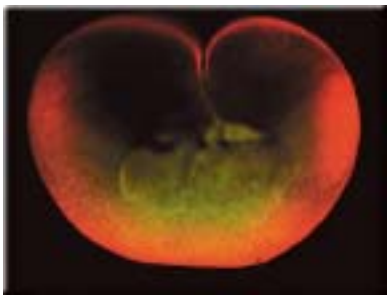
UNION COLLEGE

3800 S. 48th Street, Lincoln, NE 68506
Division of Sciences and Mathematics

BIOL 326
Developmental Biology
Chapter 16: Development of the Tetrapod Limb
Spring 2005



XVI. Development of the Tetrapod Limb

A. Introduction

1. The bones of the vertebrate limb consists of:
   a. proximal **stylopod**,
   b. middle **zeugopod**, and
   c. distal **autopod** (Figure 16.1).
2. Questions of limb development
   a. Regulation of growth rate
   b. Number of limbs
   c. Why fingers at one end of the limb and not the other?
   d. Why are the little finger and thumb on opposite edges of the limb?
3. Vertebrate limb development occurs in three dimensions:
   a. **Proximal-distal**, Stylopod to autopod; regulated by **FGF family** of proteins
   b. **Anterior-posterior**, little finger is on the posterior side while the thumb is on the anterior side; regulated by **Sonic hedgehog**
   c. **Dorsal-ventral**, palm is ventral and the knuckles are dorsal; regulated, in part, by **Wnt7a**.
4. **Morphogenesis** – how specific structures arise in particular places
5. Easy to study because the removal of a limb is not lethal.

B. Formation of the Limb Bud

1. Specification of the limb fields: Hox genes and retinoic acid

a. Identification of mesoderm that gives rise to limbs:
   (1) **Removal** of a certain group of cells and observe what does not develop in their absence.
   (2) **Transplant** a group of cells to a new regions and observing what forms in the new location.
   (3) **Marking** a group of cells and observing where their descendants take place in development.
b. Figure 16.2
   (1) Prospective forelimb area shown
   (2) If the peribrachial flank tissue, free limb, and shoulder girdle are removed, a limb will still form, only a little later in development
   (3) Also remove the ring shown around the cells and the limb will not form.
   (4) This larger region, representing all the cells capable of forming a limb, is called the **limb field**.
c. When the limb field first forms, it has the ability to regulate for lost or added parts.
d. The Freaky Frogs (Figure 16.3)

(1) In numerous ponds were found multilegged frogs and salamanders
(2) Linked to the infestation of the larval abdomen by parasitic trematode worms.
(3) Eggs of these worms split the limb buds in several places while the tadpoles were forming limb buds

e. In all land vertebrates there are only four limb buds per embryo; always on opposite sides
f. The position of the limb buds is constant with respect to the level of Hox gene expression along the anterior-posterior axis. Forelimb buds are found at the most anterior expression region of Hoxc-6.
g. The lateral plate mesoderm induces myoblasts to migrate out from the somites and enter the limb bud to become limb musculature.
h. Blocking the synthesis of retinoic acid prevents limb bud initiation. The gradient of retinoic acid along the anterior-posterior axis might activate certain Homeotic genes in certain cells specifying them to be included in the limb field. The retinoic acid comes form the Hensen's node.
i. Figure 16.4 A
(1) Tadpole tale amputated
(2) Stumps exposed to retinoic acid
(3) Regenerated several legs from the tail stump

2. Induction of the early limb bud: Wnt proteins and fibroblast growth factors
a. Mesenchyme cells proliferate from the somatic layer of the limb field lateral plate mesoderm and form the somites (Figure 16.5)
b. These cells accumulate under the ectodermal tissue to create a circular bulge called a limb bud.
c. Signals for limb bud formation come from the lateral plate mesoderm cells that will become the prospective limb mesenchyme by secreting FGF10 (Figure 16.6).
d. FGF10 is capable of initiating limb forming interactions between the ectoderm and the mesoderm.
e. Immediately prior to limb formation, FGF10 becomes restricted to the regions of the lateral plate mesoderm where the limb forms. The restriction of FGF10 to this area appears to be due to the actions of Wnt proteins (Figure 16.7).

3. Specification of forelimb or hindlimb: Tbx4 and Tbx5
a. Limb buds must be specified as either forelimb or hindlimb.
b. Tbx5 transcription factor is only expressed in the forelimbs (of mice)
c. Txb4 is expressed in the hindlimbs.
d. Humans heterozygous for the TBX5 gene have Holt-Oram syndrome characterized by abnormalities of the heart and upper limbs. The legs are not affected.
e. FGF beads were used to induce the formation of an ectopic limb between the chick hind and forelimbs. The type of limb produced was determined by the Txb proteins expressed.
f. Those that expressed Tbx4 became hindlimbs while those that expressed Tbx5 developed as forelimbs.
g. Those buds that were induced in the center of the flank expressed both Tbx5 and Tbx4 developed as chimeric structures with the anterior resembling a forelimb and the posterior resembling a hind limb (Figure 16.8).

C. Generating the Proximal-Distal Axis of the Limb
1. The apical ectodermal ridge

a. Mesenchyme cells enter the limb field and secrete FGF10, which induces the overlying ectoderm to form the apical ectodermal ridge (AER; Figure 16.9).
b. The apical ectodermal ridge runs the length of the limb bud.
c. Functions of the AER :
    (1) Maintains the mesenchyme beneath enabling it to grow linearly into a limb.
    (2) Maintains the expression of the factors that generate the anterior-posterior axis.
    (3) Interacts with the proteins that specify the anterior-posterior and the dorsal-ventral axis so that each cell is given instructions on how to differentiate.
d. The proximal-distal growth and differentiation is due to an interaction between the AER and the limb bud mesenchyme directly beneath it. This mesenchyme is often called the progress zone since its proliferation extends the length of the limb.
e. The mesenchyme cells induce and sustain the AER and determine the type of limb to be formed. The AER is responsible for the sustained outgrowth and development of the limb by keeping the mesenchyme directly beneath it in a state of mitotic proliferation and prevents them from forming cartilage.

2. FGFs in the induction and maintenance of the AER
a. FGFs are critical for the induction, maintenance, and function of the AER.
b. FGF10 can induces the AER in competent ectoderm between the dorsal and ventral sides of the embryo.
c. FGF10 induces the AER to synthesize FGF8.
d. FGF8 acts to maintain the mitotic state of the mesenchyme causing them to keep expressing FGF10.
e. Positive feedback loop: FGF10 in the mesenchyme induces FGF8 in the AER which in turn maintains FGF10 expression in the mesenchyme (Figure 16.7).

3. Specifying the limb mesoderm: Determining the proximal distal polarity of the limb
a. If the AER is removed from an early-stage wing bud, only a humerus forms. If the AER is removed slightly later, humerus, radius, and ulna form.
b. When the entire progress zone (PZ) from and early embryo is placed on the limb bud of a later-stage embryo, new proximal structures are produced beyond those already present (Figure 16.13 A).
c. When old PZ are added to a young limb bud, distal structures develop immediately so that digits emerge from the humerus without an intervening ulna and radius (Figure 16.13 B).
d. Two major models of how the mesenchyme specifies the proximal-distal axis.
    (1) Progress Zone Model (Figure 16.14 A)
        (a) Mesoderm is specified by the amount of time it spends dividing in the progress zone.
        (b) The longer a cells spends in the progress zone the more distal its specification becomes.
        (c) The PZ does not grow in size so as mitosis takes place the cells must exit the PZ.
        (d) The first cells to leave the PZ become the stylopod, and last cells to leave become the autopod (Figure 16.14A).
        (e) Removing the AER would mean that the PZ cells could no longer divide and be further specified so only proximal structures would form.

(f) PZ mesenchyme can be prevented from dividing by knocking out the genes for FGF8 and FGF4. Proximal elements are missing while the distal elements are present. Development was controlled by gene expression and not the number of mitotic divisions.

(2) Early allocation and progenitor expansion model (Figure 16.14 B)

(a) The cells of the entire early limb bud are already specified and subsequent cell division simply expands these cell populations.

(b) When the AER is taken off the limb bud, about 200 micrometers of cells undergo apoptosis.

(c) If the AER is removed off the early limb bud, this deletes the cells that would give rise to the zygopod and the autopod.

(d) If the AER is removed later in development, the 200 micrometers of apoptosis removes only the autopod.

4. Hox genes, meis genes, and the specification of the proximal-distal axis

a. The products of Hox genes specifys where limbs will form and whether a particular mesenchyme will become stylopod, zeugopod, or autopod.

b. Knock out all four loci for paralogous genes Hoxa-11 and Hoxd-11 and the ulna and radius will be lacking (Figure 16.15 A&B).

c. Knock out all four loci for Hoxa-13 and Hoxd-13 and the autopod is lost.

d. Humans with a homozygous mutation for HOXD-13 show abnormalities of the hands and feet (Figure 16.15 C); HOXA-13 – deformities in their autopods.

e. As the limb grows outward, the expression of Hox gene expression changes (Figure 16.16).

(1) When the stylopod is forming, Hoxd-9 and Hoxd-10 are expressed distally and interact with products of the meis genes to specify proximal as proximal.

(2) When the zygopod bones are forming, Hoxd-9 to Hoxd-13 are expressed (nested) in the posterior region while only Hoxd-9 is expressed anteriorly.

(3) When the autopod is forming Hoxa-13 is expressed rather than Hoxd-9 in the anterior region and in a band marking the boundary of the autopod.

D. Specification of the Anterior-Posterior Limb Axis

1. The zone of polarizing activity

a. Anterior-posterior axis specification begins before the limb bud is recognizable.

b. At the posterior junction where the limb bud meets the body there is a small block of mesodermal tissue called the zone of polarizing activity that seems to be responsible for specifying this axis (Figure 16.18).

c. Figure 16.17

(1) ZPA is removed and transplanted on the anterior region of the limb bud.

(2) The number of digits is doubled

2. Sonic hedgehog defines the ZPA

a. Sonic hedgehog is expressed in the ZPA (Figure 16.18)

b. Sonic hedgehog expression induced digit formation just as in the transplantation experiment with the ZPA (Figure 16.19).

c. Beads soaked in sonic hedgehog also caused the same duplication.

3. Specification of the ZPA
    a. Sonic hedgehog appears to be activated by FGF proteins coming from the newly formed apical ectodermal ridge (Figure 16.20).
    b. FGF8 is secreted from the AER and is capable of activating shh.
    c. Some of the mesenchyme cells may be competent to respond to the FGF signal while others are not.
    d. Two transcription factors are expressed in the posterior region and not in the anterior region of the limb bud whicn may provide competence to the FGF signal.
        (1) Hoxb-8
            (a) Normally expressed in the posterior half of the mouse forelimb bud.
            (b) When expressed throughout, instead of just the posterior half of the limb bud, both the anterior and posterior mesoderm expresses shh creating two ZPAs and mirror-image duplications.
            (c)
        (2) dHAND
            (a) Expressed in the posterior mesoderm or the limb bud just prior to shh expression.
            (b) Mice lacking dHAND do not express shh
            (c) Ectopic expression of dHAND in the anterior region of the limb bud produces a second ZPA zone by inducing expression of shh in the anterior region.
4. Specifying digit identity through cell interactions initiated by Sonic hedgehog
    a. Sonic hedgehog does not diffuse outside of the ZPA
    b. Sonic hedgehog works to initiate and sustain a gradient of BMPs which act to specify the digits.
    c. The identity of each digit is determined by the interdigital mesoderm, the webbing between the digits. This webbing will undergo apoptosis.
    d. Remove the webbing between the forming digits 2 and 3 and the second digit will change into a copy of digit 1. When webbing between 3 and four caused digit 3 to transform into digit 2 (Figure 16.21 A-C).
    e. When beads containing BMP antagonists such as Noggin were placed in the webbing between digits 3 and 4, digit 3 was transformed into digit 2 (Figure 16.21 D-F).
    f. Sonic hedgehog also alters the ratio of Gli3 repressor to Gli3 activator. Shh prevents the proteolysis that makes the repressor form of Gli3. Because proteolysis is prevented repressors are converted into activators. Mice with a homozygous mutation for Gli3 (or Gli3 and shh) form 6-11 digits per limb with no normal identity (Figure 16.21 G). Sonic hedgehog appears to work through Gli3 to specify normal identities and numbers of digits.

E. Generation of the Dorsal-Ventral Axis
1. The dorsal (knuckles) ventral (pads) polarity of the limb bud is determined by the ectoderm encasing it.
2. If the ectoderm is rotated 180◦ with respect to the limb bud mesenchyme, the dorsal ventral axis is reversed.
3. The Wnt7 gene is expressed in the dorsal ectoderm of the chick and mouse wing buds. When Wnt7a was deleted from a mouse embryo, the embryos had ventral foot pads on both surfaces of their paws.

4. Wnt7a induces activation of the Lmx1 gene in the dorsal mesenchyme. Lmx1 encodes a transcription factor that appears to be essential for specifying dorsal cell fates of the limb. If expressed in the ventral cells, they develop a dorsal phenotype. If knocked out, a syndrome in which the dorsal limb phenotype is lacking, akin to nail-patella syndrome in humans which lack nails and kneecaps.

F. Coordinating the Three Axes

1. Sonic hedgehog in the ZPA activates the expression of fgf4 gene in the posterior region of the AER (Figure 16.20).
2. FGF4 recruits mesenchyme cells into the PZ and along with FGF8 help to maintain the expression of shh in the ZPA.
3. Shh of the ZPA sustains the AER FGFs by inducing Gremlin which inhibits BMPs in the mesoderm from inhibiting FGFs of the AER. The AER and the ZPA mutually support each other (Figure 16.23).
4. FGF4 along with Wnt7a simulate shh production.
5. BMPs are responsible for shutting down the signaling from the AER and inhibiting Wnt7a signal along the dorsal-ventral axis. BMP signal eliminates growth and patterning in all three axes.
6. Gremlin work against BMP to preserve AER and the Wnt7a pathway.

G. Cell Death and the Formation of Digits and Joints

1. Sculpting the autopod
   a. Apoptosis in the webbing of feet (Figure 16.24 A&B); interdigital necrotic zone.
   b. After a certain stage, chick cells between the digits are programmed to die even if removed and transplanted elsewhere.
   c. The ulna and radius are separated from each other by an interior necrotic zone.
   d. Anterior and posterior necrotic zones shape the end of the limbs.
   e. These cells die by apoptosis associated with fragmentation of their DNA.
   f. The signal for apoptosis in the autopod is provided by BMP proteins. BMP2,4,and 7 are expressed in the interdigital mesenchyme. Blocking BMP signaling prevents interdigital apoptosis.
   g. The default state is for apoptosis. The BMP signal must be shut down by proteins such as noggin which is made in the developing cartilage of the digits and in the perichondrial cells surrounding it. If Noggin is expressed in the interdigital region, the webbing will not undergo apoptosis.
   h. Apoptosis is inhibited between the digits of duck feet by the Gremlin protein (Figure 16.25).
2. Forming the joints
   a. In developing limbs, BMPs induce Mesenchymal cells either to undergo apoptosis or to become chondrocytes depending on the stage of development. BMPs can induce death or differentiation depending on the age of the target cell.
   b. BMP7 is made in the perichondrial cells and promotes cartilage formation.
   c. BMP2 and GDF5 are expressed at the region between bones.
   d. Mice lacking Gdf5 gene also lack limb joints.
   e. Mice lacking the noggin gene also have no joints.

H. Continued Limb Growth: Epiphyseal Plates

1. Chondrocytes near the ossification fronts continue to divide and proliferate and thus the bones grow longer.
2. These cartilaginous areas at the end of long bones are called Epiphyseal growth plates.

3. Composed of three regions:
    a. region of chondrocyte proliferation
    b. region of mature chondrocytes, and
    c. region of hypertrophic chondrocytes (Figure 16.27).
4. As long as the Epiphyseal growth plates are able to produce chondrocytes, the bone will continue to grow in length.