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Evidence for an Expansion-Based Temporal *Shh* Gradient in Specifying Vertebrate Digit Identities

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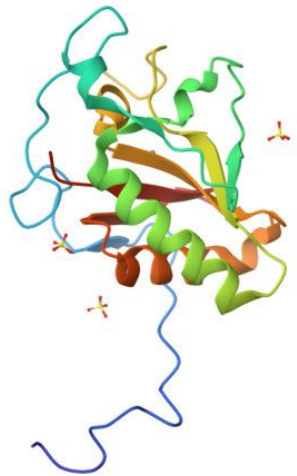

Shh signaling protein

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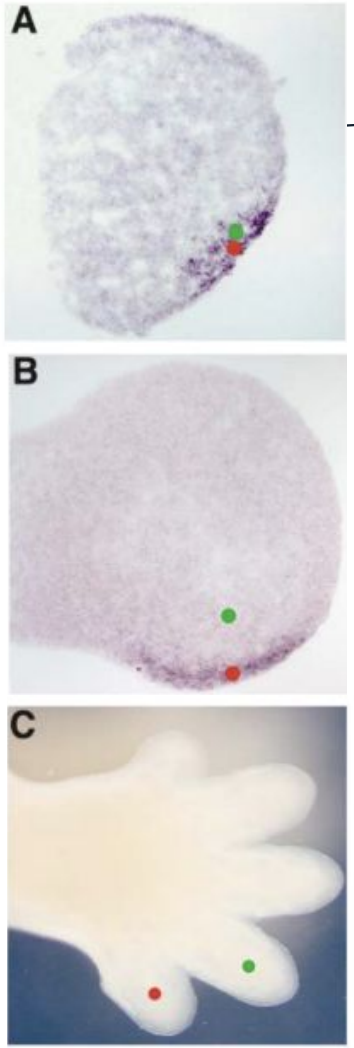
Background

Limb buds are the developing limbs of an embryo

We are looking at the signaling molecules that activate gene expression

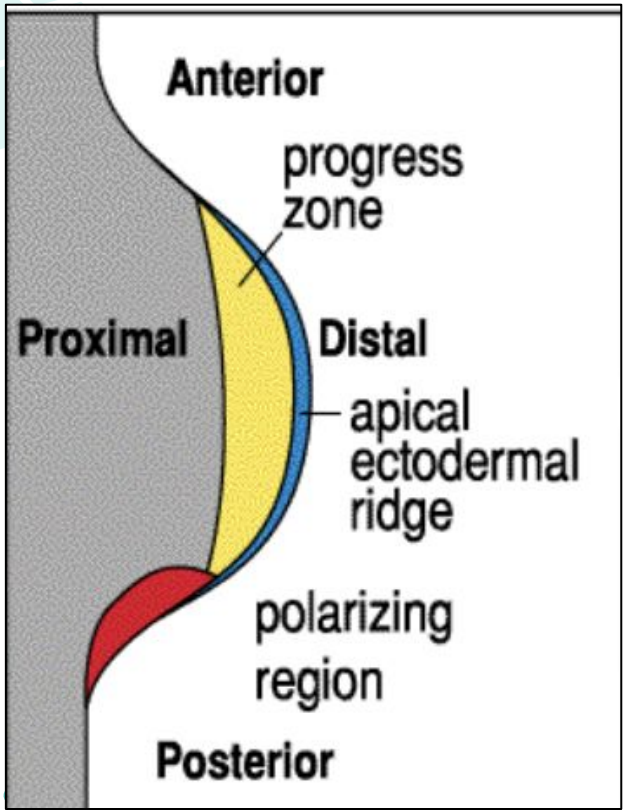
Thus allowing growth to limbs!

**We describe them by the day of development (E10.75, E11, E14)



From Fig 5

Background



AER= the ectodermal thickening of the distal/ventral edge

Mesenchyme= mesodermal embryonic tissue (develops embryo)

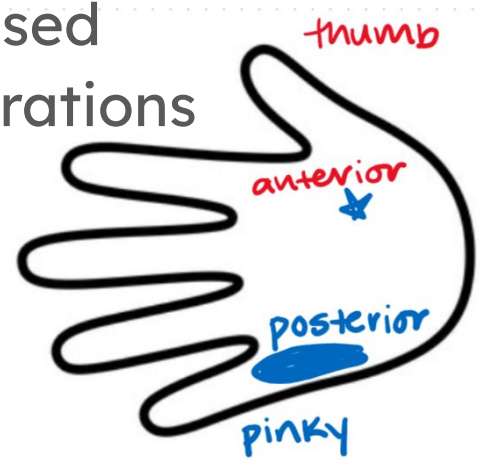
PZ= rapid division of the mesenchymal cell (*Shh* expressed)

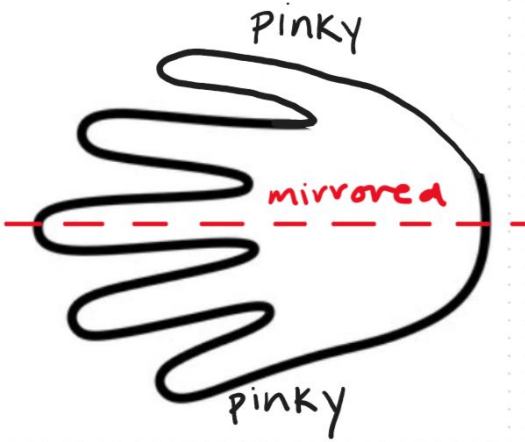
What is Shh (Sonic Hedgehog Gene)

- Gene that encodes Shh protein
- Signaling molecule in developing embryos
- Expressed in ZPA
- Morphogens
    - Embryogenesis

Morphogens

- Figuring out the idea of a morphogen was not completely clear
- First clue was Saunders and Gasseling (1968) experiment
- Polarity and patterning
- Morphogenic fate based on threshold concentrations





Where does the Shh mRNA go after being expressed and what does it become?

How does Shh actually work?

Mouse Construction

- Created altered mouse alleles in order to identify Shh descendants

Dil labeling= fluorescent neuronal tracer

FROM THIS it's concluded that at least some cells from the AER aid in making structures along proximal distal axis

**gfpcre expressed in all cells that express Shh

Mouse Construction 1

Shhgfpcre

CRE= catalyzes DNA recombination

gfp= green fluorescent protein

Shhgfpcre allele & null allele (removed 12 amino acids)

Gene targeting to insert gene that encodes gfpcre

The 3-5 digits are made of cells that have been exposed to MAX conc of *Shh* protein for different amounts of time

Mouse Construction 1

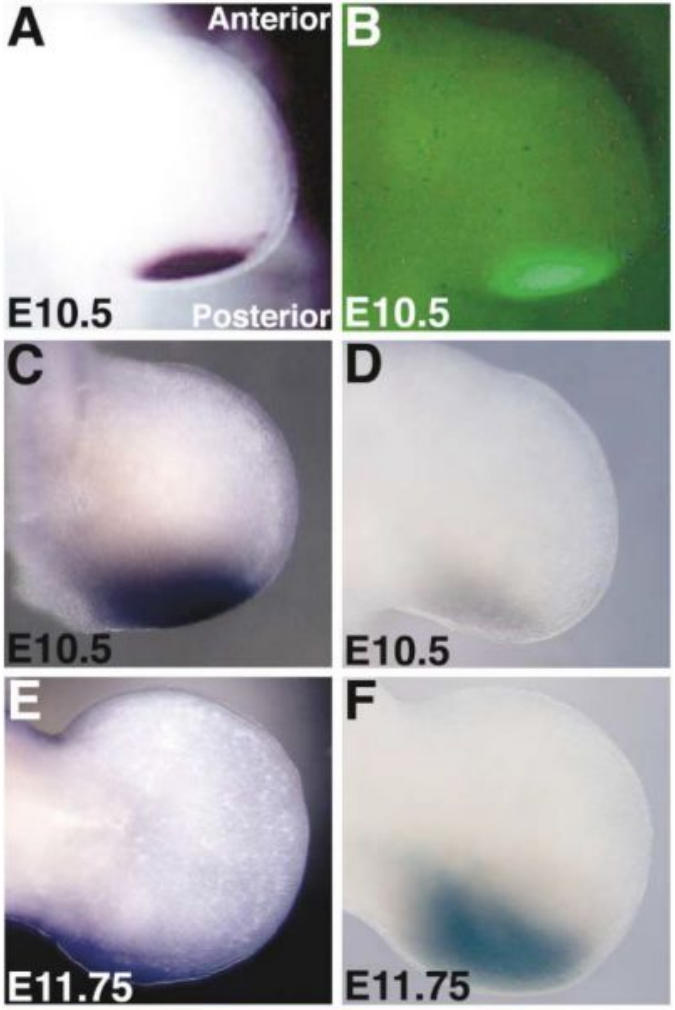
After E12 no *Shh* mRNA expression is found in the mesoderm

Cannot track the cells if there is no *Shh* mRNA (then there is no GFP)

<u>What we know as of now:</u> digits 3, 4, and 5 are dependent on the temporal gradient

- There is an overlap in Shh mRNA and GFP domains

From Fig 1



Shhgfpcre markings, fluorescence indicates Shh mRNA expression

What can't we do

Use GFP to locate and map the fate of *Shh* descendent cells

What CAN we do

Mark the *Shhgfpcre* cells using CRE controlled recombination and a reporter allele to express LacZ

Mouse Construction 2

Shhgfpcre/+;R26R/+ embryo

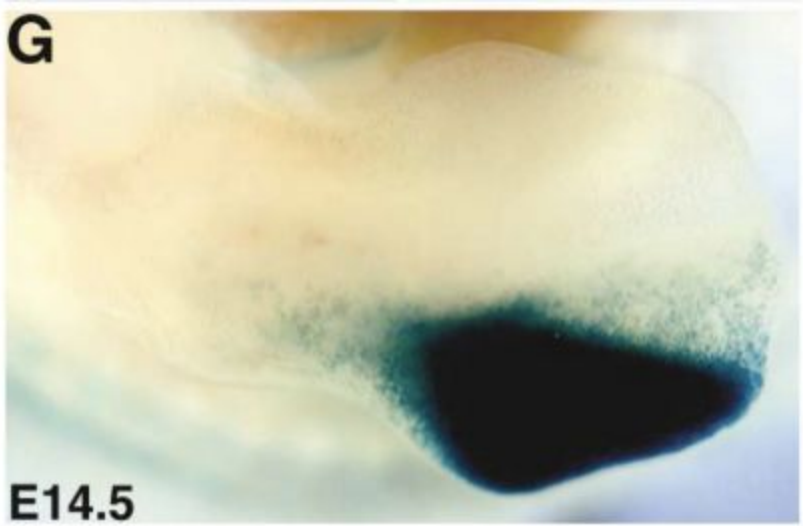
R26R= CRE inducible reporter allele

Will express LacZ in presence of CRE

Now we can map Shh expression beyond E12

What we know as of now: In E12.5-14.5, marked cells make up the interdigital mesenchyme posterior of digit 4-5

From Fig 1



Shhgfpcre/+;R26R/+ markings, fluorescence indicates Shh mRNA expression via LacZ

?

We want to know the functional significance in limb patterning of the expansion of *Shh* derived cells?

Hypothesis 1

Slow *Shh* degradation can possibly transport more anteriorly (upward) as the descendant ZPA cells expand

RULED OUT because at E10.5 there is a broader domain of Shh than in the consequent embryo stages

Hypothesis 2

Perhaps the functional purpose is to affect the distribution of signaling components downstream

Gli3 is a zinc protein that mediates downstream transcriptional effects of Shh

- Cleaved to produce repressor GLI3R

Gli3

GLI3R is concentrated in anterior

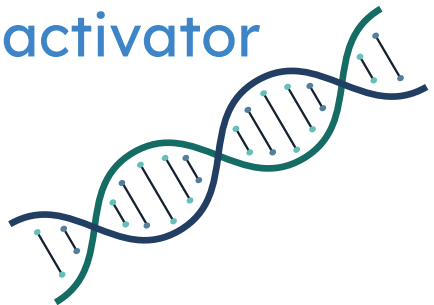
- It represses transcription in ABSENCE of *Shh*
- In PRESENCE of *Shh*, it creates activator form GLI3A

Shh activity

GLI3R repressor

Shh activity

GLI3A activator



Is GLI3R present in wild type chick limbs?

How fast does GLI3R disappear when there is Shh activity present?

How quickly does GLI3R appear when Shh activity is lacking?

Switched our thinking process

Mouse limbs >>>>> Chick limbs

Now we're looking at chick limb development to better understand Gli3 processes

Assayed Gli3 levels & bead based Western Blot

<u>What we know as of know:</u> Shh signal shown by levels of Gli3, levels out too quickly to be significantly impacted by expansion of Shh descendents

Mouse Construction 3

ShhcreER

Our first allele can only paint the picture for E9.75-E12 mice

Experimental knockout “tamoxifen inducible cre reporter cassette” into Shh locus to make a new allele

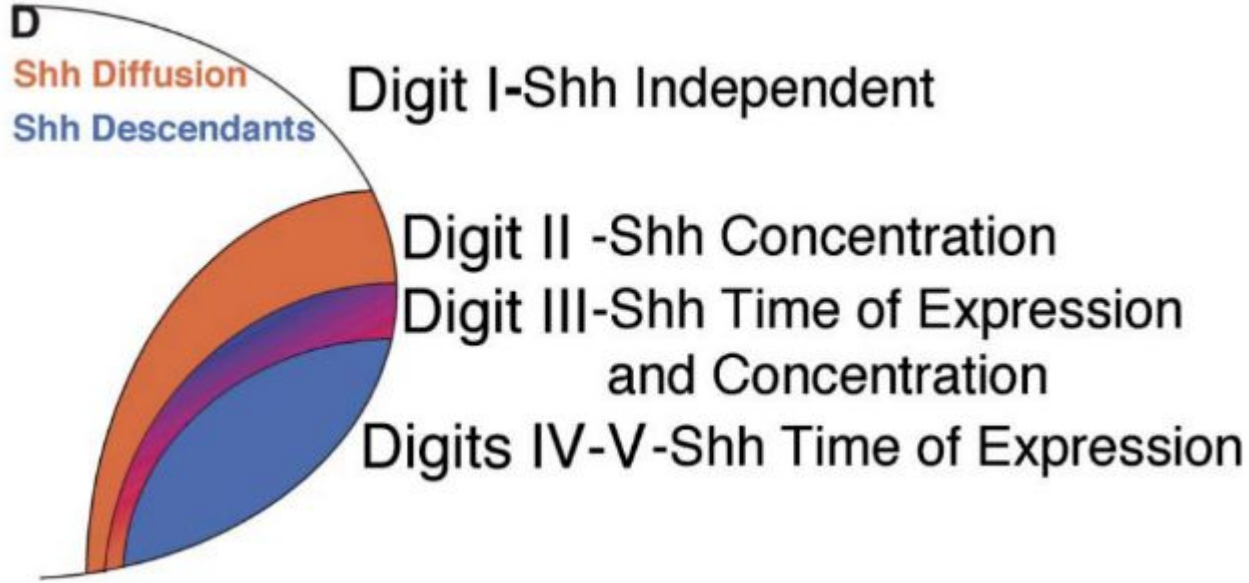
This allele produce CRE in all cells where the Shh mRNA is normally expressed BUT CREER protein cannot start up recombination in the R26R reporter locus unless injected with “tamoxifen”

Now we can map even further!

Mouse Construction 3

What we know as of now: Discovered that cells that contribute to more anterior digits stop expressing Shh at an earlier stage of limb development

From Fig 5


D
Shh Diffusion
Shh Descendants
Digit I-Shh Independent
Digit II -Shh Concentration
Digit III-Shh Time of Expression
and Concentration
Digits IV-V-Shh Time of Expression

Dispatched 1 gene

Last test just to verify that spatial expansion is what causes the patterning of digits 1-3

Disp1= required for hedgehog signaling release (modifies *Shh* availability)

There is a hypomorphic allele ($Disp1^{C829F}$) and a null ($Disp1^{\Delta 2}$)

Found that digit 2 is most vulnerable to *Shh* diffusion

Testing Memory

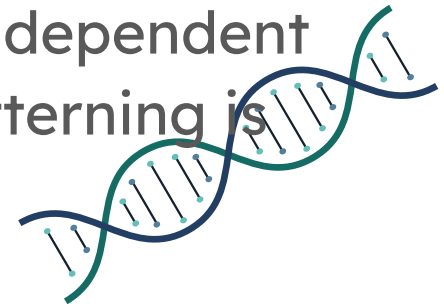
Bead based Western Blot; All digits behaved differently

Implanted beads soaked in different concentrations of *Shh*

Short amount of time and high [conc] had no differences

Second bead places for extended period of time

Found mesenchyme cells form memory to Shh independent of exposure [conc] or period AND confirmed patterning is affected by exposure length and [conc]



Conclusions

Shh is expressed by exposure level and temporal gradient

Digit 1 is Shh independent

Digit 2 is not affected by temporal gradient (dependent but never actually makes Shh) most vulnerable

Digit 3 relies on concentration and temporal gradient

Digit 4-5 relies on temporal gradient

Mesenchyme cells have memory