

**Pattern formation during development: Patterning of the vertebrate limb****Reading: 8e: Chapter 16; 9e: Chapter 13**Learning Goals Be able to:

- Explain how the limb knows where to form along the body axis, and how it knows its identity
- Explain how the A-P, D-V and proximal-distal axes of the limb are established, including molecules involved, and the interactions between these molecules.
- Compare and assess the two models for limb outgrowth: the progress zone model vs. the prepattern model.
- Given the communication among the three axes of the limb, predict the consequences when a signal is missing or present in excess.
- Interpret how BMPs and Shh are used to determine digit number and identity

Limb development is an excellent example of pattern formation: the process of establishing cells into an ordered spatial arrangement of differentiated tissues. The questions we'll address on this topic are:

- 1) Why do limbs form in particular locations?
- 2) How are forelimbs distinguished from hindlimbs?
- 3) How are the three limb axes organized?
- 4) How is the identity and shape/structure of individual limb elements (e.g. bones) determined?

**Axes of the limbs**

Proximal-distal: Proximal is closest to the body and distal is towards the fingertips.

The divisions are as follows (from proximal to distal):

Stylopod: the humerus (forelimb) or femur (hind limb)

Zeugopod: two elements radius/ulna (forelimb) or tibia/fibula (hindlimb),

Autopod: variable number of carpals (wrist/ankle bones) and three to five digits (depending on the organism).

Anterior-posterior: thumb is anterior, little finger is posterior

Dorsal-ventral: back of hand is dorsal, palm is ventral

**Limb components**

The limb grows from an area of cells called the limb field—a morphogenetic area with boundaries where cells are specified by position. (Figure 16.2) The limb also has regulative ability (if some cells are lost, can still make a whole limb), and each cell has the ability to differentiate into any kind of cell within the limb. Limb is composed of mesoderm and ectoderm: Mesoderm (mesenchyme): bones and tendons from lateral plate mesoderm; muscles from somitic mesoderm. Ectoderm: skin, feathers, scales, claws.

**Why do limbs form in particular locations along the body, and how do they know to be forelimb or hindlimb?**

*Hox gene expression.* Remember that mammals have four Hox clusters, a-d. The genes within these clusters are numbered 1 (anterior) to 13 (posterior). Hox a9, b9, c9 and d9 (for example) form a paralogous subgroup, and have similar expression patterns within the embryo. Hox b9, c9, d9 expression in flank indicates where limbs will form. Ectopic Hoxb8 expression leads to duplication of forelimbs, while Hoxb5 mutant shifts placement of the forelimb. In general, animals form their forelimb anterior to Hox6 expression, and their hindlimb posterior to Hox8 expression.

*Localized sources of FGF.* A filter placed between the intermediate mesoderm (pronephric) and limb bud mesoderm, prevents formation of the limb bud. Implanting beads with FGFs, in particular FGF8, known to be present in the limb, can induce ectopic limbs.

*Identity of forelimbs vs. hindlimb: transcription factors Tbx4 and Tbx 5.* Tbx4 specifies hindlimb and Tbx-5 specifies forelimb. A bead of FGF in between the two normal hindlimbs will be exposed to both Tbx-4 and Tbx-5, and becomes a combination of both forelimb and hindlimb structures.

### **Generation of the axes of the limb**

The two germ layers that comprise the limb, mesoderm and ectoderm, interact to pattern the limb during morphogenesis; each requires the other for normal development of the limbs.

#### Proximal-Distal Axis: the AER

Development of the limb starts proximally and moves distally. This was established by experiments in the 1940's in which the AER (an epithelium that forms at the distal tip of the developing limb bud) was removed from an early stage limb bud. Elongation ceases and only the humerus develops. Removing AER slightly later truncates the limb at the radius and ulna, still later at the carpals, and latest at the digits, suggesting that the AER is required for outgrowth.

The AER specifically forms along the D/V midline of the limb: it is placed at the boundary of expression of a molecule called *radical fringe*, present in the dorsal ectoderm, but not in the ventral ectoderm of the developing limb. The formation of the AER is necessary for development of the limb.

#### Growth of the limb requires AER

AER requires limb mesoderm, and the type of mesoderm specifies the structure. If you combine wing, leg or foot mesoderm with ectoderm, the structure that differentiates is determined by the identity of the mesoderm.

AER does not determine the stage of limb development—an AER from a late stage embryo can be grafted onto the mesoderm of an early embryo, and the limb will develop normally.

It is thought that the AER's basic function is to keep the mesoderm just behind it (a region referred to as the progress zone (PZ)) in a proliferative mode; this proliferation is required for growth of the limb. The AER and PZ reciprocally promote each other. The mesenchyme (PZ) induces the AER; once established the AER is necessary for further proliferation of the mesenchyme. And, persistence of the AER depends on a maintenance factor from the mesenchyme. Molecules involved: FGF8 (ectoderm) and FGF10 (mesenchyme).

*According to the Progress Zone hypothesis*, a cell's proximal or distal fate is determined by the time it spends in the progress zone; a short time spent in the progress zone (proliferating for a short time) defines a cell as proximal, while a longer time spent in the progress zone defines a cell as distal. The cells otherwise have no preformed identity.

*Alternate hypothesis: The "prepattern" model (proposed by the labs of Cliff Tabin and Gail Martin).* In this model, the proximal-distal identity of limb bud cells is determined before any significant limb outgrowth can occur. The identity of each cell is already established within a field of cells. Cells are pre-determined, and thus should "know" their fate if transplanted. As the limb grows, the pattern is maintained while each region of cells expands due to cell proliferation. The AER still promotes cell survival and/or proliferation, but there is no special "progress zone" where cells receive their proximal-distal identity over a period of time. When the AER is removed, cell death ensues in the region immediately underneath the AER, thus removing that section of cells from the final limb. The region of cell death has been proposed to be constant (about 200 um deep), but because each predetermined part of the limb is expanding in size, less of the limb (more distal regions) are lost with later AER removal. These two hypotheses of how the limb forms are still being actively investigated. In addition to these two models, a third model called the *reaction diffusion* model has also been proposed that hypothesizes TGFs and BMPs are expressed alternately over time, and that the distance a cell is from the TGF source determines whether it can no longer receive TGF and thus exit the cell cycle (and begin to differentiate). No one model has yet been shown to explain all experimental results, so scientists are still exploring how this works.

#### Anterior-posterior axis: The zone of polarizing activity (ZPA)

ZPA: a small region of mesodermal tissue on the posterior side of the developing limb. If this block of mesoderm is transplanted onto the anterior side of another developing limb, the digits emerge as mirror image duplications. Why does this happen?

Duplication can be mimicked by extra retinoic acid. But, there is no apparent endogenous RA gradient in the limb at this time, so scientists looked at molecules that are activated by RA. It turns out that Sonic hedgehog (Shh) is localized to the ZPA. Implanted beads that released Shh induced the same mirror image duplication that the ZPA alone did. Shh specifies the digits in a dose-dependent manner: low, more anterior, high, more posterior.

If one looks at the pattern of expression of Hox genes within the developing limb bud, it is also clear that different Hox genes are expressed differentially along the A-P axis as well. Thus, the Hox genes (activated by RA) likely induce expression of Shh differentially in the ZPA.

Patterning the dorsal-ventral axis (see powerpoint slides for more figures)

The dorsal-ventral axis arises initially as a property of the ectoderm, although the underlying mesoderm interacts with the ectoderm. If the ectoderm of a limb bud is removed and rotated 90 degrees with respect to the underlying mesoderm, the limb will be upside down, with the palms forming on the wrong side. This is NOT true of the other two axes—proximal distal axis is dependent on the mesenchyme, and so is the A-P axis.

There are several key molecule involved in dorsal-ventral patterning:

--**FGF 10**. FGF 10 signaling from the mesoderm in the limb bud allows the dorsal ectoderm to synthesize a secreted protein called **Radical Fringe** just prior to limb bud formation. The AER forms on the border of cells that express radical fringe (dorsal ectoderm) and cells that do not (ventral) (Note, this molecule is another member of the fringe family, of which Lunatic Fringe is a member. Remember that Lfng induced the formation of somite borders). The positioning of the AER is obviously critical for the development of the rest of the limb, as discussed last week. Below are the dorsal-ventral specific molecules of the limb.

--**Wnt-7a**. Wnt-7a expression is completely restricted to the dorsal ectoderm and maintains expression of **Lmx-1** in the dorsal mesenchyme, which in turn probably helps to maintain Wnt7a expression in the ectoderm. The most obvious demonstration of the role of Wnt-7a comes from looking at the phenotype of knockout mice: they do not develop any dorsal structures in the limb. For example, footpads and tendons, normally ventral structures, are present on both sides of the foot. Hair, normally present on the dorsal side, is not present on either side. (see ppt slides).

--**En-1**. En-1 is expressed in the ventral ectoderm and mesenchyme. It represses Wnt-7a in the ventral ectoderm. En-1 knockouts therefore have dorsalized limbs.

A closer look at the Wnt-7a knockout reveals the connection between the patterning of the three axes. Wnt-7a mutants have a clear loss of posterior elements in addition to loss of dorsal elements. (see figure from powerpoint slides). The ulna can also be missing. This is consistent with the connection between Wnt-7a expression and Shh expression--Wnt7a induces Shh expression (not on its own, obviously, or Shh would be expressed throughout the dorsal mesenchyme). Because the growth and development of the three axes of the limb is intertwined, usually a loss of function mutation in any of the genes involved in patterning will have broad effects on limb development. It is the coordination of all these molecules in time and space that makes the patterning of the limb so impressive.

### **Digit identity**

The anterior-posterior axis is established by asymmetrical expression of Shh in the limb bud (on the posterior side only). Shh does not just help to confer posterior identity to the digits, however; it is also important for overcoming apoptosis. This is clear when looking at experimentally manipulated limbs in which extra Shh is expressed, or in which an extra ZPA has been transplanted in: in both instances, more digits than

usual are formed. This is not due just to the presence of extra cells (possible in the ZPA transplantation), but must actually be a result of extra Shh (since it also happens in Shh overexpression). Apoptosis in the limb is mediated by BMPs (our old friends). Not surprisingly, BMPs actually have more than one role in limb patterning. Their differing concentrations across the limb bud help to determine the identity of the digits, and then later, influence which cells will die, thus helping to sculpt the shape of the limb. Thus, both Shh and BMP are involved in apoptosis and digit identity.

The gradient of Shh across the limb bud affects digit identity both by time of exposure and concentration. In the mouse forelimb, digits 4 and 5 are exposed to Shh for a long time and high concentration (they are at the site of the ZPA), while the cells of digits 2 and 3 are exposed to less Shh (gradient). Digit 1 is apparently not dependent on Shh. This is kind of a simplistic way to look at the system, however. More detailed analysis has shown the space between the digits, the interdigital space, determines the identity of the digits. These areas of cells will eventually die by apoptosis, but before they do, they help to signal the identity of the digits next to them. Specifically, the identity of a digit appears to be determined by the interdigital space POSTERIOR to the digit; thus if these cells are removed, the digit is influenced by the anterior interdigital cells. For example, if the interdigital 2 space (ID2) is removed, then digit 2(D2) is transformed into digit 1 (D1). If digit cells are transplanted into the ID2 region, these cells will make D2 no matter what their original fate.

It turns out that Shh upregulates BMP expression. Somehow Shh regulates levels of BMP such that the cells in the ID express BMPs at high levels; while the cells in the digit region of mesenchyme express BMP at low levels (but can be influenced by binding to the adjacent BMPs). Shh is higher in the posterior end of the limb; thus, the more posterior IDs express more Shh and higher BMPs. Not surprisingly, Noggin has the complementary role of inhibiting BMP expression; thus, noggin can anteriorize a digit when ectopically expressed in the ID regions.

If a bead with Shh is implanted into an ID region, the digit normally influenced by that ID is transformed to take on a more posterior fate (ie, d1 transformed to d2 if Shh put in the ID1 region).

Lastly, as mentioned above, BMPs have a second role: apoptosis. ID regions contain BMPs, therefore the cells in these regions ultimately die, leaving spaces between the digits.

The limb as a whole arises so perfectly formed due to feedback between the three sets of signals that determine the three axes of the limb: Shh induces FGF4 expression. FGF8 and FGF4 induce FGF10 expression. Ectopic Shh thus induces ectopic FGF4 and vice versa. Wnt-7a (required for the dorsal side) induces Shh expression. Thus, loss of Wnt-7a decreases Shh expression, which also effects FGF4 expression, etc!

## Review Questions

- 1) What tissues make up the limb (and what are their origins)?—this info does not need to be memorized, but helps with the big picture.
- 2) How do limbs get placed along the A/P axis of the embryo?
- 3) How does a limb know to be forelimb rather than hindlimb?
- 4) How can the ectopic addition of a single molecule like FGF make a whole limb form?
- 5) What is the AER and what are its main functions in the limb?
- 6) What are the old and new hypotheses about how the AER affects cells in the "progress zone", and what further experiments could be done to clarify the mechanism?
- 7) What is the ZPA, what is its main function, and what molecules are involved?
- 8) What experiments demonstrate that Shh acts as a morphogen along the A-P axis of the limb?
- 9) How do molecules within the three axes communicate with each other to allow limb patterning, and how does removal of one molecule then affect multiple axes?
- 10) How is the D/V axis determined within the limb and how is communication between the mesenchyme and ectoderm of the limb important?

Classes 21 and 22

11) What are Shh and noggin's roles in digit formation?

12) What role do the interdigital spaces have with regards to digit identity, and how was this demonstrated?