Chapter 5

External Appearance and Polarity

We have seen in previous chapters that in the early stages the chick embryo is a flat disc, the blastoderm, the centre of which is destined to form the embryonic body, whilst the periphery will give rise to the yolk sac and amnion. The first indication of the body proper is at the anterior end at about stage 6 (about 24 h of incubation). Anterior to the tip of the head process a crescent-shaped groove appears in the entire thickness of the blastoderm. This is the head fold (*Text-Figures 34, 50; Plate 15*). The result is that the region of the future head, together with the developing foregut, becomes raised above the flat blastoderm (*Plate 61*). Sections show that the head is covered with ectoderm and is separated at its tip from the blastoderm. The folds continue to expand posteriorly and at the same time the growing head extends further anteriorly, becoming more and more defined. The lateral body folds (*Text-Figure 35*), which start to form as longitudinal grooves of the ectoderm and somatic mesoderm on either side of the embryo, are continuous with the posterior extensions of the head fold. There is a high level of cell division in the ectoderm of the folds (Miller, 1982); by stage 15 the lateral folds extend to the anterior wing level (somites 15–17) and by stage 16 have reached the level of somites 17–20. Meanwhile, the tail fold has begun to form at the posterior end (*Text-Figure 51*). By stage 17 the head fold (*Text-Figure 50*), lateral body folds (*Text-Figure 35*) and the tail fold have met to form a continuous groove around the periphery of the body, lifting it up above the surface of the yolk sac. By stage 20 the body is no longer continuous with the extra-embryonic tissues except in the region of the umbilicus where the head, tail and lateral body folds converge.

Although the foldings are most conspicuous in the ectoderm and somatic mesoderm, they also include the endoderm and the splanchnic mesoderm, so that they lead to the early stages in the formation of the gut. The foregut (*Plate 61*) forms as the head fold develops and the hindgut is initiated by the tail fold (see Chapter 8).

Once the body starts to become raised above the blastoderm, changes in the external appearance begin. The cranial flexure (*Text-Figure 30*) is visible from about stage 12 or 13 (*Plate 77*), though measurements of the cranial angle show that it begins as early as stage 10 (Goodrum and Jacobson, 1981; Pikalow *et al.,* 1994). It starts with the head bending ventrally toward the yolk sac, and there is evidence that this is correlated with the ventral bulging and elongation of the prosencephalon (Goodrum and Jacobson, 1981). The infundibulum at the anterior end of the neural tube is anchored to the foregut by Rathke's pouch (*Plate 105*) so that as the brain elongates, it has to bend around the foregut. Meanwhile, the head has begun to rotate also so that its left side comes to lie against the yolk sac and its right side to lie uppermost (see Appendix II; *Plate 88*). At this stage the trunk region has not yet turned and its ventral side still lies against the yolk sac with its dorsal side uppermost, but gradually the rotation spreads down the body until at about stage 20 the entire embryo has rotated and lies on its left side.

Two other flexures occur that affect the shape of the body. In the cervical flexure (*Text-Figure 30b*), which forms between about stages 14 and 23 (2–4 days), the head bends round in the neck region so that it comes to lie at right angles to the trunk, its ventral side lying against the ventral side of the pharynx. Experimental evidence suggests that the cervical flexure is related to looping of the heart, though whether the contraction of the heart from a straight tube to a looped one is responsible for pulling the head around in an arc (Flynn *et al.,* 1991) or, conversely, whether the head flexures are responsible, at least in part, for cardiac looping (Männer *et al.,* 1993), is not yet clear. Meanwhile the trunk itself becomes curled

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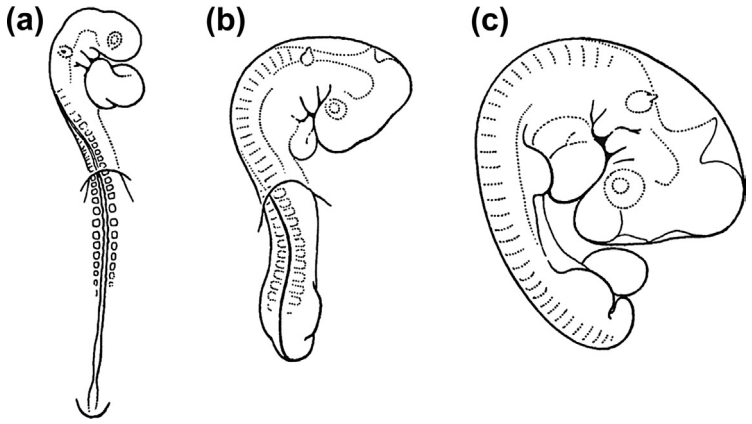


Text-Figure 30. Body flexures. (a) ***Cranial flexure****, about stage 14: the head has turned to the right but the trunk has not. (b)* ***Cervical flexure****, about stage 18: the head now lies at right angles to the trunk. (c)* ***Trunk curvature****, about stage 22: the trunk has now turned so that the whole embryo lies on its side and the tail bud is close to the head.*

around so that the tail bud comes to lie close to the head (*Text-Figure 30c*).

From this stage onwards the body shape is an indicator of many of the events taking place beneath the surface. These include the formation of the limb buds (*Plates 29, 30*), starting at about stage 17, with their enlargement and elaboration until the major features of the wing or leg have formed at about 10 days. The tail bud (*Plate 31*), the developing eyes, the beak and the pharyngeal pouches are all conspicuous aspects of the developing embryo, as is the bulge caused by the increasing size of the heart. These and other external features are categorized in the Normal Tables of Hamburger and Hamilton (1951; see Appendix II).

FACE

Before the face starts to form, the anterior part of the head consists of an ectodermal layer over the forebrain with a few scattered mesenchyme cells. The facial structures are derived from neural crest cells which migrate between the forebrain and the ectoderm during day 4, or earlier, of incubation (after the stomatodaeal plate has ruptured; see Chapter 8), and form a group of outgrowths. These are the left and right **maxillary** and **mandibular** processes of the first pair of pharyngeal arches which lie on either side of the stomatodaeal opening, and the medial **fronto-nasal process** which lies above it. The nasal placodes (*Plate 27*) become visible as shallow pits at stages 15–16 and indent further by stage 18 on either side of the fronto-nasal process. The maxillary processes grow towards the midline of the face during days 4 and 5 and fuse with the sides of the fronto-nasal process. The fronto-nasal process itself becomes extended forward to form the upper jaw, whilst the two mandibular processes also fuse in the midline and become extended forward to form the lower jaw (*Text-Figure 79*). The maxillary processes meet in the midline and form the palate and, together with the fronto-nasal process, the upper lip (beak). The expansion of these processes is largely due to cell proliferation, especially at the tips of the maxillary and mandibular processes, but also at the base of the fronto-nasal process (McGonnell *et al.*, 1998). Apoptosis takes place in the **periderm** (see Chapter 11, p. 120) at the site of fusion and the epithelial cells proper break down to form mesenchyme at the region of junction (Sun *et al.,* 2000).

The facial outgrowths form as a result of an interaction between the overlying epithelium and the neural crest mesenchyme. The bone morphogenetic proteins BMP2 and BMP4 appear to play a role in the outgrowth of these structures (Francis-West *et al.,* 1994) as well as FGF signals emitted from the nasal pits (Szabo-Rogers *et al.*, 2008). High levels of *Msx* gene transcripts have been correlated with regions of outgrowth (Brown *et al.,* 1997). The presence of the epithelium is essential, since without it the neural crest mesenchyme cells will not form the primordia (Saber *et al.,* 1989) but the type of facial prominence that does develop is determined by the mesenchyme. The evidence comes from recombinant experiments in which the epithelium has been separated from the mesoderm of one primordium and been replaced by the epithelium of another (Richman and Tickle, 1989), and is supported by molecular studies showing that signals from the ectoderm of the fronto-nasal process regulate the BMP proteins in the neural crest-derived cells (Hu and Marcucio, 2009a,b).

The site at which a particular primordium forms, however, appears to be dependent on events in the

ectoderm. For example, before the cranial neural crest cells have arrived in the facial region, the ventral head ectoderm expresses FGF8 in two domains that correspond to the future mandibular processes, and BMP4 at the site of the future maxillary processes. The spatial relationship between the forebrain and the facial features appears to be coordinated by local retinoid signalling that maintains the expression of FGF8 and *sonic hedgehog* (Schneider *et al.,* 2001; Le *et al.,* 2001). A region of the fronto-nasal process, the fronto-nasal ectodermal zone (FEZ), has been identified as a signaling centre expressing SHH controlling the dital tip of the upper jaw (Hu and Marcucio, 2009b); it is expressed in the forebrain prior to the outgrowth, but then becomes activated in the adjacent epithelium of the FEZ.

A morphometric analysis of changes within the fronto-nasal process was published by Patterson and Minkoff (1985). The development of the primary palate was described by Yee and Abbott (1978). Extirpation of the fronto-nasal process leads to the reduction of the upper beak, agenesis of the primary palate and impaired development of the maxillary processes and the palatal shelves (McCann *et al.,* 1991).

ORIGIN OF THE LIMBS

The potential limb regions become visible from about stage 15 (50–55 h) (Appendix II) as slightly thickened ridges of the somatic lateral plate mesoderm, though, according to Stephens *et al.* (1992) a limb-forming region can be recognized as early as stage 11. The wing buds (*Plates 94, 107d*) form at the level of somites 15–20 and the leg buds (*Plate 96*) at the level of somites 26–32, these levels apparently being determined by the distribution expression of various *Hox* genes. For example, the forelimb buds form at the most anterior level of expression of *Hox-6* (Burke *et al.,* 1995; Burke, 2000).

As the lateral body folds form, the limbs come to lie along the sides of the body wall. At 3 days of incubation each limb bud is about 1 mm in length by about 1 mm in width (*Plate 5* of Appendix II). Each consists of an envelope of ectoderm enclosing a core of mesoderm; the ectoderm is derived from the ectoderm of the lateral body wall and the mesoderm is formed from the somatic lateral plate, although this subsequently becomes supplemented by cells migrating in from the somites. The somatic mesoderm gives rise to the tendons, skeleton, dermis and connective tissues of the limbs, whilst the somitic cells form the muscles.

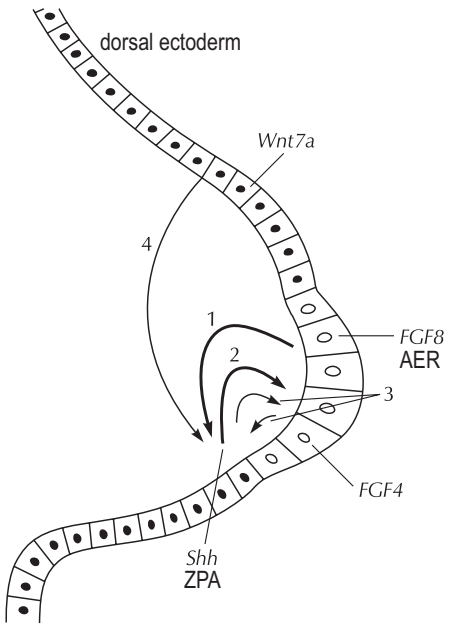
The first morphological step in the formation of the limb bud is the proliferation of the lateral plate mesoderm cells, and this appears to be brought about by the production of the paracrine factor FGF10 by the lateral plate mesoderm itself. When beads soaked in FGF10 were inserted under ectopic ectoderm supplementary limb buds were induced (Sekine *et al.,* 1999). The FGFs themelves appear to be induced by members of the *Wnt* family of growth factors. In particular, *Wnt2b* is expressed in the wing region and *Wnt8c* in the leg (discussed by Tickle and Munsterberg, 2001). The mesenchyme cells become polarised along the proximo-distal axis of the limb bud as they migrate (Wyngaarden *et al.,* 2010; Gros *et al.,* 2010).

In their early stages of development, the wing and leg buds are similar to one another morphologically, but by about stage 24 they have begun to acquire their individual characteristics. These events are controlled, at least in part, by the transcription factors TBX5, which is present in the wing buds, and TBX4, which is found in the leg buds. Experimental introduction of these factors into a developing limb bud has been shown to influence its future development into either a wing bud or a leg bud.

At the tip of the limb the ectoderm becomes thickened and is then known as the **apical ectodermal ridge** (*Plates 141, 142*). Important interactions take place between the ridge and the underlying mesoderm. The ridge itself is induced to form by the mesenchyme cells, largely through the secretion of FGF10, and possibly also FGF8 (Johnson and Tabin, 1997), but the mesenchyme is subsequently dependent on the presence of the ridge. If the ridge is removed, the limb fails to differentiate further, whereas if an additional apical ectodermal ridge is grafted onto the limb bud a supernumerary limb structure develops (Saunders *et al.,* 1957). The development of the ridge and the signalling pathways involved are reviewed by Fernandez-Teran and Ros (2008).

The mesenchyme immediately beneath the **apical ectodermal ridge** is a region of high mitotic activity, being the source of the additional cells that are needed as the limb bud elongates. It is known as the **progress zone** and consists entirely of cells that are as yet undifferentiated. It appears to be maintained by FGF8 secreted by the apical ectodermal ridge. There is also evidence that *Wnt* signalling is involved (Kawakami *et al.,* 2001). Gradually, the most proximal cells in the progress zone leave it, being replaced by more cells distally as mitosis continues. The first cells to leave the progress zone form the most proximal structures of the limb, and successively, as more and more

cells leave the progress zone, they become destined to form progressively more distal structures. The specification of the tissues that form along the limb bud is due to the activity of a sequence of *Hox* genes. In the wing bud the humerus forms first, apparently under the influence of *Hox-d9* and *10,* which is expressed at the time throughout the limb bud. This first phase is followed by the formation of the radius and ulna (middle phase) and then by the metacarpals and digits (third phase), and during these periods there is a rearrangement of the expression of these genes and the expression of additional *Hox* genes. These skeletal structures are formed initially in cartilage and become ossified later (see Chapter 10). Once the regions of the limb have become laid down, the apical ectodermal ridge disappears (Wolpert, 2002). During this period when the proximo-distal axis is already present, two further axes become established. The first is that of the anterior-posterior axis. This has been shown by a series of experiments to be controlled by the so-called ZPA (zone of polarizing activity), a region of mesenchyme at the posterior border of the early limb bud near its junction with the body wall (*Text-Figure 31*). If this region is transplanted to the anterior border of another, intact limb bud it leads to the formation of additional, duplicated limb skeletal elements with a polarity defined by the graft usually in mirror image to that of the host limb. Similar duplications were seen in which beads soaked in retinoic acid were implanted in the same region, the degree of duplication being dose dependent (Tickle, 1991). It appears that retinoic acid is synthesized in the proximal mesenchyme and then passes distally along the limb bud forming a gradient of concentration, the higher levels initiating more proximal structures whilst the lower levels cause more distal ones (Bénazet and Zeller, 2009).



Text-Figure 31. *Some of the molecular interactions at the apical ectodermal ridge which are involved in the initiation and maintenance of the limb bud. Stage 1: the apical ectodermal ridge (AER) expresses FGF8, which induces sonic hedgehog (Shh) in the zone of polarizing activity (ZPA). Stage 2: ZPA expresses Shh, which induces FGF4 in the posterior apical ectodermal ridge. Stage 3: Shh and FGF4 reciprocally maintain each other. Stage 4: the dorsal ectoderm expresses Wnt7a which helps maintain Shh in the ZPA.*

Riddle *et al.* (1993) demonstrated that the ZPA was characterized by the gene *sonic hedgehog* and that similar duplications could be obtained when cells that were capable of secreting *sonic hedgehog* protein were grafted into a chick limb bud. In the normal limb bud the *sonic hedgehog* also controls the level of FGF4 in the posterior region of the apical ectodermal ridge. The role of *sonic hedgehog* in controlling the antero-posterior axis is not, however, a straightforward one, as BMP2 and BMP4, as well as retinoic acid, play a part. Moreover, SHH is expressed not only in the mesenchyme, but also in the overlying ectoderm (Harfe, 2011).

Each limb also develops a dorso-ventral axis so that, for example, the dorsal (upper) side of the foot differs from the ventral (plantar) side. This appears to be due mainly to the activity of events in the ectoderm. In particular, *Wnt7a* is active in the dorsal ectoderm and is responsible for a cascade of events.

The autopod (hand or foot) is the final region of each limb to be laid down. TGF32 and activin signalling are involved in the skeletogenesis of the digits (Merino *et al.,* 1999a). (For a recent review of digital development see Sanz-Ezquerro and Tickle, 2003). The 'hand' of the wing is highly modified in birds, and it is generally considered that the digits of the avian wing develop in positions 2, 3 and 4, though there is some evidence that these three digits should be reclassified as 1, 2 and 3 (discussed by Young *et al.,* 2011). The patterns of gene action in the development of individual digits have been mapped by Fisher *et al.* (2011).

By contrast, the foot retains the characteristic vertebrate plan of five digits. It initially forms as a flattened paddle-like structure (Appendix II) in which the cartilaginous elements are clearly distinguishable yet still connected by mesenchyme (*Plate 174*). In the duck and other web-footed birds this interdigital region remains as a web, but in the chick it is removed by apoptosis except at the base, so that the toes become separated.

Other specialized areas of the limb bud, in addition to the ZPA, are the **anterior necrotic zone** (ANZ) and the **posterior necrotic zone** (PNZ), which not only play a role in the shaping of the limbs, but are centres from which morphogens appear to diffuse and interact with other tissues, playing an essential role in the patterning of the limbs. There is evidence that retinoic acid is an active principle secreted by these zones (reviewed by Paulsen, 1994). The relationship between SHH and FGF has attracted much attention (see Harfe, 2011). The development of the limb in the chick and other vertebrates has attracted many investigations, perhaps largely because of its ease of access compared with that of internal organs. But the complexity of limb development with its interacting axes (dorso-ventral, antertior-posterior and proximo-distal) and its eventual differentiation into sharply distinct regions has led to many theoretical attempts to provide an over-arching concept. Earlier ones were based on the idea of simple gradients of diffusing substances, but with the ever-increasing molecular evidence now available, new ideas embracing modern knowledge of the genes involved and their interacting products (e.g. Bénazet and Zeller, 2009; Cooper *et al.*, 2011; Fisher *et al.*, 2011), more sophisticated concepts are being put forward.

These changes in the external appearance of the limbs are illustrated and described in the Normal Table of Hamburger and Hamilton 1951 (see Appendix II).

GROWTH OF THE EMBRYO

Growth, a process usually defined as the increase in mass of an organism, is brought about mainly by the continual rise in the population of cells present in the embryo.

Changes in cell shape and size, as well as the accumulation of extracellular materials, also affect the shape and size of developing organs. Some regions undergo a more rapid rate of cell division than their neighbours, leading to a build-up of tissue. Growth factors, such as FGF, play an important controlling role. Schellpffer *et al.* (2005), using high-frequency ultrasound imaging, published charts and tables of the normal growth *in ovo* of purebred White Leghorn chick embryos. Seven morphological structures were measured (e.g. crown-rump length, femur length). Kim *et al.* (2011), using microcomputered tomography to study 3D aspects of chick growth between 4 and 12 days of incubation, found that whereas some organs showed constant growth (e.g. eye and heart), others grew in spurts (e.g. forebrain and limbs).

APOPTOSIS

As well as regions of high proliferation, there are also areas that have a high rate of cell death. Probably in all embryonic tissues there is a continuous loss of cells through death, but in certain parts of the developing body the death rate outstrips the proliferative rate. This means that just as some regions become enlarged by rapid cell division, others become eroded away. The dying cells undergo a process of breakdown and gradually become phagocytosed by other cells. Apoptosis is also known as 'programmed cell death' because in many cases the patches of cells die in a particular location of the embryo at a specific time in development and play an important role in morphogenesis. Two classical examples in the chick occur in the shaping of the wing bud and of the toes. Apoptotic cells are, however, found in normal embryos even as early as gastrulation (Bellairs, 1961), and after that in many well-defined sites in the differentiating tissues, e.g. in the mesonephros (Chapter 7), in the heart (Chapter 6), in the sclerotome (Sanders *et al.* 1997), in the nervous system (Oppenheim *et al.,* 1999), in the neural crest (Jeffs and Osmond, 1992) and in the tail bud (Sanders *et al.,* 1986), branchial arches and lateral body wall (Hirata and Hall, 2000). In most of these examples, cell death is focused on a highly localized region and occurs within a restricted period of time, e.g. cell death within the endocardial cushions of the developing heart occurs only between about days 5.5–7.5 (Keyes and Sanders, 1999).

Regions of cell death play a specific role in the shaping and patterning of organs, though they can sometimes be 'rescued' from death if treated with an appropriate growth or differentiation factor. For example, regions of programmed cell death in the early nervous system of the chick embryo can be counteracted by the application of *sonic hedgehog* protein (Charrier *et al.,* 2001). Hirata and Hall (2000), who have reviewed the temporo-spatial patterns of cell death from stages 1 to 25, have concluded that cell death is a feature of development at all these stages, but that there are changing patterns, depending on the specific stages of growth, differentiation and morphogenesis.

POLARITY: SYMMETRY AND ASYMMETRY

We have already seen that the embryonic axes (dorso-ventral and antero-posterior) begin to be established even before the egg is laid, but the finalization of this

polarity does not take place until later. Although the antero-posterior polarity is initiated by the effects of gravity during cleavage, it is still possible to overcome it until the primitive streak has begun to develop. At that time a series of *Hox* genes comes into play which interact with one another so that each level of the body becomes categorized in a unique way. It acquires its own 'positional information' (Wolpert, 2002). There are a number of markers of antero-posterior polarity, e.g. *Gata2* protein is expressed in the area opaca epiblast prior to primitive streak formation as a gradient along the antero-posterior axis, being highest anteriorly (Sheng and Stern, 1999) and disappears at stage 3+ (though it reappears later in other regions, such as the lateral part of the somites).

With the establishment of antero-posterior polarity, the embryo acquires a left and a right side. The two sides are asymmetrical, being mirror images of one another (e.g. a left wing and a right wing), which essentially remain so throughout development. With the differentiation of the internal organs, however, the two sides diverge and certain regions become more highly asymmetrical, as shown by the heart, liver and gut.

The first signs of left–right asymmetry appear to be associated with Hensen's node and involve a molecular cascade. Sonic hedgehog produced by Hensen's node plays a critical role in establishing the left cascade, which includes *Nodal–Lefty–Pitx* (Levin *et al.,* 1995). By contrast, *sonic hedgehog* ceases to be produced on the right side where actin, FGF and BMP play major roles. The establishment of asymmetry between left and right is dependent on a sharp boundary along the midline, and it has been suggested that the individual cells are themselves orientated in respect to that boundary. This is supported by evidence that Vangl2, a core planar cell polarity protein, is 'consistently polarised', giving cells in the blastoderm a vector pointing toward the primitive streak (Zhang and Levin, 2009). It is possible to disrupt the left–right arrangement of the body by interfering with the cascade. For example, if cells secreting *sonic hedgehog* are transplanted to the right of the node, nodal is induced symmetrically and the looping of the heart may occur randomly to the left or right (Levin *et al.,* 1997). BMP signaling also plays a role in left–right asymmetry in Hensen's node by inhibiting DAN, which is normally expressed on the left side (Katsu *et al.*, 2011). Nodal plays a significant role in the essential communication that occurs between the left and right sides; this process is a complex one (discussed by Katsu *et al.*, 2013). Many genes have been implicated in the establishment and maintenance of asymmetry, many of which propagate signals in small populations of cells that subsequently result in left- or right-sidedness in the future organs (Levin, 2005).