Vertebrate Limb Patterning

What makes limb patterning an interesting/useful developmental system
How limbs develop
Key events in limb development
  positioning and specification
  initiation of outgrowth
  establishment of the patterning axes
Regulating expression of genes – limb enhancers
All tetrapod limbs exhibit the same basic structure but a wide range of morphologies.

Forelimbs and hindlimbs as well as limbs from different species exhibit the same basic structure but a wide range of morphologies.

Historically, the two most common model systems to study limb development have been mouse and chick.
What makes chick a good model system for studying limb development?

Ease of physical manipulation
Development of Limb Tissues Is From Five Sources

- Lateral Plate Mesoderm
  - Skeleton
  - Tendons
  - Ligaments
  - Vasculature

- Somites (Dermo-myotome)
  - Migrate
  - Musculature
    - Schwann cells
    - Dorsal root ganglia
    - Sensory axons

- Neural Crest
  - Migrate
    - Motor Axons

- Neural tube
  - Grow
    - Skin, hair
    - feathers etc.

- Ectoderm
Lateral Plate Mesoderm


Tanaka (2013) Dev. Growth Diff 55, 149
Pathways of limb development are conserved

Tetrapod limbs originate as two pairs of limb buds that appear at species-specific levels of the embryonic flank. In mouse, forelimb buds appear first (~E9 @ ~somite 8-9) followed by hindlimb buds ~ 0.5-1 day later (@ ~somite 23-24).

Each limb bud consists of lateral plate mesoderm (LPM) covered by ectoderm.

E10 mouse

Duboc (2011) Dev Dyn 240, 1017
Scanning EMs of a series of mouse embryos

Martin (1990) Int. J. Dev. Biol 34, 323
Key events in limb development

(1) Specifying limb positioning (Hox genes, Tbxs & Hand2) and identity (forelimb versus hindlimb) (Pitx1 affects hindlimb; no comparable gene in forelimb)

(2) Initiation of limb bud outgrowth (Tbxs and Fgfs)

(3) Establishment of the patterning axes PD (AER-Fgfs), DV (BMPs, Wnts, Lmx1, En1) & AP (Hand2 & Gli3 & Shh). There are both positive and negative feedback loops between these proteins that regulate their expression.
In a variety of vertebrates the anterior expression boundaries of a number of Hox genes in the LPM occur exactly at the forelimb level (Oliver et al. 1990, Rancourt et al. 1995, Nelson et al. 1996)
Tbx3, Gli3 and Hand2 and limb bud positioning

Tbx3 misexpression by retrovirus injection shifts limb anteriorly (see above)

Hox gene expression is unaltered.

Limb position determined by hox genes acting upstream of Tbx3, Gli3 and Hand2 expression.
Specification of limb identity (forelimb versus hindlimb)

Tbx4 is expressed in hindlimbs and Tbx5 in forelimbs.

Tbx5-/- animals fail to form a FL bud.

BUT the patterning of the forelimb field occurs appropriately in the mutant.

Pitx1 specifies skeletal elements of HL identity

Minguillon (2005) Dev Cell 8, 75

Tbx4 can rescue forelimb growth in Tbx5<sup>fl/fl</sup> Prx1-cre mice.

Tbx4 and Tbx5 do NOT specify limb identity.

Duboc1 (2011) Dev 138, 5301
Regulation of limb bud outgrowth

AER is required for limb bud outgrowth

Limb mesenchyme specifies limb identity

Inductive interactions between AER and limb mesenchyme similar to the inductive interactions that take place between metanephric mesenchyme and ureteric bud.
What is the role of the AER?

AER functions by providing FGFs required for outgrowth of the limb bud.

Placing a bead soaked in FGF anywhere along the flank of the embryo will induce formation of an ectopic limb.

Cohn (1995) Cell 80, 739
### AER, FGFs and limb bud outgrowth in mice

<table>
<thead>
<tr>
<th>Fgf10</th>
<th>LPM</th>
<th>k/o</th>
<th>limb deformities</th>
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<tr>
<td>Fgf4</td>
<td>AER</td>
<td>ck/o</td>
<td>no effect</td>
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<tr>
<td>Fgf8</td>
<td>AER</td>
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<td>Fgf17</td>
<td>AER</td>
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![Image of limb bud outgrowth](image_url)
Fgf10 and limb bud outgrowth

Fgf10 is expressed in the mesenchyme. In its absence:
- There is no Fgf8 expressed in the forelimb bud AER (although Fgf8 is expressed elsewhere in the embryo)
- There is no Shh in the FL or HL mesenchyme
- Tbx5 is induced appropriately (E9.5) but expression is rapidly lost.

Fgf10 is required for the expression of Fgf8 and formation of AER
Fgf10 is NOT required for inducing expression of Tbx5 but is required for maintaining it.

Sekine (1999) Nat Gen 21, 139
Fgf8 and Fgf8/Fgf4 and limb bud outgrowth

Fgf8\(^{-/-}\) is early embryonic lethal. Therefore conditional k/o made using Msx2-cre (Fgf8\(^{-/-}\)).

Msx2-cre is expressed in both FL and HL but it is turned on after Fgf8 expression has initiated in the FL but before expression is initiated in HL.

Fgf8\(^{-/-}\) FL are smaller but relatively normal while proximal elements of hindlimb (i.e. stylopod) are reduced or missing.

Lewandoski (2000) Nat Gen 26, 460

Fgf8\(^{-/-}\); Fgf4\(^{-/-}\)

Fgf8\(^{-/-}\); Fgf4\(^{-/-}\) FL are abnormal (zeugopod/autopod) and HL are absent.

The role of Tbx5 in forelimb initiation and outgrowth

Tbx5-/- embryos fail to form a FL bud

Fgf10 is not expressed in Tbx5-/-
Does Tbx5 regulate Fgf10 expression?

Tbx5 activates reporter construct containing Fgf10 5’ region.

Tbx5 and beta-catenin (Wnt signaling) co-operate to activate Fgf10 expression.

Tbx5-/- mice fail to form forelimbs because they fail to induce Fgf10 expression
How is Tbx5 expression induced? Identification of a Tbx5 enhancer

Identification of a limb specific enhancer within intron 2 of Tbx5 using B-gal reporter transgenes.

This intron 2 enhancer contains 6 Hox binding sites and mutation of 4/6 of those sites causes loss of expression of reporter construct

Expression of Hox4 and Hox5 PG activate reporter construct containing Tbx5 CREs

Minguillon (2012)

Binding of FL-expressed hox genes to the intron 2 enhancer in the FL activates Tbx5 expression while binding of HL-expressed Hox genes to the same repressor inhibits Tbx5 expression in the HL

Nishimoto (2014)
Genomic Knockout of Two Presumed Forelimb Tbx5 Enhancers Reveals They Are Nonessential for Limb Development

Cunningham (2018)
Establishing the limb patterning axes

The developing limb bud is patterned along three axes:
- Proximal-Distal
- Dorsal-Ventral
- Anterior-Posterior
Misexpression of RA affects patterning along the Proximal Distal Axis

Mercader (2000) Dev 127, 3961
Signaling by the non AER ectoderm controls dorsal ventral patterning

normally expressed in ventral ectoderm
En-1
Bmp4 & Bmp7

Normally expressed in dorsal ectoderm
Wnt7-a

Normally expressed in dorsal mesenchyme
Lmx-1 & Shh

Normally expressed in AER
Fgf8

*In situ* for Shh (molecular marker for ZPA) & Fgf8 (molecular marker for the AER)

Pizette et al. (2001) Dev.128,4463
Wnt – BMP regulate DV patterning

Downregulate BMP(v) signaling by misexpression of Noggin (BMP antagonist) by retroviral injections to the ectoderm leads to loss of en1(ve) and expansion of Wnt-7a (de), Lmx1 (dm) & Shh (dm) into ventral region of bud (i.e. dorsalization of bud). Also leads to misexpression of Shh and Fgf8 (AER) (i.e perturb PD & AP patterning)

Upregulate BMP(v) signaling by misexpression of a constitutively active Bmp receptor leads to expansion of en1, and loss of Wnt-7a & Lmx1(d) (i.e. Ventralization of bud). Also leads to loss of AER

Pizzare et al. (2001) Dev. 128, 4463


Wnt7a-/- mouse exhibits formation of dorsal tendons and footpads

En-/- mouse shows loss of ventral pads and formation of nails on both dorsal and ventral side
Anterior-posterior patterning is controlled by signals from the mesenchymal cells of the ZPA (zone of polarizing activity).
Shh\(^{-/-}\) mice exhibit defects in AP patterning

Shh\(^{-/-}\) mice form truncated limbs with single digits. The stylopod looks normal.
A number of genes previously shown to be regulated by Shh are either expressed at low levels (Bmps 2&4) or turned on but rapidly downregulate (Gremlin).
Gli3 expression is extended (i.e. Gli3 expression is negatively regulated by Shh).

In the absence of Shh, AER formation is initiated but doesn’t persist as long as in wt.

A/P patterning occurs prior to Shh induction but requires Shh to be maintained.

Shh expression is also required to maintain the AER.

Chiang et al. (2001) Dev. Biol. 236,421
Regulation of Shh expression and the identification of a long range limb bud enhancer (ZRS or MCFS1)

Sharpe (1999) Curr Biol 9, 97 Mouse mutation Ssq causes partial duplication of distal hindlimb, secondary to induction of an anterior Shh expression domain and increased expression of Fgf8. This is caused by the random insertion of a transgene. The site of the transgene insertion was mapped to the vicinity of Shh (within a gene ~1Mb distant. The transgene itself showed an expression pattern similar to Shh within the limb bud. Note that Shh is expressed in multiple sites within the developing embryo while the transgene was only expressed within the limb bud.

Lettice (2002) Human mutations with similar phenotype were mapped to the homologous region of the human genome (within Lmbr1 gene).

Additional mutations with similar phenotypes were also found in mice and other animals.
A cosmid library was created from Ssq genomic DNA. A cosmid containing the transgene plus ~8.5kb of flanking sequence at least partially recapitulated the expression pattern of the reporter in the original Ssq mouse. Sequence comparison of mouse and human genomic DNA identified an ~800bp segment of highly conserved sequence most of which was included in the cosmid.

Based on this data a transgene was constructed consisting of 800 nucleotides of highly conserved sequence, placed upstream of a minimal promoter driving expression of β-galactosidase.

Transgenic mice containing this transgene express β-galactosidase in the same posterior region of the limb bud where Shh is expressed. β-galactosidase is not expressed elsewhere in the embryo.

This sequence contains a limb-specific enhancer for Shh.
Multiple enhancers regulate Shh expression but only one affects expression in the limb

Deletion of the ZRS causes loss of Shh expression in the limb and results in limb truncations. The ZRS enhancer does not affect expression of Lmbr1 or Rnf32.
Multiple mutations within this highly conserved sequence have been found in mice, humans and cats with polydactyly.

Single nucleotide transversions and transitions as well as small insertions and deletions within the ZRS lead to ectopic expression of SHH and polydactyl and other limb anomalies.

Ectopic expression is maintained in transgene

Lettice (2008)
What snakes have to teach us about Shh expression in limbs

Kvon (2016)
The Conserved Sonic Hedgehog Limb Enhancer Consists of Discrete Functional Elements that Regulate Precise Spatial Expression

Graphical Abstract

Highlights

- The ancient vertebrate enhancer, the ZRS, shows sequence plasticity
- Discrete regulatory activities are assigned to specific sites in the enhancer
- The number of HOXD binding sites determines the level of Shh expression
- Robust expression is a collective of regulatory and redundant information

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In Brief

Lettice et al. examine the composition of a highly conserved limb-specific enhancer, the ZRS, by dissecting the endogenous sequence using genome editing. Analysis of the resulting phenotype gives insights into the complex composition of the enhancer, which integrates discrete expression activities and redundant elements to drive accurate spatiotemporal gene expression.

Note there are 7 figures plus 2 supplemental figures and 1 supplemental table. You don’t need to provide figure facts for any of the supplemental material.

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