8. LIMB DEVELOPMENT

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RECOMMENDED READING: Larsen's Human Embryology, 3rd Edition, pages 315-328, 335-342

LEARNING OBJECTIVES:

You should be able to:

1. Compare the contribution made by lateral plate (somatopleure) mesoderm and somitic (paraxial) mesoderm to the formation of the limb.
2. Follow the consequence of limb rotation on the innervation pattern of adult limbs.
3. Discuss the signaling mechanisms between the zone of polarizing activity and the apical ectodermal ridge in the anterior-posterior patterning of hand.
4. Describe the novel biochemistry whereby sonic hedgehog establishes a concentration gradient in the limb.

GLOSSARY:

Apical ectodermal ridge (AER) - most distal rim of epithelium of the limb bud. It is a major signalling center in regulating patterning of the limb and apoptosis in underlying mesoderm (see lecture on Apoptosis).
Fibroblast growth factor (FGF) - FGF-4, a secreted protein from the AER overlying the ZPA, regulates the expression of SHH.
Induction: the change in a cell or tissue's fate due to a signal from another tissue or cell.
Morphogen: A secreted molecule that regulates induction. A concentration gradient of the molecule is frequently established.
Progress Zone (PZ) - mesoderm below AER where cellular proliferation takes place.
Sonic hedgehog (SHH)- a member of the "hedgehog family" of secreted signalling proteins. SHH is made by the ZPA (below) and regulates anterior/poterior patterning.
Zone of Polarizing Activity (ZPA) - mesenchyme just below the AER on the posterior boundary of the limb bud. Major signalling center for the regulation of anterior/posterior patterning.

TEXT:

Anatomical aspects of limb formation

A. Initial appearance

1. Limb buds first appear in the fourth week of gestation as small elevations on the ventrolateral body wall. The upper limb buds are visible on day 24 at **C5-C8** and the lower buds are recognizable around day 28 at **L3-L5**. The differentiation of the upper bud into arm/hand and lower into leg/foot is wholly dependent on their positional location (see latter). The later development of the lower limb is consistent with the pattern of cranial to caudal differentiation that we saw in somite differentiation (Fig. 8-1A & B)

2. During early stages of development, the limb bud consists of homogeneous appearing mesenchymal cells derived from the somatic body wall (i.e., **lateral plate mesoderm**) and a thin ectodermal covering. At the distal tip the ectoderm condenses into the **APICAL ECTODERMAL RIDGE** (AER). The AER marks the doral-ventral boundary of the limb. Three steps are involved in AER formation: induction of precursors, migration of precursors and compaction of ridge. FGF10 from the mesenchyme induces the AER. The AER then produces FGF8 (as key morphogen, see below). The mesenchyme immediately below the ridge is called the **PROGRESS ZONE** (PZ, labeled 'M' in Fig. 8-1D) and is the site of cell division/elongation of the limb. We shall see that the AER has an inductive influence on the PZ and is essential for the elongation process (Fig. 8-1C & D).

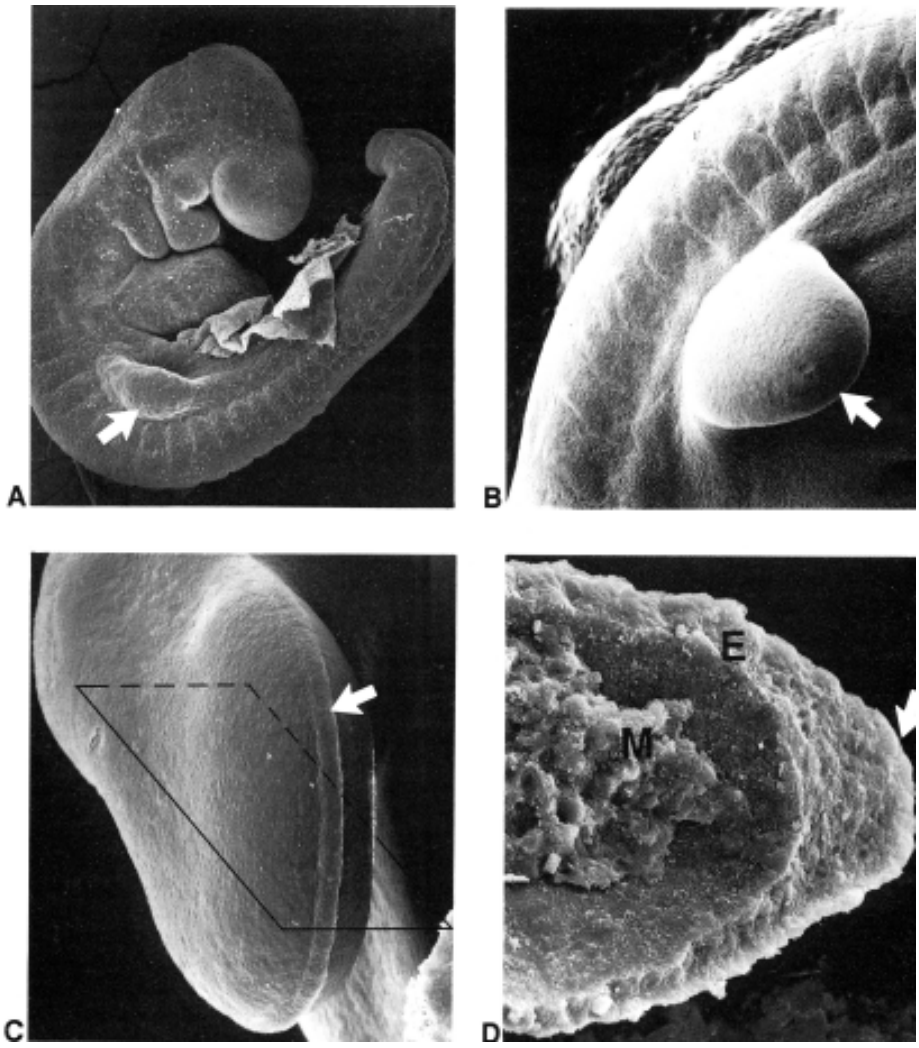

Fig. 8-1. Scanning electron micrographs showing limb buds. The limb buds are formed when somitic mesoderm induces the proliferation of overlying lateral plate mesoderm. (A) Embryo with newly formed upper limb bud (arrow). (B) By day 29, the upper limb bud (arrow) is flattened and paddle-shaped. (C) By day 32, the apical ectodermal ridge (arrow) is visible as a thickened crest of ectoderm at the distal edge of the growing upper limb bud. Rectangle indicates plane of sectioning of Fig.D. (D) Limb bud sectioned to show the inner mesenchymal core (M) and the outer ectodermal cap (E). Arrow: apical ectodermal ridge. (Figs. A, C, D from Kelley RO. 1985. Early development of the vertebrate limb: an introduction to morphogenetic tissue interactions using scanning electron microscopy. Scanning Microsc 11:827, with permission.)

B. Origin of limb components.

1. The limb buds elongate by the proliferation of the mesenchyme. This mesenchyme condenses to form cartilage/bone and connective tissue of the dermis (See SBPM/D).

2.The muscles of the limb are derived from the somites opposite the appropriate somatopleure.

3. The motor innervation originates from the spinal cord; the myelinating glia (Schwann cells), from the neural crest.

C. Limb elongation (Figs. 8-1, 8-2, 8-3).

1. The presence of the AER is essential for the proliferation of the mesenchyme and the elongation of the limb (see below).

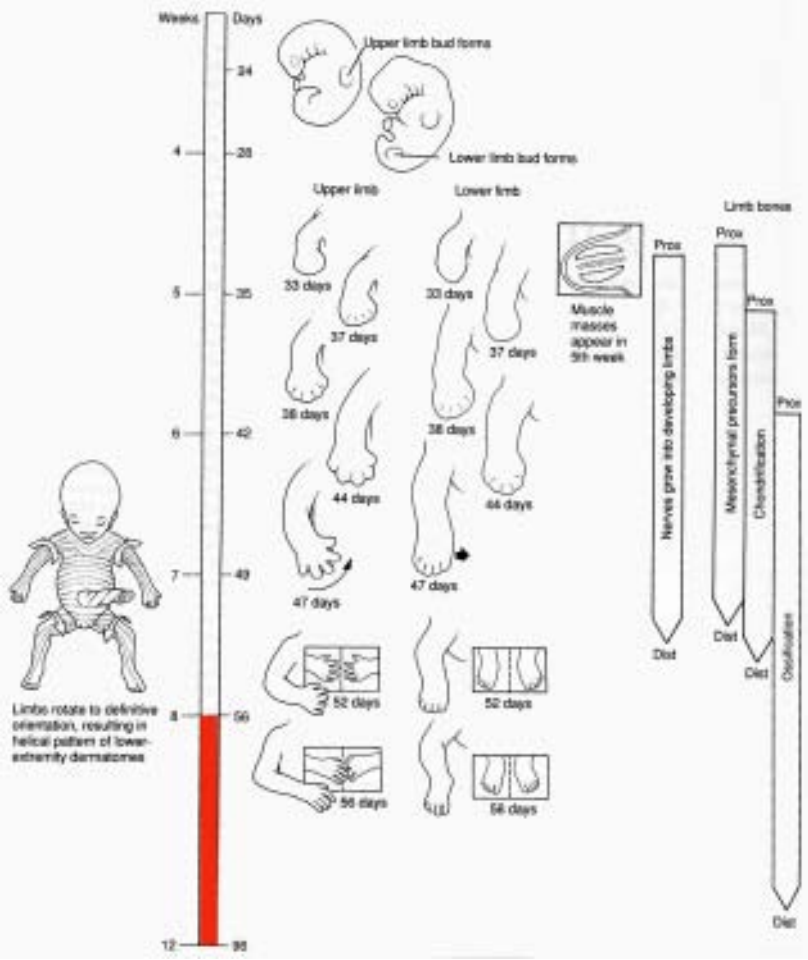

Fig. 8-2. Timeline. Development of the limbs.

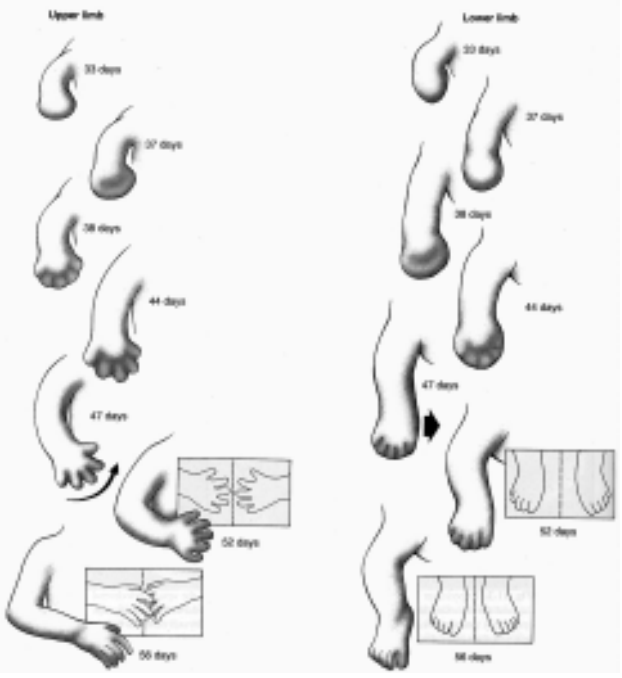

Fig. 8-3. The development of the upper and lower limb buds occurs between the fifth and eighth weeks. Nearly every stage in the development of the lower limb bud takes place several days later than in the upper limb bud.

2. At early bud stages, the limbs have the appearance of flippers. The upper buds appear disproportionately low on the trunk due to the initial dominance of the head and neck region of the embryo.

3. Initial elongation begins around day 33 (Fig. 8-2, 8-3.) and results in a paddle shaped limb. Elongation continues into the 7th week. The mesenchymal precursors of the limb skeleton form in a proximal to distal fashion.

4. Condensation of the mesenchyme is followed by the differentiation of these cells into the cartilagineous lineage. The cartilage cells form a model of the limb bones which is replaced by bone (discussed in detail in SBPM/D; review Histology laboratory on this subject).

5. Transformation of the cartilage model into bone is nearly complete by the 12th week although some bones such as the carpels in the wrist will not ossify until the first year after birth.

6. Programmed cell death (see Chapter 15 for mechanism) plays an important role in shaping the limb. This is particularly noticeable in the hand and foot where mesenchyme between the digital rays must be sculpted to separate the individual fingers and toes.

D. Entry of muscle progenitors

As the appendicular skeleton forms, myoblasts migrate in from the somites (5th week; Fig. 8-4) and cells aggregate into dorsal and ventral muscle masses in each limb. In general the dorsal mass will develop into the extensors and supinators of the upper limb; extensors and abductors of the lower. The ventral components give rise to flexors and pronators in the upper/ flexors and adductors in the lower (Table 8-1). This rule is not absolute.

Table 8-1.
Muscles Derived from the Ventral and Dorsal Muscle Masses of the Limb Buds

VENTRAL MUSCLE MASS	DORSAL MUSCLE MASS	
Upper Limb	*Upper Limb*	*Lower Limb*
Anterior compartment of the arm and forearm	Posterior compartment muscles of the arm and forearm	Anterior compartment muscles of the thigh and leg
All muscles on the palmar surface of hand	Deltoid	Tensor fasciae latae
Lower Limb	Lateral compartment muscles of the forearm and hand	Short head of the biceps femoris
Medial compartment muscles of the thigh	Latissimus dorsi	Lateral compartment muscles of the leg
Posterior compartment muscles of the thigh except for the short head of the biceps femoris	Rhomboids	Muscles of the dorsum of the foot
	Levator scapulae	Gluteus maximus, medius, and minimus
	Serratus anterior	Piriformis
Posterior compartment muscles of the leg	Teres major and minor	Iliacus
	Subscapularis	Psoas
All muscles on the plantar surface of the foot	Supraspinatus (?)	
	Infraspinatus (?)	
Obturator internus		
Gemellus superior and inferior		
Quadratus femoris		

Data from Crafts RC. 1985. A Textbook of Human Anatomy. 3rd Ed. Churchill Livingstone, New York.

E. Rotation of the limb.

1. Axons grow into the dorsal and ventral muscle masses before they split into primordia of individual muscles (Figs. 8-2, 3, 5a, 5b). The nerves for the upper limb are derived from C5-C7 (grow to the craniodorsal aspect of the limb) and from C8-T2 (grow into the ventrocaudal parts of the upper limb). Axons from these segmental nerves enter the branchial (or for the lower limb, lumbosacral) plexus and diverge at that point as indicated. Eventually decisions are made as to the precise muscle that each nerve will innervate (Fig. 8-5a,b). Note the arrangement of the dermatomes (organization of innervation in Fig. 8-6).

2. By the end of the 6th week the limbs have extended and lie in a coronal plane (Fig. 8-6A). The buds will undergo two rotations to bring them into their final position.

3. Both limbs rotate from their original coronal orientation (Fig. 8- 6A) into a roughly parasagital orientation (Fig. 8-6B). See Fig. 8- 2 for timing.

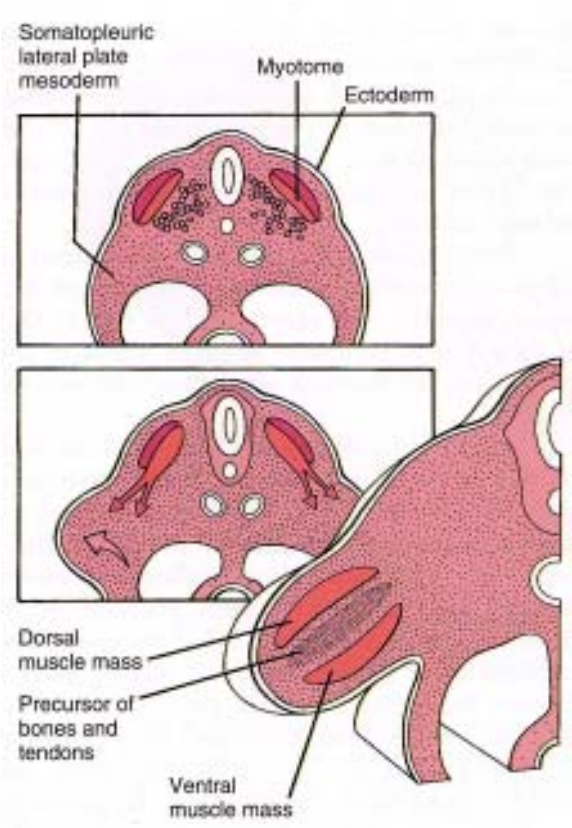

Fig. 8-4. Somitic mesoderm initially forms two major muscle masses in each limb bud. The ventral mass gives rise mainly to flexors, pronators, and adductors, whereas the dorsal muscle mass gives rise mainly to extensors, supinators, and abductors.

4. The limbs then rotate along their long axes.

a. The developing upper limb rotates LATERALLY through 90 degrees on the longitudinal axis, so that the elbows point caudally.

b. The lower limb rotates MEDIALLY so that the knees point cranially.

5. As seen in Fig. 8-6 B and C this rotation causes the originally straight segmental pattern of innervation to twist into a spiral. The rotation of the upper limb is less extreme than that of the lower limb. The former is accomplished partly through the caudal migration of the shoulder girdle. Some of the dermatomes in the upper limb bud exhibit overgrowth and come to dominate the limb surface.

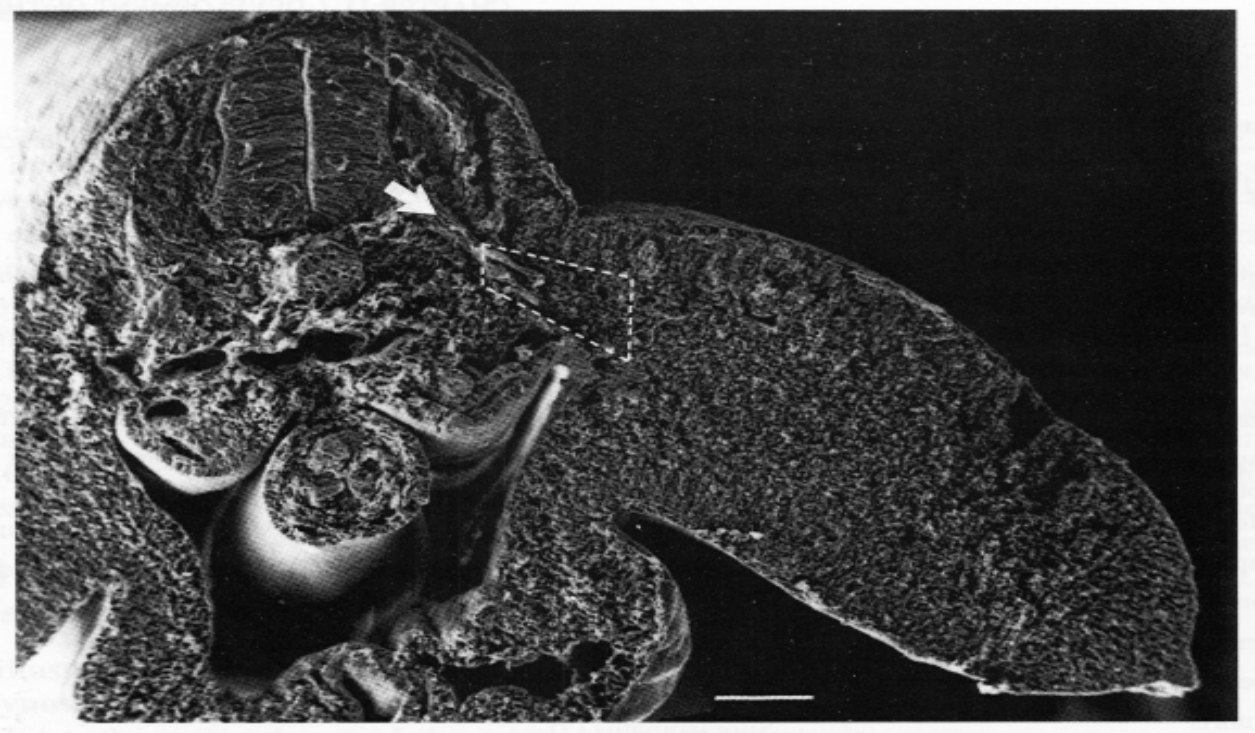

Fig. 8-5a. Scanning electron micrograph of a sectioned embryo showing axons entering the limb bud (dotted area). (From Tosney KW, Landmesser LT. 1985. Development of the major pathways for neurite outgrowth in the chick hindlimb. Dev Biol 109:193, with permission.)

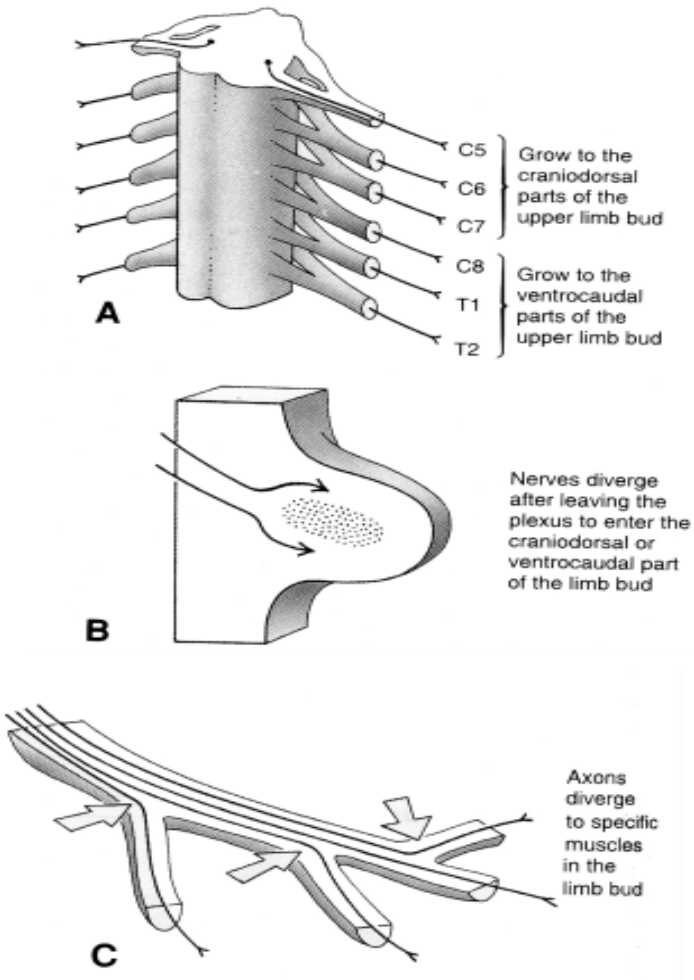

Fig. 8-5b. Growth of spinal nerve axons into the limb buds. (A, B) Axons grow into the limb buds along permissive pathways. As the axons of the various spinal nerves mingle at the base of the limb buds to form the brachial and lumbosacral plexuses, each axon must "decide" whether to grow into the dorsal or ventral muscle mass. Factors that may play a role in directing axon growth include areas of dense mesenchyme or glycosaminoglycan-containing mesenchyme, which are avoided by outgrowing axons. (C) Once the axons grow into the bud, decision points (arrows) under the control of "local factors" may regulate the invasion of specific muscle anlagen by specific axons. (Modified from Tosney K, Landmesser LT. 1984. Pattern and specificity of axonal outgrowth following varying degrees of chick limb bud ablation. J Neurosci 4:2518, with permission.)

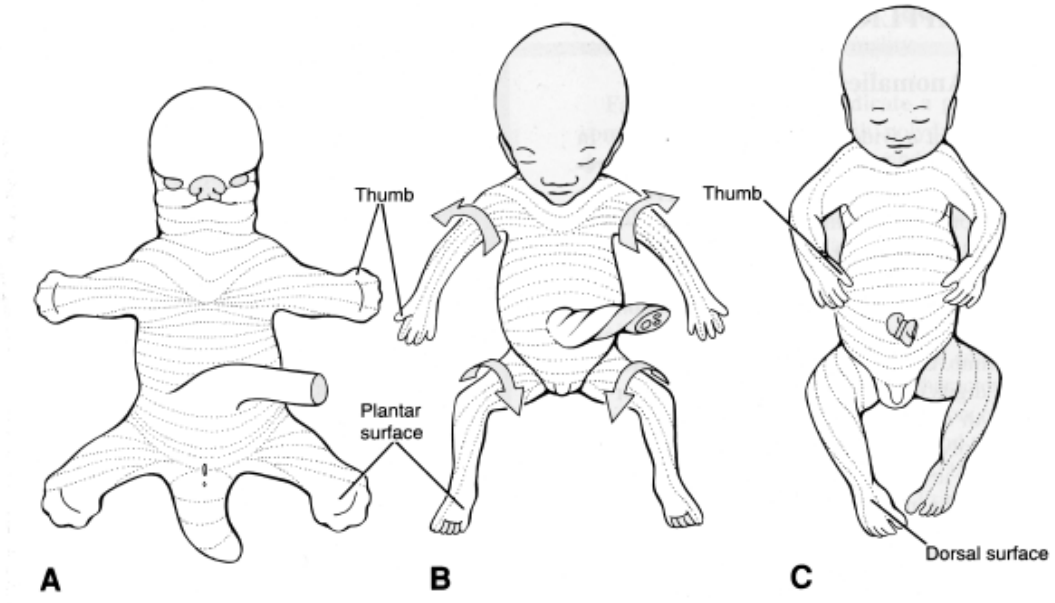

Fig. 8-6. Rotation of the limbs. The dramatic medial rotation of the lower limbs during the sixth to eighth weeks causes the mature dermatomes to spiral down the limb. The configuration of the upper limb dermatomes is partially modified by more limited lateral rotation of the upper limb during the same period.

Pattern formation

A. Introduction.

Limbs illustrate, most graphically, the need for molecular cues that provide positional information. With these cues undifferentiated mesenchyme produces sequentially different structures (e.g., humerus, ulna/radius, wrist and digits) (Fig. 8-7). In the past lectures we have looked at the induction of specific cell types. In the limb it is not a matter of just turning mesenchyme into muscle, bone and connective tissue but into the right bones, in the right order. This is called pattern formation. Cells are "assigned" a characteristic appropriate to their position (a phenomenon that we first encountered in compaction in the regionalization into inner and outer cells) so that the simple tissues of bone, cartilage, muscle and connective tissue are formed into a complex spatial pattern.

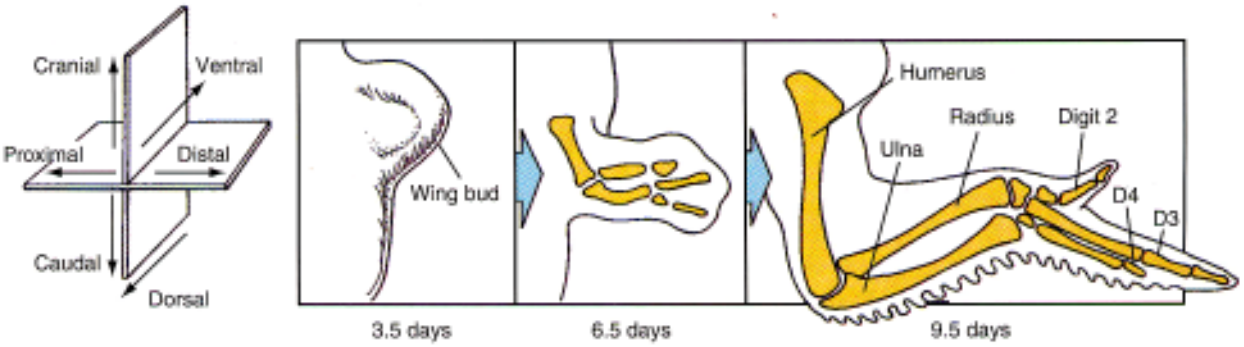

Fig. 8-7. The limb buds of birds and other vertebrates grow with respect to three axes of symmetry: craniocaudal, dorsoventral, and proximodistal. (Modified from Alberts B. Bray D, Lewis Jet al. 1983. Molecular Biology of the Cell. Garland, New York, with permission.)

B. Limb fields (Fig. 8-8)

1. Forelimb bud and hindlimb bud are not equivalent. This is true even before the limb bud emerges.

2. Spatial information has been imparted to the mesenchyme. Therefore the cells that give rise to each bud CARRY POSITIONAL INFORMATION.

3. The concept of specific limb fields on the body wall, prior to the formation of the limbs themselves, originated from work of Ross Harrison in1918 (he was also the first person to use tissue culture). He showed that as early as tailbud stage (newt tadpole), a region of lateral plate mesoderm acquired the potential to become forelimb and would express this characteristic when transplanted to another region of the embryo.

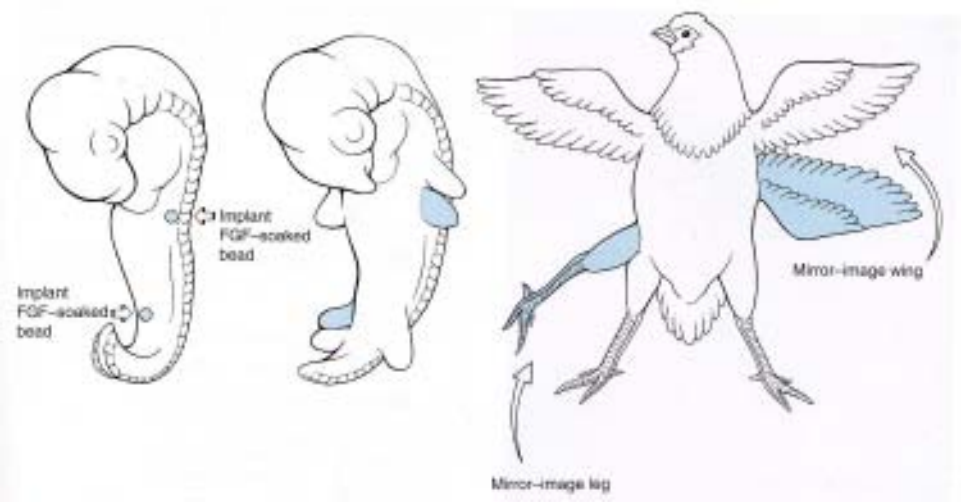

Fig.8-8. FGF-soaked bead produces supernumerary limb. FGF = fibroblast growth factor.

4. This positional information resides in the mesoderm itself and is independent of the AER (defined above). If mesoderm from a chick embryo **leg** bud is transplanted under the AER (needed for elongation) of the wing bud, that mesenchyme will differentiate into a leg, the prior positional information is retained. This experiment highlights the competence of the leg bud mesenchyme to respond to signals from wing AER, suggesting that once the memory of leg vs wing is established, either AER will result in appropriate proximal to distal production of the skeletal elements (see more below).

5. In a mouse mutant known as **limbless,** there is no apical ectodermal ridge and therefore no limb. BUT limbless mesoderm, placed under normal AER, develops into a limb.

6. Not only does a limb field know it is upper or lower limb, it also already knows directionality. Hence if you rotate the limb field 90 degrees the outgrowth will also rotate so that the limb is oriented 90^{o} (Fig. 8-8).

Concept: Mesoderm has the clue. The transplantation experiments demonstrate that the signals used by both limbs to specify all three axes must be the same, but they interpret the information (e.g, "distal") according to their prior history. Therefore one must invoke the concept of cell memory. Using such memory the body can use a small number of signaling molecules in generating patterns.

C. Elongation - laying down of proximo-distal axis (Fig. 8-9)

1. Sequential establishment of skeletal elements. As mentioned above, proliferation in the progress zone of the mesenchyme depends on the presence of the AER. If the AER is removed, the limb is truncated due to cell death in the progress zone below (PZ). Depending on the time of AER removal the degree of truncation varies but distal structures are always the ones lost.

a. Remove AER early you might get only a humerus.

b. Remove AER later, the limb is less severely truncated, humerus and radius/ulna but no digits, etc. See text book for effect of thalidomide, a teratogen, on limb elongation.

2. Is AER instructive or just permissive? To test this requires a mix-match experiment in which either the age of the mesenchyme is varied or the age of the AER is varied (Fig. 8-9). The bottom line is that the age of the AER is irrelevant. The mesenchyme "remembers" how long it has been exposed to AER signals. This suggests that spatio-temporal development of limb structures is intrinsic to mesoderm.

3. Does the positional information along prox/distal axis depend on length of time mesenchyme progenitors spend in the progress zone (PZ)? This can be answered with another variety of transplantation with different aged PZ and AER grafts as illustrated in Fig. 8-9. There are three concepts to derive from this experiment: (1) The PZ mesenchyme does indeed know how old it is; (2) the AER is essential for limb elongation and (3) the age of the AER is irrelevant vis-a-vis the differentiation of the mesenchyme into specific skeletal elements (Fig. 8-9).

4. More recently this "progress zone model" has been challenged (see Niswander, Nature Review Genetics 4:133, 2003 for review) but the newer model, for which there is still scant data, appears to be a more philosophical (to me) change than substantive one.

Digit formation

A. Introduction.

In discussing limb elongation (proximal-distal pattern) we focused on signals between the AER and the PZ. To understand anterior-posterior pattern in digit formation we must introduce a new signaling center - the zone of polarizing activity (ZPA). This is a specialized zone of posterior mesenchyme, just below the AER. Most of intensive investigation in this area was first carried out in chick or amphibia because of the easy access to the embryo. However, new surgical paradigms and in vitro culture systems have demonstrated that the principles are applicable to mammalian embryos (Fig. 8-10).

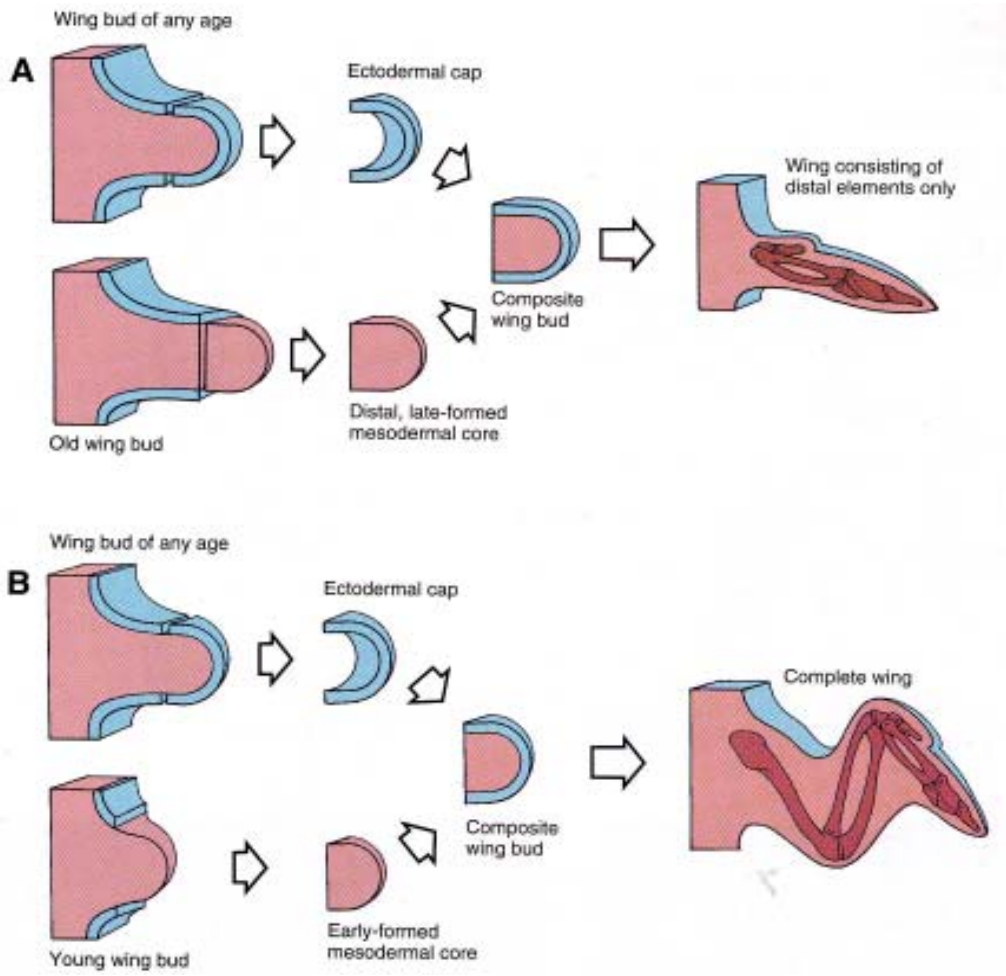

Fig. 8-9. Experiment on chick embryos demonstrating the roles of the apical ectodermal cap and the mesodermal core in limb bud development. (A) If an ectodermal cap of any age (even very young) is recombined with the distal end of an old mesenchymal core, the hybrid bud will form only distal elements of the limb. (B) If an ectodermal cap from a bud of any age (even very old) is recombined with a complete mesodermal core from a young limb bud, the hybrid bud will form an entire limb. (Modified from Rubin L, Saunders TW. 1972. Ectodermal-mesodermal interactions in the growth of limb buds in the chick embryo: constancy and temporal limits of the ectodermal induction. Dev Biol 28:94, with permission.)

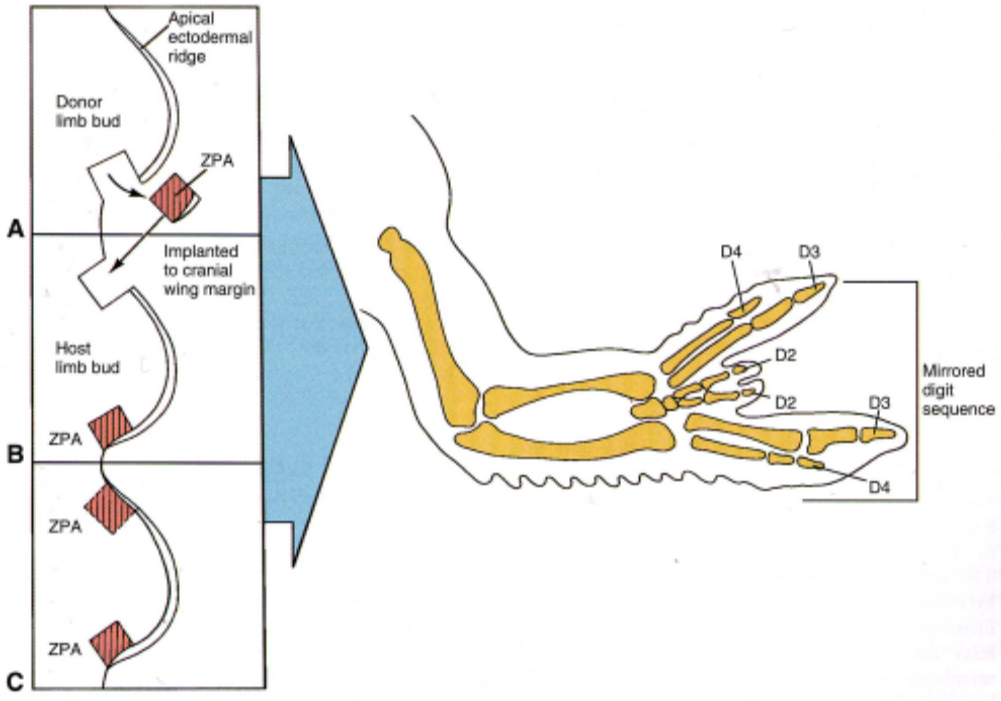

Fig. 8-10. Transplantation of the zone of polarizing activity (ZPA) of one limb bud to the cranial edge of another will induce mirror polydactyly. (Modified from Alberts B, Bray D, Lewis J et al. 1983. Molecular Biology of the Cell. Garland, New York, with permission.)

B. Proof of ZPA as signaling center (see Fig. 8-10)

1. When we studied gastrulation, transplantation of an extra dorsal lip of the blastopore or Hensen`s node resulted in a second, mirror image embryo by inducing host tissue. The same thing happens if a 2nd ZPA (from a same aged donor) is transplanted to the anterior region of a bud. The limb bud expands in the anterior -posterior dimension and a complete mirror image duplication occurs.

2. The newly induced structures are of host origin, hence the tissue host is respecified.

3. Because the respecification is a mirror image, one suspects that signal from ZPA might establish a concentration gradient of diffusible substance such that tissue closest will become little finger, fartherest away the thumb.

4. The idea that the signal is diffusible and that the mesenchyme responds to differences in amounts is also suggested by transplantation experiments. Here a donor ZPA is placed in different anterior/posterior (A/P) sites, resulting in different patterns of digit formation. The graded nature of the signal is best demonstrated by grafting different numbers of cells - 30 cells give an extra #2 digit, 80 cells both an extra #2 and # 3 while 130 cells produces a complete mirror duplication. We shall return in a moment to the chemical nature of this signal.

5. The specification by the ZPA of the A/P axes occurs in a very narrow frame of time, very early in development (approximately day 3 in chick over a few hour time span).

6. The ZPA is universal among quadruped vertebrates and equal in fore and hindlimbs. The signal is universal but the mesenchyme responds according to its genome and prior cellular history.

C. What induces the ZPA?

1. If culture the ZPA alone, it loses its polarizing activity.

2. If culture with ZPA with the AER, polarizing activity is maintained.

3. In ablation experiments if one removes the AER, no ZPA.

4. The AER can be replaced with a bead soaked in FGF-8.

5. FGF-8 is made in posterior aspect of AER and is a secreted molecule. This appears to be the signal that at least maintains ZPA activity, see below. FGF`s are limited in their diffusion because they bind tightly to ECM components.

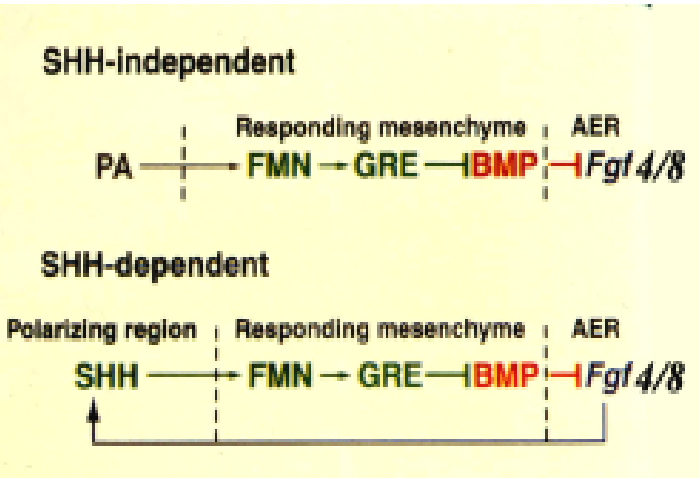

Figure 8-11 . A diagram of the interactions between limb-bud mesenchyme and AER. These interactions lead to the establishment of a positive feedback loop for SHH secretion.
In the top panel an unknown (to date) polarizing activity (PA) acts on the presumptive ZPA mesenchyme to induce the sequential secretion of two proteins (FMN and GRE). The main point is that the release of these proteins INHIBITS the release of bone morphogenetic protein (BMP) from the mesenchyme. This INHIBITION of BMP permits the activation of the FGF gene and secretion of FGF from the posterior AER.
The bottom panel illustrates two major additional points. When the AER secretes FGF, this stimulates the posterior mesenchyme to make SHH. SHH in turn acts like the unknown PA in the top panel to inhibit the production of BMP in the mesenchyme, resulting in more FGF release and more SHH. This is then a positive feedback loop. The presumptive ZPA is now a fully functional ZPA.
The diagram suggests that the major FGF is FGF4; current work suggests FGF8 is more critical.

D. Interaction of ZPA/SHH and AER/FGF4/8

In recent work (Fig. 8-11) a mechanism is presented for the establishment and maintenance of the AER and ZPA. In this outline an unknown polarizing activity emanates from the presumptive ZPA. This results in the secretion of proteins which can block the effect of BMP. When BMP signaling is inhibited the AER makes FGF-8. FGF-8 stimulates the production of SHH.

SHH from this ZPA is essential to maintain the mesenchymal signaling cascade which inhibits BMP production and so continues to stimulate the production of FGF-8. FGF-8 in turn maintains the production and secretion of SHH, hence making a positive feedback loop (Fig. 8-11).

E. What is the signal from the ZPA?

1. There is a large literature suggesting that retinoic acid (RA), a derivative of vitamin A, was the signal. It is made in the ZPA at the right time. Beads soaked in RA produced a mirror image duplication. So for a long time this was the candidate morphogen. Although RA may function in early limb development it is not the essential morphogen .

2. Instead our favorite hedgehog, sonic hedgehog, is the current molecule of choice.

a. It is produced in ZPA at the right time. It is also in the notochord and floor plate of the CNS, both of which have polarizing activity.

b. If fibroblasts are transfected with SHH they can act as ZPA's.

c. Is SHH secreted? Yes.

F. Diffusion of SHH.

THE DETAILS OF THE INFORMATION IN THIS SECTION ARE FOR YOUR INFORMATION ONLY. HOWEVER, IT DOES INDICATE MECHANISMS BY WHICH MORPHOGEN GRADIENTS CAN BE ESTABLISHED.

It is clear, that the distance along the A/P axis from the source of SHH, is a critical feature of axis establishment. This further suggests that the distance over which SHH diffuses must be carefully regulated. All Hedgehog (Hh) proteins are made as presursors. After removal of the signal sequence the Hh is cleaved into an N terminal domain (Hh-n) and a C terminal domain. The N terminal has all of the biological activity. However, the C terminal section before being cleaved autocatalyzes the addition of cholesterol to Hh-n. This lipophilic cholesterol tail plays a role in limiting the degree of diffusion (Fig. 8-12).

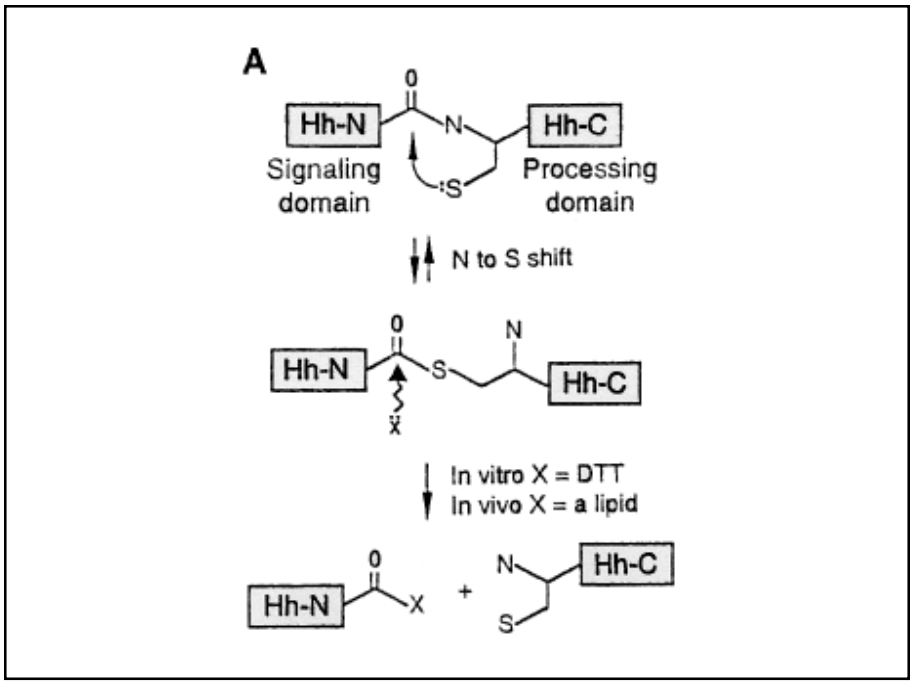

Fig. 8-12.