fetal prevalence of 47.9% decreased only to 42.3% by PND 40 (Chernoff et al., 1991). About half of the supernumerary lumbar ribs in mouse fetuses were more than half the length of the thirteenth rib; nearly all of the supernumerary ribs remaining on PND 40 were greater than half the length of the thirteenth rib. In a review, the range of cervical ribs among control mouse fetuses was 3%–62%, and the range of lumbar ribs was 1%–28% (Chernoff & Rogers, 2004). The strains giving rise to these data were CD-1, CF-1, Jcl:ICR, and Sic:JCR, with similar variation among the strains.

6.2.2 | Rats

In a teratology study of halothane, 16% of Sprague-Dawley rat fetuses had an extra rib, described by the author as unrelated to treatment (Lansdown, 1976). Data were not shown. In a Wistar-derived rat strain, supernumerary ribs occurred in about 20% of control animals, and the incidence was almost doubled by treatment with aspirin (Wickramaratne, 1988; Wickramaratne, Richards, Kinsey, & Kilmartin, 1985). By PND 60, all supernumerary ribs in the controls and all but 1 in the aspirin-exposed animals had resolved and had largely been replaced by ossified transverse processes on the first lumbar vertebra. The author postulated that stress associated with treatment might alter vertebral specification in the

highly labile thoracic-lumbar boundary and that alterations were readily remodeled postnatally.

After gavage treatment of Sprague-Dawley rats on GD 6–15 (sperm = GD 0) with corn-oil vehicle, 16.6% of fetuses had supernumerary ribs all of which had resolved by PND 40 (Chernoff et al., 1991). The fetal prevalence of supernumerary ribs was increased by gavage treatment on GD 6–15 with bromoxynil 15 mg/kg/day, a dose level associated with maternal body weight changes. The fetal prevalence of 68.4% was reduced to zero by PND 40 (Chernoff et al., 1991). Most of the supernumerary ribs in the rat offspring were considered rudimentary and characterized by small amounts of ossification lateral to the fourteenth thoracic vertebra.

After gavage treatment of Sprague-Dawley rats with ethylene glycol 2,500 mg/kg/day on GD 6–15, among findings scored as malformations, fused rib was noted on PND 1 in 18/50 fetuses (36%); by PND 63, fused rib was noted in 5/39 fetuses (12.8%) (Marr et al., 1992). Rib agenesis was found in 40% of GD-20 fetuses but in only 1 of 39 adults. In a review, the range of cervical ribs among control rat fetuses was 0%–69%, and the range of lumbar ribs was 1%–14% (Chernoff & Rogers, 2004). In control Sprague-Dawley rats from an inhalation developmental study, cervical ribs were present in 3 fetuses in 2 litters (litter prevalence 1%) on GD 21 but in 29 pups in 11 litters (litter prevalence 12.7%) on PND 65 (Mukerji, Glatt, Gannon, & Lewis, 2017). The authors had no explanation for this finding, but wondered if maternal stress associated with the inhalation technique might have contributed to the postnatal appearance of cervical ribs.

6.2.3 | Rabbits

There are normally 12 or 13 ribs in rabbits (Makris et al., 2009). Among Dutch belted rabbits, 36% of control fetuses had at least one extra rib (J. P. Gibson, Staples, & Newberne, 1966), but it is not clear if these authors meant a thirteenth rib or a fourteenth rib. Bipartite ribs occurred in 23% of 249 control fetuses. Palmer (1968) reported that control rabbits in his colony had 12 ribs in 54% of fetuses and 13 ribs in 46%. Bipartite, hemicentric, or fused ribs occurred in 1.29% of about 8,000 NZW control rabbit fetuses. In a review and other papers, the range of cervical ribs among control rabbit fetuses was highly variable, ranging from 0% to 63%, and the range of lumbar ribs was 0% to 16% (Chernoff & Rogers, 2004; Holson et al., 2006; Palmer, 1968; Stump et al., 2012). Another review article cited the fetal prevalence of supernumerary ribs, not otherwise defined, in rabbits as 37%–47% in three studies (Tyl et al., 2007). A paper cited in this review gave a fetal prevalence in rabbits of extra ribs as 21% and of rudimentary ribs as 11%, with "extra" meaning greater than half the length of the thirteenth rib and "rudimentary" meaning less than half the length of the thirteenth rib. In adult rabbits, a radiographic examination found a prevalence of 13 ribs of 49% (Pitt et al., 2002).

6.2.4 | Developmental field alteration

The appearance of lumbar ribs in laboratory animals has been associated with so-called anteriorization of the first lumbar vertebra after certain exposures, and the appearance of cervical ribs has been associated with posteriorization of the seventh cervical vertebrae. The anteriorization or posteriorization may involve caudal or cranial shifts in the expression domains of *Hox* genes, shown in fetal rats with sodium salicylate and boric acid (Wéry, Foulon, Blacker, Picard, & Gofflot, 2005; Wéry et al., 2003) and in fetal mice after exposure to retinoic acid (Kawanishi et al., 2003; Kessel, 1992; Kessel & Gruss, 1991), valproic acid (Faiella et al., 2000; Kawanishi et al., 2003), bromoxynil (Kawanishi et al., 2003), and hyperthermia (Li et al., 1997). These shifts were associated with cervical or lumbar supernumerary ribs and sometimes associated with abnormalities of vertebral shape and vertebral or rib fusions. The appearance of cervical or lumbar ribs in these instances, and perhaps in all instances, has been described as a homeotic transformation of a cervical or lumbar vertebra into a thoracic vertebra, rather than just being an instance of an extra rib. However, disabling of *Hox* gene functions would be expected to produce characteristic abnormalities in addition to the supernumerary ribs (reviewed by Augustine-Rauch [2007]). For example, an anteriorizing mutation of the cervico-thoracic boundary in *Hox*b-5 would be expected to be associated with abnormalities of the shoulder girdle.

In Sprague-Dawley rats, sodium salicylate treatment during gestation was associated with a more than 70% per-fetus/per-pup prevalence of fourteenth ribs (Foulon, Jaussely, Repetto, Urtizberea, & Blacker, 2000). Some of the rudimentary ribs (defined as less than half the length of the thirteenth rib) resolved by PND 54, whereas none of the extra ribs (more than half the length of the thirteenth rib) persisted to PND 54. The ribs were assessed by X-ray, and the same pups were serially assessed during the experiment.

In rabbits, variations in the number of ribs and lumbar vertebrae, representing posterior shifts in thoracolumbar and lumbosacral segmentation, were evaluated by crossing different strains (Sawin, 1945, 1946; Sawin & Gow, 1967; Sawin & Hull, 1946). The tendency toward such posterior shifting was consistent with inheritance of parental genes in such a manner that the dose of key genes determined the location of segmental boundaries, that is, a number of genes appear to contribute to determination of segmental boundaries. The authors proposed that posterior shifting occurred in larger offspring and that body size was genetically determined in rabbits in a manner similar to the determination of segmental boundaries.

6.2.5 | Human supernumerary ribs

A review article cited three papers showing cervical ribs in 19%–33% of human fetuses and one paper showing a 63% prevalence of cervical ribs in stillborn infants (Chernoff & Rogers, 2004). The prevalence of cervical ribs in human children is 4.5% and in human adults, up to 2% (Viertel et al., 2012). In another study, 199 embryos and fetuses were evaluated after elective abortion between 10 and 21 (probably menstrual) weeks after clearing and alizarin red staining (Bots et al., 2011). Almost 40% of the conceptuses had cervical ribs in the absence of evident malformation. The authors wondered if the use of alizarin red staining identified ossification sites lateral to the fifth cervical vertebra that might have been missed in studies using X-ray, but they noted that in 12.6% of conceptuses (23.6% of those with cervical ribs), the twelfth rib was missing, consistent with a homeotic shifting in developmental fields. This finding echoed that of Schumacher, Mai, & Gutjahr (1992) who reported that half of children with missing twelfth ribs had cervical ribs, although most children with cervical ribs were not missing twelfth ribs. Bots et al. (2011) also reviewed other reports of high (10%–63%) prevalences of cervical ribs among fetuses, although most of these studies involved deceased and therefore presumably abnormal fetuses. An association of childhood cancer with cervical ribs has been reported more than once (e.g., Merks et al., 2005; Schumacher et al., 1992). These associations raised the question of whether cervical rib is a marker for alteration in *Hox* gene or gene products involved in malignancy. It is not known whether cervical ribs associated with xenobiotic treatments in laboratory animals are markers for an increase in malignancy.

Lumbar ribs were found in about 1%–2% of human fetuses and 0.04% to 16% of human adults (reviewed by Chernoff & Rogers, 2004). Although cervical rib in humans has been associated with thoracic outlet syndrome, in which the neurovascular bundle to the upper extremity is compressed, most instances of thoracic outlet syndrome (>99%) involve compression between the first rib, clavicle, and associated soft tissues rather than a cervical rib (reviewed by Kuhn, Lebus, & Bible, 2015; Sanders & Hammond, 2002). In most patients, neck trauma precedes the development of thoracic outlet symptoms with a cervical or atypical first rib.

Most studies cite an incidence of lumbar ribs as 2% or less in human fetuses or adults (Chernoff & Rogers, 2004). Lumbar ribs may be without clinical significance; although a study in which 100 radiographs of the lumbar spine were reviewed suggested that a rudimentary rib in the lumbar region was associated with disc degeneration between the fourth and fifth lumbar vertebrae (MacGibbon & Farfan, 1979). The basis for this suggestion was not explained, and presumably all the radiographs in this study were available for review because patients had back pain. A 2016 review of lumbar ribs raised the question of whether lumbar ribs caused symptoms or were simply found coincidentally in some people with back pain (Aly, Chapman, Oskouian, Loukas, & Tubbs, 2016). Alteration in *Hoxa10* or *Faciogenital dysplasia 1* (*FGD1*) expression was proposed as the basis for at least some lumbar ribs, which might represent anterior homeotic transformations, although the authors pointed out that these ribs were anatomically dissimilar from normal thoracic ribs based on their lack of cartilage and their resemblance to vertebral transverse processes, although longer. A thirteenth rib is common in great apes, which have four lumbar vertebrae instead of the five seen in most humans.

6.3 | Sternebrae

Among CD-1 mouse dams that were treated with a control substance, fused sternebrae occurred in 0.1% of fetuses, and extra sternebrae occurred in more than half of fetuses (Perraud, 1976). One-quarter to two-thirds of untreated CD-1 mice evaluated on postnatal day 60–64 had "malformed" sternebrae, although these alterations were not otherwise described (Beck, 1983). After gavage treatment of Sprague-Dawley rats with ethylene glycol 2,500 mg/kg/day on GD 6–15, among findings scored as malformations, abnormal sternebrae were noted on PND 1 in 20% of pups; these abnormalities resolved by PND 63 (Marr et al., 1992).

Sternebral findings in Dutch belted control rabbit fetuses were described by J. P. Gibson et al. (1966). Among 249 control fetuses (64 litters), the fifth sternebra was missing or atrophic in 23.7% of fetuses. Bipartite and/or asymmetrical sternebrae occurred in 15 fetuses from 10 litters. An extra sternebra was present in 0.8% ($n = 2$) of fetuses and usually appeared between the fifth and last sternebrae, was atrophic, and was often fused to the fifth sternebra. Sixteen fused sternebrae occurred in 13 fetuses (5% of fetuses) from 11 litters. One of these fetuses had fused fourth and fifth ribs, and one fetus had fusion of the right half of the twelfth thoracic and first lumbar vertebrae.

In human fetuses, mineralization starts with the manubrium, followed by mineralization of the second sternal segment. The ossification sites can vary in size and are often irregular (McCormick & Nichols, 1981). Ossification centers in the human sternum can be paired or multiple, but are usually single. When ossification centers are paired, malalignment is not unusual, and bridging between adjacent ossification centers is common. It is also common to see variability in the schedule of fusion of ossification centers, both horizontally and vertically (Delgado, Jaimes, Gwal, Jaramillo, & Ho-Fung, 2014).

6.4 | Vertebrae

In mice, there may be 25, 26, or 27 vertebrae between cranium and sacrum; inbred strains differ in number of

vertebrae, and there are asymmetric combinations of vertebrae in which the sacrum is attached to one vertebra on one side and to a different vertebra on the other side (Green, 1962). Twenty-five presacral vertebrae were seen in 3.6%–91.7% of mice, and 26 presacral vertebrae were seen in 2.6%–89.8% of mice, depending on strain. Asymmetric vertebrae occurred in 4.3%–21% of mice, again depending on strain. Based on crosses between strains, Green (1962) suggested that there was a large amount of non-genetic variability. McLaren and Michie (1958) transferred early embryos of one strain to recipient uteri of another strain and showed that the number of lumbar vertebrae (5 or 6) was influenced by the recipient dam rather than by the donor dam, suggesting a non-genetic effect of the uterine environment.

A variable number of presacral vertebrae is the normal condition in the rabbit. In an evaluation of 10,000 control rabbit skeletons from 79 studies, the mean litter percentage of fetuses with 27 presacral vertebrae was 17.4%, with a range of 5%–32% (Stump et al., 2012). Presacral vertebrae persist into adulthood and appear to be asymptomatic, as radiographic examination found that 24% of adult female New Zealand rabbits exhibited this condition (Pitt et al., 2002).

After gavage treatment of Sprague-Dawley rats with ethylene glycol 2,500 mg/kg/day on GD 6–15, among findings scored as malformations, abnormal vertebrae (missing, extra) or centra (misshapen, unilateral, bilobed) were noted on GD20 or PND 21 but no adult animals had abnormal findings on PND 63 except for one with a bipartite thoracic centrum (Marr et al., 1992). The lack of permanence of the findings suggests that they do not represent meaningful alterations of development.

A study in Sprague-Dawley rats treated with 5-fluoro-2′-deoxyuridine or vehicle evaluated vertebral findings on GD 21 (sperm = GD 0), PND 7, and PND 21 (Chahoud, Talsness, Walter, & Grote, 2015). The results were expressed as a percentage of vertebrae within the cervical, thoracic, lumbar, and sacral segments, without regard to fetus or litter of origin. Based on resolution by PND 21, unossified, bipartite, hemicentric, and asymmetric centra were considered variations except in the cervical spine where there was persistence of the findings. Prevalence of findings per vertebra in the cervical spine on GD 21 was 0%–85% and on PND 21 0.68%–5.46%. Dumbbell-shaped cervical centra occurred in 2.46% of cervical vertebrae on GD 21 and 1.26% of cervical vertebrae on PND 21 and were also considered to be malformations on the basis of persistence. Misshapen centra were considered variations in thoracic vertebrae but malformations in lumbar vertebrae based on a prevalence per lumbar vertebra of 25.56% on GD 21 and 1.76% on PND 21. These conclusions cannot be accepted due to the inappropriate analysis per vertebra and the lack of evidence that PND 21 is the appropriate date for assessment of permanence.

6.5 | Carpal flexures

6.5.1 | Deformations

The purpose of a developmental toxicity study is to determine whether exposure of pregnant animals to an agent during pregnancy interferes seriously with genesis of offspring during the critical period when the rudiments for organ systems are being first laid down. The recognized endpoints for alterations in development are congenital malformation and anatomical variations. There is, however, a third type of structural alteration, although it is less often observed. The third type of alteration is termed a "deformation" (Browne, 1955, 1967). Deformations are not initiated during the period of organogenesis, but rather occur due to abnormal compression of a limited area of the fetus during a later stage of gestation. Depending on the type of deformation, some of these alterations may spontaneously resolve. We recommend that deformations be considered in glossaries and guidances used by evaluators and regulators.

6.5.2 | Occurrence of carpal flexures in animals

Flexures of the forelimbs are seen occasionally in the offspring of many species, including horses (Adams & Santschi, 2000; O'Grady & Poupard, 2003), sheep (Keeler & James, 1971), dogs (Cetinkaya, Yardimci, & Sağlam, 2007), and even elephants (Croze, 2007), as well as in progeny of experimental animals, especially rabbits (Palmer, 1968, 1972, 1978). Pliable forelimb flexures often resolve shortly after birth providing there is no associated osseous fragility with minimal or no intervention in foals (Adams & Santschi, 2000; O'Grady & Poupard, 2003), puppies (Vaughn, 1992), lambs (Keeler & James, 1971), and elephants (Croze, 2007). In rabbits, when there is no underlying skeletal defect, carpal flexures have been found to resolve spontaneously within 72 hr after mild exercise (Fratta, Sigg, & Maiorana [1965]—data; Palmer [1968, 1972]—report of personal observations and impressions). Because these alterations are seen with some frequency in rabbits, they deserve further discussion.

6.5.3 | Attributes of rabbit fetuses with carpal flexures

There are a number of physical characteristics that are often present in rabbit fetuses that present with carpal flexures. These include fetuses with small size or body weights (Sawin & Crary, 1964), fetuses located in positions that restrict movement (Palmer, 1968, 1972), fetuses experiencing presumptive compression due to a smaller than usual amount of amniotic fluid (DeMeyer & Baird, 1969; DeSesso, 1979), and postmortem handling such as prolonged time left in the

uterus prior to fetal examination (Harris & DeSesso, 1994). Palmer (e.g., 1968, 1972) made the point numerous times that temporary flexures of the forelimb in rabbit fetuses have been frequently mistaken for the more serious defect arthrogryposis, which is a condition of stiff, extended joints that present with malformed joints or underlying thickened joint capsules (Warkany, 1971).

6.5.4 | Carpal flexures as deformations

Carpal flexures in rabbit fetuses without underlying skeletal changes should be classified as (reversible) deformations as opposed to malformations, an important distinction. In contrast to malformations, deformations of fetuses occur after organogenesis is completed during the latter phase of gestation when the fetus is undergoing its growth phase. Deformations are usually caused by mechanical forces placed on the offspring due to compression or to unusual anatomical obstructions (e.g., uterine septa, amniotic bands, interference of fetal growth by the pelvic brim in humans; Browne, 1955, 1967; Cohen, 1990; Miller, Dunn, & Smith, 1979). While not all deformations are spontaneously reversible, it is important to note that their underlying causes differ from those of malformations and should not send the same signal with respect to the potential risk from chemical exposure.

The interpretation of reversible findings in fetuses is not straightforward. With respect to carpal flexures, the designation of "malformation" is incorrect because the condition of the wrist of affected offspring will likely become normal within a short time (e.g., Fratta et al., 1965). Furthermore, carpal flexures that resolve spontaneously are unlikely to cause other untoward health impacts in later life. Nevertheless, the fetuses are not normal at birth. A more appropriate classification for carpal flexure with normal skeletal anatomy in rabbits is that of (reversible) deformation. It is suggested not to classify them as variations (as indicated by Palmer, 1972) because carpal flexures are conditions that often result from a challenged uterine environment having an effect late in gestation after the rudiments of the bones and joints of the carpus have been established, whereas variations tend to be minor changes in structure that appear occasionally in unchallenged control animals and may have their origin during organogenesis.

In view of these literature data, it is the premise of this report that carpal flexures observed in rabbit fetuses without underlying skeletal defects are deformations that will resolve spontaneously.

7 | SYNTHESIS AND CONCLUSIONS

Evaluation of the development of the skeleton is a standard part of the embryofetal assay required by regulatory bodies. However, evaluation of the skeleton during the days prior to parturition is a snapshot in time of a system that normally does not complete its development until well after birth in laboratory species and in humans. Interpretation of skeletal findings should be performed with an understanding of embryological events to avoid interpreting transient developmental findings as representing a disruption of normal development. We draw the following conclusions from the rich published experience of skeletal development:

- Ossification delay is a transient finding that may indicate a delayed schedule of events but does not indicate disrupted development;
- Wavy ribs and bent long bones are manifestations of delayed ossification and can be expected to resolve as the organism matures;
- Changes in the size, shape, or symmetry of sternebrae or vertebral centra are transient and have no implications for the health or survival of the offspring;
- Short supernumerary ribs represent ossification centers lateral to the vertebrae and are not of developmental importance. They usually resolve as the animal matures;
- Long supernumerary ribs may represent developmental field alterations and appear to be permanent, at least in some studies. However, it is not clear that they have implications for the health of the offspring.

It is possible that food intake and weight gain are inadequately sensitive indicators of maternal toxicity. Maternal toxicity is a potentially confounding factor and is best avoided. The use of toxicokinetic data to set dose levels is an alternative that can avoid this potential confounder (Saghir et al., 2012).

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