[Frontiers in Bioscience 16, 2734-2746, June 1, 2011]

Induction and patterning of intramembranous bone

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1. ABSTRACT

The primary focus of this article is to review intramembranous bone development, that is, ossification that takes place directly. Comparisons with endochondral ossification (ossification with a cartilage precursor) will be made in order to illustrate the differences between these two modes of ossification and to highlight the comparatively sparse information that is available about intramembranous ossification. Despite decades of research into understanding skeletal development, there is still much to learn. Most of the research in this area has focused on the development of the calvariae (or skull bones) as typical intramembranous bones and the development of the limb bones as a typical endochondral bones. Few studies investigate other skeletal elements or compare these processes in a systematic manner. In this review, I focus primarily on condensation formation and skeletal patterning with specific examples from different organisms.

2. INTRODUCTION

Intramembranous bones are bones that develop intramembranously, that is they develop directly within membranous tissue and no cartilage phase is involved. Intramembranous bones include most of the vertebrate skull (the calvarial bones of the skull roof and most of the facial bones including the jaws) as well as parts of the pectoral girdle. In the lower jaw, the mandibular skeleton, which consists of Meckel's cartilage as a central element, is surrounded by membrane bone which develops from within the perichondrium of the cartilage. Within the pectoral girdle, part of the clavicles of mammals forms directly via intramembranous ossification and part forms indirectly via a cartilage precursor (1). In the classical reptilian model organism, the chicken, the interclavicles (also called the furcula) are considered intramembranous dermal bones (2). In mammals and some marsupials, parts of the scapula are also deposited intramembranously (1, 3). In zebrafish, the

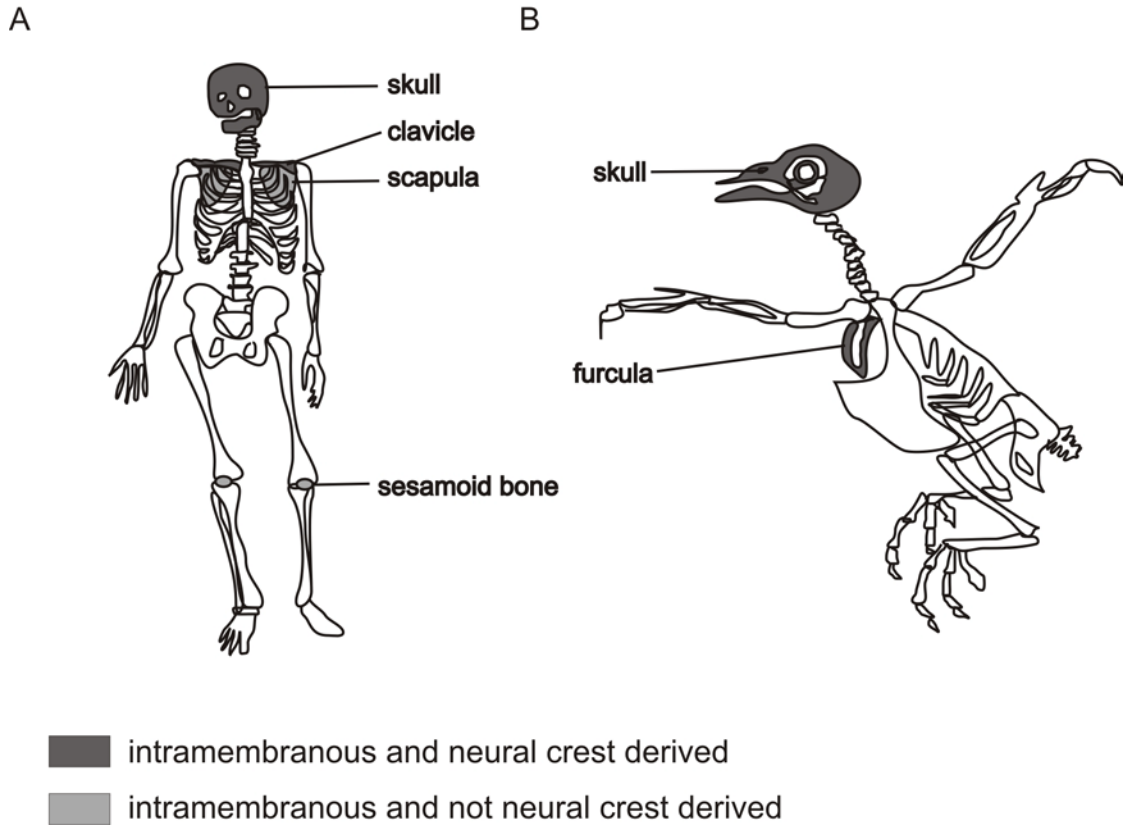

Figure 1. A schematic of the neural crest derived and intramembranous bones of the human (A) and chicken (B) skeleton. Intramembranous bones include most of the skull roof, facial bones and part of the clavicle (or furcula), parts of the scapula, sesamoid bones such as the patella, and periosteal bone around the long bones (latter is not shaded). Neural crest derived bones include the skull roof, facial bones and parts of the clavicle. For clarity, only the major skeletal elements are shaded to illustrate their origins or mode of ossification.

cleithra and opercula are the first intramembranous bones to form (4). These examples demonstrate that there are some significant differences in the ossification modes of skeletal elements amongst vertebrates. Other less obvious intramembranous bones include the sesamoid bones (e.g. the patella in humans) and the periosteal bone that forms around long bones during bone growth (see Section 2.1 below). In this review I will only discuss some of these elements (Figure 1) as many have not been well studied.

In addition to this difference in modes of ossification within the skeletons of vertebrates, there are differences in the germ layer origins of these intramembranous bones. The three primary germ layers (endoderm, ectoderm and mesoderm) of the embryo give rise to all the tissues of the adult organism. A fourth major cell population of the early embryo that many researchers consider as a fourth germ layer is the neural crest. The neural crest cell population arises from the crest of the developing neural tube. These cells acquire mesenchymal properties that enable them to migrate out of the neural ectodermal layer to other parts of the embryo where they give rise to neurons and ganglia of the peripheral nervous system, pigment cells (melanocytes) and bone cells (osteoblasts) (Figure 2). Interestingly, only neural crest cells from the anterior portion of the neural tube (the cranial neural crest cells) are able to differentiate into bone cells. Most of the bones in the vertebrate skull are therefore neural crest derived, whereas most of the bones of the axial (vertebral column and ribs) and appendicular (girdles and limbs) skeletons are derived from mesoderm (Figure 1). In the mammalian skull, for example, the entire frontal and parietal bones are neural crest derived but only parts of the temporal and occipital bones are derived from the neural crest. Currently, there is debate as to how much of the posterior portion of the avian skull is neural crest derived (5-7). Exceptions to this "rule" that the only neural crest derived bones are found in the skull, are the clavicles. In mammals, the clavicles are the only neural crest derived bone outside the skull. The furcula or interclavicles of birds is also neural crest derived. The scapula to which the clavicles/furcula articulate is not neural crest derived although it does partly ossify intramembranously. In teleosts (such as zebrafish), mapping the neural crest derived bones is underway by several research groups.

2.1. An overview of ossification

Regardless of the origin of the cells that contribute to intramembranous bones, the mechanism of their ossification is similar. During intramembranous

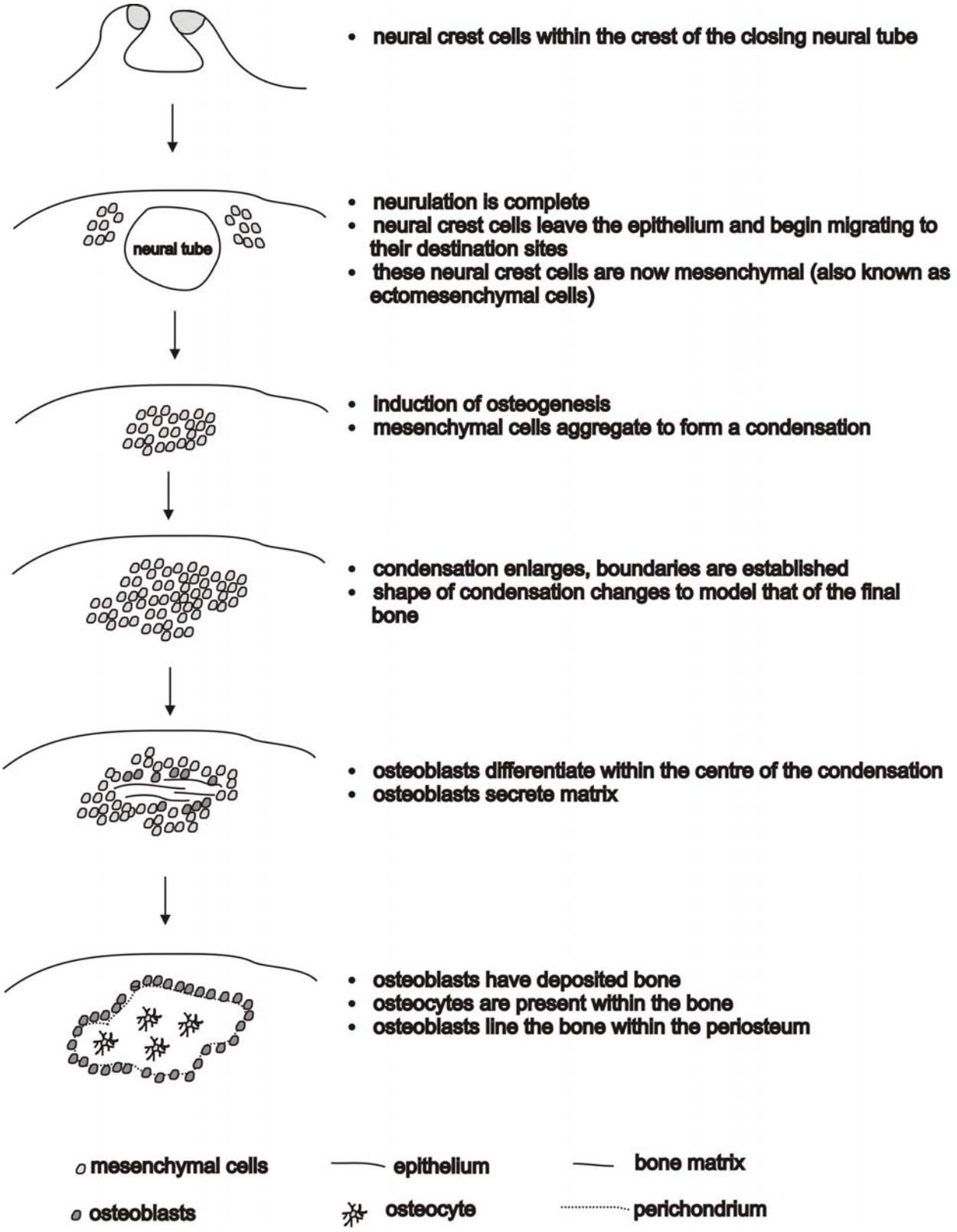

Figure 2. A schematic showing the steps involved in intramembranous ossification. See text for further details.

ossification, osteochondroprogenitor cells differentiate directly into osteoblasts to form intramembranous (or membrane) bones (Figure 2). In contrast, during endochondral ossification these cells differentiate into chondroblasts (which mature into chondrocytes) and ultimately form a cartilage template of the future bone. In both modes of ossification, the osteoblasts secrete bone matrix and are ultimately entrapped in this matrix. Once entrapped these cells are called osteocytes, the mature bone cell. This classic description of bone cell entrapment or burial is not clearly understood (8, 9).

Other modes of ossification include perichondral ossification and periskeletal ossification (10). The term periskeletal is used to encompass all forms of direct ossification from the perichondrium or an extension thereof, and therefore includes perichondral ossification as a subtype. Since the perichondrium is the connective tissue sheath (or membrane) around the cartilage template, both perichondral and periskeletal ossification are considered as forms of intramembranous ossification. The perichondrium is the connective tissue sheath that surrounds the cartilage matrix; it plays a vital role in the maintenance of the skeletal element by providing a passage for the vascular system which supplies nutrients to cartilage cells (chondrocytes) deep within the cartilage matrix. Also located within the perichondrium are osteochondroprogenitor cells, which can differentiate into chondroblasts (and chondrocytes) or osteoblasts (and osteocytes).

A detailed description of the genes and factors expressed by preosteoblasts, osteoblasts, transitional cells, and osteocytes is given in (8). Briefly, we determined that one has to be very careful when interpreting the expression of genes/proteins in the skeletogenic lineage as differential gene and protein expression profiles might reflect particular modes of ossification, types of bone, or the location of the bone (8). The species, age and sometimes the gender of the individual organism can also affect these profiles. It is for this reason that distinguishing the cell types within the osteocyte lineage relies heavily on the cells' association with the bone matrix and their location with respect to the bone matrix. Some of the major signaling molecules involved in induction and patterning of bone are discussed below.

The three key phases of osteogenesis are (a) induction of cells to the skeletogenic lineage, which typically occurs via an epithelial-mesenchymal interaction, (b) condensation formation and (c) differentiation of the cells. Ossification (the secretion of bone matrix) proceeds thereafter (Figure 2). Each of these phases will be discussed below, with special focus on what is known during intramembranous ossification.

3. INDUCTION

The textbook definition of induction is that it is the process whereby one cell or tissue influences another (usually adjacent) cell or tissue in such a way as to change its fate. There is some evidence that neural crest cells are determined prior to reaching their final destination (discussed below in Section 6.1). That is, while these cells are migrating to the site of, for example, skeletal formation, their fate is being restricted such that on arrival at this location they are fated to become skeletogenic cells. That is, they are already osteochondroprogenitor cells destined to the skeletogenic lineage.

Once at their final destination, an epithelial-mesenchymal induction event occurs that results in osteogenesis. Or put another way, the osteogenic differentiation pathway is activated. This process often involves reciprocal epithelial-to mesenchymal interactions and a number of factors. The reciprocal signaling between and among mesenchymal cells (regardless of origin) and the overlying epithelial cells ultimately establishes the pattern of the skeleton (11).

An example of an epithelial-mesenchymal induction of an intramembranous bone is during development of the flat bones of the skull (the calvariae). Here, neural crest derived mesenchyme (ectomesenchyme) interacts with the overlying epithelium (the dura mater) to induce osteogenesis (12, 13). Similarly, during mandible development, the epithelium is vital to promote a skeletal fate in the mandibular mesenchyme (14). This reciprocal signaling is temporally and spatially dependent.

The importance of the location and timing of this inductive signaling can be further illustrated by the following example of endochondral ossification in the chicken skull. Early FGF signaling from the pharyngeal endoderm and the ventral forebrain is required for pharyngeal skeletogenesis (15, 16). Later, FGF from the surface ectoderm regulates outgrowth of the frontonasal skeleton (17, 18). Finally, Bmp4 expression in the mesenchyme of the embryonic beak primordium regulates the size and shape of the beak. That is, signals from the endoderm, ectoderm and mesenchyme all play a role in craniofacial patterning in the avian head; however each signaling mechanism occurs at a particular time and in a particular location within the craniofacial tissue. The context of the signaling is thus an important aspect to consider.

Typically, after induction of the cells within the mesenchyme to the skeletogenic fate, the epithelium is no longer required. The distance over which these epithelial-mesenchymal signals operate may be small (i.e. less than 50 μm) as in the induction of the calvariae, or may be large (i.e. 150-300 μm) as in induction of scleral ossicles within the reptilian eye (19). Further details on the patterning of skeletogenic cells are provided in Section 6.1.

4. CONDENSATIONS

Once mesenchymal cells are induced to undergo osteogenesis, they aggregate to form, a skeletogenic condensation (Figure 2). By definition condensation formation requires an increase in cell numbers within the condensation and/or a decrease in cell numbers surrounding it (1). After induction and initiation of the formation of

Table 1. Major genes and gene products involved in the different phases of skeletogenic condensations (1)

Initiate condensation	Cfkh-1**	Regulates TGF beta
	Tgf-beta1	Regulates fibronectin
	FN (fibronectin)	Regulates NCAM
	N-CAM	Cell adhesion molecule
	Prx1 and Prx 2	Regulates NCAM
Stop initiation	Syndecan	Binds to tenascin and fibronectin, can inactivate N-CAM
Condensation boundaries	Syndecan	Binds to tenascin and fibronectin, can inactivate N-CAM
	Tenascin	Extracellular glycoprotein binds to syndecan
	Hoxa2	Upregulated by BMPs, downregulates Runx2
Cell adhesion	Hoxa13	Alters cell adhesion
	N-CAM	Cell adhesion molecule
	N-Cadherin	Cell adhesion molecule
Cell proliferation	Cfkh-1**	Regulates TGFb
	Mfh-1	Mesenchymal transcription factor
	Sox9	Regulates collagen I and II
	Scleraxis	A basic-helix-loop helix protein
	Hoxd-11	Upregulated by BMPs
	FGF2	Regulates N-CAM
Condensation growth	Hoxa2	Upregulated by BMPs, downregulates Runx2
	Hoxd-11	Upregulated by BMPs
	Bmp2, 4, 7	Regulates hox genes in response to Shh; regulates Msx1, and 2
	Shh	Regulates hox genes indirectly via BMPs
Stop condensation growth	Noggin	Inhibits BMPs
Within condensations	Pax1, Pax9	Regulated by BMP7
	Sox9	Regulates collagen I and II
Cell differentiation	BMP2, 4, 5	Regulates hox genes in response to Shh; regulates Msx1, and Msx2
	MSX 1, 2	
	Hoxa11, 12, 13	Transcriptional activation
	Osf2/cbfa1/runx2**	

* Chicken forkhead helix transcription factor, **acronyms for the same gene (osteoblast stimulating factor 2, core-binding factor 1, mammalian member of runt-domain family of core-binding transcription factors).

condensations, the aggregation of cells can come about through a number of different processes – namely:

1. Increased rate or amount of cell proliferation inside compared to outside the condensation or vice versa,
2. Migration of cells into the condensation area or lack of movement away from the centre,
3. Decreased cell death inside versus outside the condensation,
4. A shorter cell cycle time of cells within the condensation or a longer cell cycle time in cells outside the condensation.

Various studies have shown that the primary cellular processes responsible for condensation formation are the migration of cells towards a centre or the lack of movement away from a centre rather than the more obvious process of altering cell proliferation, cell death or cell cyle rates. This was shown in avian limb buds and in amphibian limb regeneration. See (1) for a detailed discussion of how these processes operate during condensation formation. In 2008, Franz-Odendaal, similarly reports that neither cell death nor cell proliferation appear to play a role in the condensation formation of the scleral ossicles, intramembranous bones in the avian eye (20).

Regardless of which of these processes act to form the aggregation of cells, the condensation needs to reach a critical size in order for differentiation to take place. The condensation boundary also needs to be established. A number of genes and gene products are known to play a role in condensation initiation, formation and in the transition to differentiation. These are summarised in Table 1. Essentially, progressing from condensation to differentiation requires down-regulation of genes associated with adhesion, migration and proliferation (e.g. N-CAM, syndecan) and upregulation of genes associated with arresting condensation growth and those associated with promoting skeletogenic differentiation (e.g. Sox9, Runx2).

In summary, condensation formation is a critical step in osteogenesis responsible for the patterning of the skeleton and necessary for its differentiation. In most vertebrates, skeletogenic condensations have three distinct origins – paraxial mesoderm (for the axial skeleton), lateral plate mesoderm (for the appendicular skeleton) and neural crest derived (for the skull). Most bones of mesodermal origin undergo endochondral bone formation, the exception are elements within the pectoral girdle (Figure 1).

4.1. Timing of condensation formation

The timing between the three key phases of skeletogenesis (induction, condensation formation, differentiation) is not the same for all intramembranous bones. Ossification, which follows cellular differentiation to osteoblasts, will only begin once the condensation has reached its critical size.

In chickens, the clavicles, which are part of the pectoral girdle, develop as a pair of condensastions. These clavicular condensations appear at HH stage 31-32 (day 7-7.5) and ossification follows rapidly at HH stage 33, only 12-18 hours after condensation formation (21). By HH stage 34 (day 8) the entire condensation is ossified. The epithelial induction event that signals formation of the clavicular condensation is completed by HH stage 29 which

Table 2. A comparison of the timing of induction, condensation formation and ossification in two intramembranous bones of the chicken

	Clavicle	Scleral ossicles
Induction completed	HH 29 (day 6-6.5)	HH stage 36.5 (day 10.5)
Distinct Condensations	HH 31-32 (day7-7.5)	HH stage 38 (day 12)
Ossification	Completed at HH 34 (day 8)	Begins at HH stage 41(day 15)
Total days:	2 days	5 days

HH stages are according to (22)

is 1-1.5 days before condensations are evident and 1.5-2 days before ossification begins. Similarly, when compared to scleral ossicles, another dermal bone in the chicken, epithelial induction starts at stage 35 (day 8.5-9) and is complete by late stage 36 (day 10) (20). Condensations are first visible at stage 38 (day 12), which is about 1.5 days after induction. Ossification begins a few days later (stage 41, day 15). This comparison is summarized in Table 2, which clearly demonstrates that different neural crest derived intramembranous bones from the same organism are induced and ossify according to different temporal schedules.

Careful consideration of the timing of events should be taken into account when comparing the development of skeletal elements, even if they are derived from the same germ layers or ossify by the same mechanisms. That is the spatial expression patterns of genes as well as their temporal patterns are important.

4.2. Condensations splitting and fusing

One of the remarkable characteristics of condensations is their ability to split up or to fuse with other condensations. These latter condensations may be derived from different cellular origins or germ layers and may ultimately ossify in different ways (endochondrally or intramembranously). The condensation in the forearm that ultimately gives rise to the ulna and radius in vertebrates, for example, first develops as a single condensation which then splits horizontally to give rise to two condensations. Conversely, the frontal, parietal and interparietal bones in humans, each develop as two condensations that then fuse as ossification progresses (23). In these latter cases of two condensations for a single bone, each condensation has its own ossification centre. This example of one bone having two ossification centres may be restricted to elements that arise in two halves, one for each side of the body. The human scapula forms from eight ossification centres, however many of these are for the endochondral portions of this element (24). Bones that ossify via different ossification modes can also fuse together. For example, the interparietal fuses with the supraoccipital bone to form the squamosal part of the occipital bone – the interparietal is formed by intramembranous ossification while the supraoccipital is formed by endochondral ossification. In summary, the fusion and splitting of condensations is common within the vertebrate skeleton, yet few studies have investigated how these processes occur. Presumably they would involve an array of mechanisms and genes, such as altering the condensation boundary genes (e.g. syndecan), activating cell death or cell migration, or changing the activity of matrix metalloproteinases.

4.3. Direction of ossification

The direction in which ossification takes place from within the condensation has largely been overlooked by skeletal biologists. The clavicles which are cylindrically shaped bones ossify laterally; the calvariae which are large flat triangular or rhomboid bones, similarly ossify in a lateral to medial direction (25). This is in contrast to scleral ossicles, which are flat square-shaped bones within the eye that ossify from the centre of the condensation outwards (Figure 3). Detailed studies investigating the differences in gene expression and osteoblast activity across a single condensation are lacking, yet in order to fully understand skeletal development more detailed analyses at the level of the condensation is needed.

5. SKELETAL PATTERNING

5.1. Before condensations arise

Several research groups have shown that cranial neural crest cells possess intrinsic patterning information that controls the timing, the location and the formation of the skeletogenic condensations (e.g. 28). That is, skeletal identity is acquired during early development *before* the mesenchymal condensation forms through a process called pattern formation. This patterning is controlled by several major signaling pathways which are mediated by Wnts, Hedgehogs, BMPs, FGFs and Notch/Delta. Later these same pathways control cell fate determination, proliferation, and maturation in the skeleton. During limb patterning, for example, three signaling centres are active in the early limb primordium (limb bud) before mesenchymal condensations (29). Fibrillin-1 and fibrillin-2 are abundant in the extracellular matrix before condensation formation in the limb bud. Some evidence suggests that fibrillins maintain the physiological thresholds of TGFb and BMP signals during osteoblastogenesis by balancing the pools of committed and mature osteoblasts (30, 31). Other evidence indicates that gap junctions are important in patterning skeletogenic tissues since these junctions are important for cell-to-cell communication (32). Furthermore, it has also been shown that the otic vesicle can affect the pattern of neural crest cell migration in the hindbrain (33, 1).

External factors can also affect the final shape and size of the bone (26, 27, 34). Transplantation experiments between quail and chick have concluded that the capacity to form species-specific craniofacial skeletal elements is an inherent property of neural crest cells (6, 34). For the mandibular arch elements, this patterning information (including both skeletal identity and orientation) is acquired from external signals, e.g. from the endoderm (6). Other researchers have shown a role for mesoderm in patterning cranial neural crest cells (35).

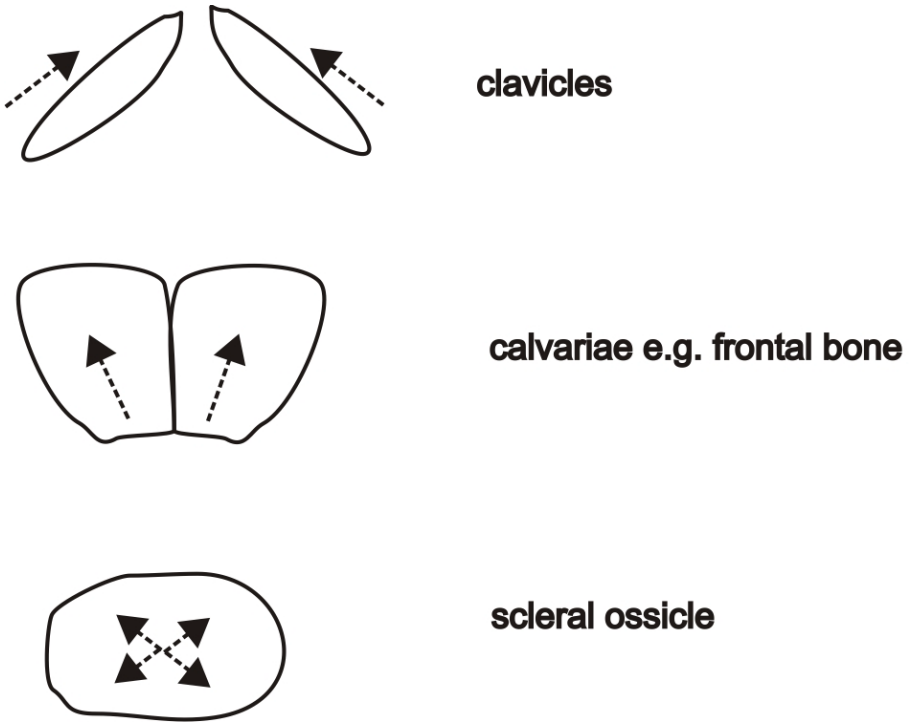

Figure 3. A schematic showing the direction of ossification within skeletogenic condensations.

More recently, it was shown that the neural crest cells themselves also provide some of this patterning information (e.g. for shape), and are not passive players in the patterning process (34). Importantly in this study, it was shown that the transplantation was population dependent – and size matched. That is, the skeletogenic condensation needs to be a certain large size before the cells with it follow the molecular cues that they themselves generated and that are maintained by the entire group of cells. Only at this point do the cells within the condensation disregard signals from the local environment. We still do not know what these cues are or what the critical size is that is needed to maintain condensations.

Another classic example demonstrating the importance of timing on pattern formation can be found during tooth development (Table 3). In one study, tissue recombination experiments indicate that the dental mesenchyme is the source of patterning information. In a different recombination experiment, involving different sources of mesenchyme, teeth are however also produced. It turns out that the outcome of each experiment depends on the timing of the experiment and the degree to which the tissues that are to be recombined have already received patterning signals. Before E11, the source of patterning is the oral ectoderm; after E11 or later, the source of patterning is the mesenchyme. That is, the mesenchyme acquires the patterning ability after it itself has received patterning cues from the epithelium. Facial morphogenesis is therefore a cumulative result of reciprocal signaling between and among all these tissues. We will continue with this example in the next section when we discuss signaling.

5.2. Condensation signaling

Little is known about the way in which signaling from epithelium to mesenchyme occurs during intramembranous ossification. That is, signaling may occur via direct cell-cell interaction, or via diffusible factors or both. In calvaria, it is believed both mechanisms occur. In addition, the diffusible factors may act in an autocrine manner, affecting the same cells that secreted the factor, or may act in a paracrine manner, affecting neighbouring cells. Paracrine signaling molecules tend to be small and are often called growth or differentiation factors; they generally fall into one of the four key families (Fibroblast growth factors, Transforming growth factor-beta, Hedgehog, and the wingless family). Signaling molecules can also act in an endocrine manner, affecting cells at some distance away from the secreting cells and travelling via the circulation.

The development of a particular tissue may involve all of these families or only some and the signaling may occur from epithelium to mesenchyme or vice versa. One classic example in which this is demonstrated is during tooth development in the mouse (40). The oral ectoderm gets a pre-pattern through a nested expression of Fgfs, Shh, and Bmp4. This is evident as early as neurulation – long before pharyngeal arches have formed (41). It appears that the pharyngeal endoderm sets up this framework for tooth development with Fgf8 as a key player (11). The mesenchyme then interprets these signals into spatially restricted domains of homeobox gene expression. These transcription factors then regulate other signaling molecules (Bmp, Wnt, Fgf proteins) which induce epithelial folding and invagination.

5.2.1. Hedgehog family

The vertebrate hedgehog family comprises Indian hedgehog (Ihh), desert hedgehog (Dhh) and sonic hedgehog (Shh). They are involved in left-right asymmetry, cartilage differentiation, limb morphogenesis, myotome, hair follicles and sclerotome and neuronal specifications (e.g. 29). These vertebrate proteins are homologues of the *Drosophila* Hedgehog which encodes secreted proteins and are involved in cell-cell communication. Interestingly, placental mammals have all three Hh members, whereas it appears as if other vertebrates (e.g. reptiles such as chicken, zebrafish finch and *Anolis*) are missing Dhh (based on genome searches). In vertebrates, all Hedgehog signaling is mediated by two transmembrane proteins Ptc and Smo. In the inactive signaling pathway, when HH is not bound to Ptc, Ptc inhibits Smo. When HH binds to Ptc, it prevents this inhibition allowing the signal to be transduced. This leads to a cascade that upregulates Hh target genes (e.g. Gli1, Hip1, Ptc1)(29).

5.2.2. Ihh signaling

Ihh signaling is required for osteoblast differentiation by activating Runx2 expression, however this factor is only required for endochondral ossification. In intramembranous ossification Runx2 expression is independent of Ihh. It is not known what controls Runx2 expression in intramembranous ossification but it has been proposed that the function of Ihh is compensated for by Shh (29, 42, 43). This has been demonstrated in the calvaria with Ihh knockout mice. These mice can still form intramembranous bones although their growth is slightly impaired (44).

5.2.3. Wnt signaling

Wnt signaling within the condensations is higher during intramembranous ossification than during endochondral ossification as it promotes osteoblast differentiation while inhibiting chondrocyte differentiation (29). During endochondral ossification, the levels of Wnt are kept low to promote chondrocyte differentiatiation. Later, Wnt is upregulated in the periochondrium where osteoblasts differentiate; the perichondrium will ultimately transform into the future periosteum (29). Wnt/B catenin signaling can also act downstream of Hh signaling in promoting bone formation.

5.2.4. Bmp signaling

Bmp signaling promotes differentiation of osteoblasts and chondroblasts from mesenchymal progenitors and therefore plays a role in the early commitment to osteogenic and chondrogenic lineages. BMPs are considered to be of key importance during mesenchymal condensations (45). At least five TGF-beta family members (BMP4, BMP5, BMP7, GDF5, GDF6) play a role in patterning skeletal elements. Bmp signaling is transduced through at least two distinct pathways: canonical Smad-mediated and a mitogen-activated protein kinase (MAPK) pathway (46). These activities are modulated by secreted inhibitors – Noggin and Chordin. In several different developing systems (including the skeleton) BMPs act in response to Shh (47-49) or via FGF/FGFR mediated pathways (50).

Condensation size is also regulated in part through BMP signaling pathways, specifically BMP 2 and 4. Overexpression of both of these growth factors in the developing chick beak results in dramatic increase in both size and shape of skeletal elements (51). BMP2 may play a role in modulating expansion of condensation size since it is expressed in the mesenchyme surrounding condensations (1).

5.2.5. FGF signaling

FGF ligands (FGFs) and FGF receptors (FGFRs) are both expressed in the developing skeletal system as well as in many other developing tissues. There are 22 FGF ligands in total in mammals. Each of these contains a core of 120 amino acids that permits binding to heparin and heparin sulphate proteoglycans, such as syndecan (53). Binding to these molecules is believed to restrict the distance FGFs are able to travel. FGFRs have a transmembrane and an intracellular domain. Each FGFR can make a variety of splice variants. In addition, soluble FGFRs can form; these compete with membrane bound forms as they can bind ligands. It has been shown that cell adhesion molecules can signal via FGFRs although the mechanism is not clear (54). Some FGFRs (e.g. FGFR1 and FGFR2) have been found to play a role in mesenchymal cell condensations of intramembranous bones (reviewed in 55). There is evidence that Fgf signaling acts in mesenchymal condensations to control osteoblast differentiation during intramembranous ossification however the mechanisms remain to be elucidated (29). Specifically, FGF18 and FGF20 have been found in mesenchymal condensations of calvarial bones while FGF 2, 4 and 9 have been found within sutural mesenchymal (reviewed in 55). The latter has a mesodermal and neural crest contribution.

The function of FGF signaling in mesenchymal condensations and in chondrocyte differentiation from progenitors remains to be elucidated. Complete inactivation of FGF signaling in mesenchymal condensations has not been achieved. Mutant mice lacking some FGFs and FGFRs do not show phenotypes in mesenchymal condensations or in chondrocyte differentiation. That is there is no bony phenotype, although they do exhibit defects in neurons and vasculature. Overexpression of FGFs (e.g. FRGR3) in mice results in chondrodysplasia with shortened long bones and macrocephaly. Despite shortening of long bones that form via endochondral ossification, the occipital bone which partly ossifies intramembranously and partly endochondrally is enlarged (56). Fgf signaling can promote or inhibit osteoblast proliferation and differentiation depending on context. This is done either directly or by acting with Wnt and Bmp pathways (28).

5.3. Target genes: MSX1 and 2

Msx1 and Msx 2 are expressed in a number of vertebrate tissues including in neural crest cells, bone and teeth. They are often associated with epithelial-mesenchymal interactions where they are targets of FGF and BMP signaling. Msx1 and Msx2 are associated with

Table 3. Tissue recombination experiments involving dental mesenchyme and epithelium from mouse and chicken demonstrating the importance of timing (11, 36-39)

Mesenchymal tissue	Epithelial tissue	Source of patterning
Dental mesenchyme	Non-dental ectoderm	Dental mesenchyme
Naïve mesenchyme	Presumptive dental epithelium	Dental epithelium
CNCCs (mouse)	Chicken dental epithelium	Makes teeth with avian epithelium and murine ectomesenchyme

Table 4. Summary of the skeletogenic phenotype of runx2 and sox9 mutants in mouse and zebrafish

	Mouse	Zebrafish
Runx2*	Runx 2 mutant: No osteoblasts plus no intramembranous ossification and no endochondral ossification (64, 65)	*Runx2b* depletion (morpholinos) – affects craniofacial cartilage formation by affecting the transition from pre-chondrocytes within condensations to cells undergoing chondrogenesis (4). *Runx2a* does not seem to play a role in this; neither does runx1. *Runx2b* (and also *runx3*) is essential for endochondral ossification.
Sox9	Sox 9 mutant: No cartilage formation (66).	Sox9a mutants (morpholino): chondrocytes don't stack; Sox9b mutants: insufficient cell numbers (67); Sox 9a mutants (morpholino), some dermal bones (dentary, maxillary, opercular) are reduced in size others are unchanged (cleithrum). In sox9b mutants, most dermal bones are missing, some are reduced (cleithrum, opercle). (67); Sox9 null mutants: massive cell death in trunk neural crest at around the time that neural crest cells delaminate (68).

*although Runx2 is key player, some compensation from other members of Runx family of proteins (e.g. from Runx1 and Runx3) are known in both mouse and zebrafish (4, 69-71, 77).

intramembranous ossification in the skull (57, 58) and with tooth development in mice (59). In calvaria, the level of Msx2 expression decreases with increasing osteoblast differentiation to the extent that this gene might negatively regulate collagen I and osteocalcin, two differentiated osteoblast markers (e.g. in 60-62, and others). Msx2 has been suggested to be an upstream regulator of Runx2 in osteoprogenitor cells (in mice) (58), and plays a crucial role in maintaining the balance between osteoprogenitor differentiation and proliferation during calvarial development, particularly at the sutures (55, 63).

5.4. Two important regulators: Runx2 and Sox9 within condensations

Runx2 and sox9 are two critical genes for osteogenesis across vertebrates. Table 4 summarizes the phenotype of the mouse and zebrafish null-mutants for these genes and shows that although mutants of both species show severe skeletal defects, these defects are not the same in each organism.

Runx 2 (also known as Cbfa1) plays a critical role during osteoblast differentiation. This gene is one of the mouse homologs of the *Drosophila* runt protein. *Runx2* has been found to be necessary for bone and tooth formation and its expression is largely restricted to osteoblasts and mesenchymal condensations that give rise to these elements (72-74). Mutant mice lacking the *Runx2* gene have no osteoblasts, and also no intramembranous or endochondral ossification (64, 65). Much of this data, however, comes from cell culture work; and whether Runx2 is sufficient for inducing intramembranous ossification *in vivo* remains to be elucidated (75). Interestingly, in the Runx2 knockout mice, molar development arrests at the late bud stage, with the lower molars more severely affected than the upper molars (76).

In zebrafish, there are two *runx2* genes but only one *runx1* and one *runx3* gene; that is only part of the Runt-family was duplicated during the teleost genome duplication event. *Runx2a* and *runx2b* are expressed in primordia of intramembranous and endochondral bones; however they are expressed in different patterns (4, 76). At 2 dpf, *runx2a* is strongly expressed in the opercula and at the base of the cleithra, whereas *runx2b* is expressed at low levels in the opercula and over the entire length of the cleithrum at this age (64). Similarly in cartilages within the head different expression patterns are observed at 2 dpf. *Runx2a* is expressed in the trabecula crania and is diffuse in the parachordal cartilages, whereas *runx2b* is strongly expressed in the trabecula, in the ethmoid plate; and in the parachordals (4, 76). *Runx2a* is expressed in the first pharyngeal arch (mandibular) and is low in the second arch (hyoid), but is absent in ceratobranchial 1-5. In contrast, all seven arches express *Runx2b* in condensations; although the expression is low at first.

The Sry-related transcription factor Sox9 is required for differentiation of persistent and replacement cartilages. It activates expression of genes associated with cartilage, such as *col2* and *aggrecan*. Similarly to runx2, the two sox 9 orthologues partitioned spatially and temporally in zebrafish, and have been shown to regulate other neural crest specifiers (*foxd3, sox10, crestin*) as well as the cartilage gene *col2a1* and the bone gene *runx2*. Expression of *Tfap2-alpha* was unchanged in sox9a and sox9b single and double mutants (62). In Sox9a mutants, chondrocytes fail to stack and in sox9b mutants they fail to attain proper numbers. These authors further conclude that *sox9a* is more important for endochondral bone formation whereas *sox9b* is important for both endochondral and intramembranous bones.

In a ground breaking study in 2004, Eames and colleagues showed using *in situ* hybridization in chicken embryos, that permanent cartilages, replacement cartilages and direct bone have different expression profiles of *runx2* and *sox 9* in their condensations (75)(Table 5). They

Table 5. Expression of Runx2 and Sox9 in condensations for permanent cartilage, replacement cartilage and in directly ossifying bones in chicken embryos (75)

	Permanent cartilage (meckel's)	Replacement cartilage (ceratobranchial)	Direct ossifying bone (surangular)
Runx2	-	+	+
Sox9	+	+	-

Table 6. Expression of *runx2a, runx2b, sox9a* and *sox9b* in condensations for permanent cartilage, replacement cartilage and in directly ossifying bones in zebrafish (4, 78-81)

	Permanent cartilage (meckel's, parasphenoid)	Replacement cartilage (ceratobranchial)	Direct ossifying bone (opercle, cleithrum)
Runx2a	+	-[1]	+
Runx2b	+	+	+
Sox9a	+	+	-?[2]
Sox9b	+	+	-?[2]

[1]But does have *runx3* which plays a role in chondrocyte differentiation (4), [2]The cleithra are not shown as positive in images of wildtype pectoral fin in 81, Figure 1.

showed that in the condensations of permanent cartilages *runx2* is down regulated while *Sox9* is upregulated; in condensations for replacement cartilages (i.e. cartilages that will undergo ossification to form bone), *runx2* and *sox9* are upregulated; and finally in condensations for directly ossifying bones (i.e. intramembranous bones), *runx2* is upregulated while *sox9* is down regulated. This analysis indicates that based on the expression profiles of these two genes at the condensation stages, the fate of the condensation can be predicted. This study was conducted in chicken and its applicability to other organisms remains to be determined. Table 6 shows the expression profiles for these three types of condensations (permanent cartilage fate, replacement cartilage fate, or direct bone fate) in zebrafish. From the published literature to-date, it appears as if the expression profiles noted by Eames and colleagues are not observed in zebrafish (71). This difference may explain the different phenotypes observed in null-mutants for *runx2* and *sox9* in mouse compared to zebrafish. It appears therefore that in zebrafish, although the same genes (and cells) are involved in skeletogenesis, different mechanisms and pathways of osteogenesis take place. This might be due to the genome duplication event that took place in teleosts, which enabled subfunctionalisation and neofunctionalisation of the skeletogenic genes (82).

6. SUMMARY AND PERSPECTIVE

A myriad of factors are involved in skeletogenesis and numerous studies have explored these factors. Unraveling the expression pattern of these genes must however involve an awareness by researchers of the different cellular origins and ossification modes of the skeletal elements as well as the dynamics of condensation formation. Pathways of osteogenesis may vary between organisms, as well as between skeletal types, locations and origins. There are many fundamental aspects of condensation dynamics that are not understood well, even in popular model organisms such as the chicken and mouse. A systematic comparison of skeletogenesis within an organism and across organisms would greatly enhance our understanding of the intricacies of skeletal development. Although most of the research on intramembranous ossification has revolved around the calvariae, there are other intramembranous bones that should not be overlooked and which could provide important insight into skeletal induction and patterning.

7. ACKNOWLEDGEMENTS

I acknowledge the Natural Science and Engineering Research Council of Canada for awarding me a University Faculty Award and a Discovery Grant (305401). In addition I thank the Nova Scotia Health Research Foundation and Mount Saint Vincent University for funding and infrastructure. I would also like to thank wBrian Hall for his guidance over the last few years and the Editor of the Frontiers in Bioscience for the invitation to write a review on this remarkable field of biology.

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Abbreviations: HH: Hamburger and Hamilton

Key Words: Dermal Bone, Intramembranous Ossification, Skeleton, Signaling, Bone, Ossification, Neural Crest Cells, Condensations, Review

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