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Extended exposure to Sonic hedgehog is required for patterning the posterior digits of the vertebrate limb

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Summary

Sonic hedgehog (Shh) is a key signal in establishing different digit fates along the anterior-posterior axis of the vertebrate limb bud. Although the anterior digits appear to be specified by differential concentrations of Shh in a traditional, morphogen-like response, recent studies have suggested that posterior digits are specified by an extended time of exposure to Shh rather than, or in addition to, a threshold concentration of Shh. This model for digit patterning depends upon continued Shh signaling in the posterior limb through mid-to-late bud stages. We find that cyclopamine, a potent antagonist of Shh signaling, can down-regulate hedgehog target genes in the posterior limb throughout the time *Shh* is expressed, indicating continued active Shh signaling indeed takes place. To further explore the relative roles of time and concentration of Shh during limb development we carried out two additional series of experiments. To test the effect of limiting the time, but not the amount of Shh produced, we treated chick embryos with the hedgehog antagonist cyclopamine at various stages of limb development. We find that short exposures to Shh result in specification of only the most anterior digits and that more posterior digits are specified sequentially with increasing times of uninterrupted Shh activity. To test the effect of limiting the level of Shh produced, but not the time of exposure, we genetically modified Shh production in mice. As previously shown, reducing both the concentration of Shh produced and the duration of Shh exposure results in a loss of posterior digits. We find that maintaining a low level of Shh production throughout the normal time frame of ZPA signaling results in a near complete restoration of the posterior-most digits. These data are consistent with, and lend additional support to, the model that concentration of Shh seen and duration of exposure both contribute to the dose-dependent specification of digit identities, but for the posterior-most digits the temporal component is the more critical parameter.

Keywords

Sonic hedgehog; patterning; digits; limb; morphogen; temporal gradient

Introduction

In the chick limb bud system, exposure to ectopic Shh can repattern anterior mesenchyme, producing mirror image digit duplications with an altered polarity such that digits with the most posterior-like morphology form in closest proximity to the source of Shh (Riddle et al., 1993; Yang et al., 1997). Experiments varying the time of ectopic exposure indicate the longer limb bud cells are exposed to Shh, the greater the duplication and the more posterior in character are the digits that form (Harfe et al., 2004; Yang et al., 1997). Moreover, even relatively short

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times of Shh exposure below the threshold for altering positional fates causes subsequent Shh exposure to be more effective in posteriorizing the mesenchyme (Harfe et al., 2004), indicating there is some sort of cellular memory of Shh signaling. A mechanism by which this could occur was first proposed by Cheryll Tickle (1995) who suggested a "promotion" model, in which digit primordia are first specified to an anterior fate and are then promoted sequentially to more posterior fates with continued Shh exposure. Experimental evidence for this mechanism was provided by studies in which cells were labeled with diI adjacent to a bead soaked in Shh and digits forming from marked cells were assayed after different times of exposure to the bead (Yang et al., 1997). Depending on the length of exposure to Shh, equivalent, marked cells developed into an ectopic digit 2 (with short exposure) or digit 3 or 4 (with longer exposure).

In these experimental models the duration of Shh exposure was manipulated by varying the length of time Shh-carrying beads were implanted and/or removed, and limbs treated for different lengths of time were compared. To put time of exposure into the normal *in vivo* context, it was noted that there is extensive expansion of the posterior mesenchyme during the period of Shh signaling (Vargesson et al.,1997). Thus over time, some cells that were initially close to source of Shh would be pushed into a more distant location within the limb bud, therefore seeing a lower concentration of Shh, which would translate into a shorter duration of seeing a high concentration (Tickle, 1995; Yang, 1997). An unexpected twist on this model came from recombinase-based fate mapping experiments in the mouse (Harfe et al., 2004). A mouse line was constructed in which cre recombinase is produced in all cells that normally express *Shh*, irreversibly marking the cells by virtue of a histochemical marker expressed from a cre-inducible promoter. The resultant fate map of *Shh*-expressing cells shows that descendents of Shh-expressing cells end up populating the entirety of digits 5 and 4 as well as contributing to a portion of digit 3. Thus, the expansion of the posterior mesenchyme, in conjunction with the continued restriction of active Shh to the extreme posterior margin, means that the most posterior cells make Shh for longer than their anterior neighbors (Figure 1). Presumably, the cells producing Shh are also the cells seeing the highest concentrations through autocrine signaling. A variant of this experiment using an inducible cre ER^{T2}, verified that the digit 3 primordia makes Shh for a shorter time than digit 4, which in turn makes Shh for a shorter time than digit 5 (Harfe et al., 2004).

This view of digit specification can be applied to understanding mouse mutant phenotypes where the spatial gradient of Shh has been perturbed. For example, significant reduction of paracrine Shh signaling in the mouse limb bud ($Disp1^{\Delta 2/C829F};Shh^{+/-}$) results in loss of the anterior digit 2 only (Harfe et al., 2004). The presence of morphologically distinct posterior digits in this mutant, in the absence of significant functional transport of Shh protein, suggests their differential specification is less dependent on a classical diffusion gradient. However, this phenotype can be explained by reference to different times of exposure. As the posterior mesenchyme still expands in this mutant, the precursor cells of digits 4 and 5 make Shh and hence see maximal Shh-signaling in an autocrine manner for different lengths of time, as in wildtype. The same logic can be applied to a second mutant where the spatial distribution of Shh in the limb bud is perturbed by producing the ligand in the absence of cholesterol modification, although in this instance both the biochemical effect on protein distribution and the resultant phenotypes remain controversial (Lewis et al., 2001; Li et al., 2006).

Taken together these experiments have led us to propose the following model for digit specification: Digit 1 is believed to be independent of Shh because a biphalaneal digit forms with appropriate metatarsal association in Shh-deficient mouse limbs (Chiang et al., 2001; Ros et al., 2003). Digit 2 is specified by a low level of Shh, requiring diffusion or transport of Shh protein, since this digit primordium never makes Shh itself (Harfe et al., 2004). Digit 3 may be established by the combination of concentration and time exposure to Shh, while digits 4 and 5, are specified by different times of exposure to high levels of Shh signaling (Figure 1).

The model is one where the cumulative dose of Shh is integrated through time. However, for the posterior-most digits, where autocrine signaling is presumably at equivalent levels in the primordia of each digit, the length of time of exposure is a more significant parameter than the amount produced for determining the cumulative dose.

In spite of the strong experimental evidence for time of exposure to Shh (an expansion-generated temporal gradient of exposure) being a key parameter in digit specification, this idea has not been examined *in vivo* in the context of formation of the normal digits. Moreover, there are other data which present a challenge to this model. In particular, it has long been known that the Shh target gene *Gli1* is down-regulated in the posterior limb bud at later times of *Shh* expression (Marigo et al., 1996a). This fact was highlighted in a recent study (Ahn and Joyner, 2004), which interpreted these data as indicating that Shh signaling ceases in the posterior limb bud approximately 24 hours after *Shh* expression begins. If true, this would be problematic for the model where continued high-level Shh activity is the basis for posterior digit specification over a several day period.

These considerations led us to undertake a series of experiments in which we use pharmacological and genetic approaches to manipulate either the time of exposure to Shh or the concentration of Shh within the limb bud mesenchyme during the patterning of the endogenous digits. Our results are consistent with a model where time of exposure to Shh is the most critical parameter for specifying the posterior digits.

Materials and Methods

Cyclopamine Treatment

Eggs were incubated to the appropriate stage and windowed. Embryos were treated with 5 micro liters of 1mg/mL solution of cyclopamine (Toronto Research Chemicals) in 45% 2-hydropropyl-ß-cyclodextrin (HBC; Sigma) as previously described (Incardona et al., 1998). The embryos were reincubated until the appropriate stage and then processed for skeletal staining or for whole amount in situ hybridization. Staging was according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Skeletal Staining

Wholemount Alcian Blue staining of the E9 chick limbs was done as previously described (Goff and Tabin, 1997). E16.5 and E18.5 mouse limbs were stained with Alcian blue and alizarin red as previously described (McLeod, 1980).

Wholemount In Situ Hybridizations

Wholemount in situ hybridizations were performed as previously described (Dietrich et al., 1997) with minor modifications. DIG-labeled probes were detected with NBT/BCIP (Sigma). Probes included *cPtc1* (Marigo et al., 1996b), *cPtc2* (Pearse et al., 2001), *cGli1* (Marigo et al., 1996a), *cGli2* (Marigo et al., 1996a), *cShh* (Riddle et al., 1993), *cBmp2*, *cFgf4* (Niswander et al., 1994), *mPtc1* (Goodrich et al., 1996), *mShh exon2* (Lewis et al., 2001), and *mFgf4* (Niswander et al., 1994).

Bead Implants

Affygel beads were soaked in 1.0 or 0.1 mg/mL recombinant but properly processed (i.e. cholesterol-modified) Shh protein (Curis) for 1 hour on ice. To look at the effects of exposure to Shh on *Gli1* expression, eggs were incubated to stage 24, windowed, and a Shh bead was implanted into the limb mesenchyme. The embryos were reincubated for 12 hours and then processed for whole mount in situ hybridization. Chick eggs were incubated to stage 20 (E3.5),

windowed, and a Shh bead was implanted into the limb mesenchyme. Embryos were reincubated to day 10 and processed for skeletal staining.

ß-galactosidase Detection

ß-galactosidase detection was performed as previously described (Harfe et al., 2004).

Quantitation of percentage of ß-galactose stained cells

To get a very rough estimate of the relative number of cells descended from Shh-expressing cells in wildtype versus Shh-deficient limb buds, ß-galactose staining was examined at E12.5. Serial sections across wildtype and mutant limb buds were stained and photographed (representative sections shown in Figure 5C, D). In each wildtype section the lacZ-positive domain, including the region entirely blue and the region salt-and-pepper, blue and white, was carefully cut out manually from the photograph, as was the completely white area of the image. These were then weighed and the values summed for all sections across the limb bud. From this it was determined that 27.67% of the tissue contained blue cells. A similar procedure was carried out with a sectioned Shh null limb, except that in this case only the solid blue tissue was cut from the rest of the limb, leaving the salt-and-pepper and white tissues together. This gave an estimation of 42.91% blue cells. The procedure we used, grouping salt-and-pepper with unmarked mutant tissue and with marked wildtype tissue, results in an over-estimate of marked wildtype cells and an under-estimate of marked mutant cells. None-the-less, there are clearly more marked cells in the mutant (42% vs. 27%). We used this procedure because, in the context of the current study, the exact difference does not matter (and indeed the exact percentages will change as the limb bud grows) but rather the point of the quantification was merely to verify the visual impression that there are relatively more marked cells in the mutant.

Mouse Breeding and Genotyping

Shhgfpcre/+;R26R males (Harfe et al., 2004; Soriano, 1999) were crossed to $Shh^{c/c}$ females (Lewis et al;., 2001) to generate $Shhgfpcre/Shh^{c}$ mice. *Prx1Cre* males (Logan et al., 2002) were crossed to $Shh^{c/c}$ females to generate *Prx1Cre;* $Shh^{c/c}$ mice. *Shhgfpcre/+;R26R* mice were mated together to generate *Shh* nulls, since the knock-in causes a null allele. Mice were genotyped by PCR and by phenotype.

Results

Signaling in the late posterior limb bud

The lack of expression of the Shh target gene *Gli1* in the posterior of the limb bud at later stages of patterning (Marigo et al., 1996a) and in particular the lack of late stage expression of this gene in the primordia of digit 5 in the mouse (Ahn and Joyner, 2004) would seem to stand in contradiction to any model requiring continued posterior Shh signaling. However, a number of other Shh-responsive genes in the limb bud, including *Bmp2*, *Ptc1*, and *Ptc2* continue to be expressed most strongly at the posterior margin at these same stages (Marigo et al., 1996b; Pearse et al., 2001). The difference in regulation between *Gli1* on the one hand and *Ptc1*, *Ptc2* and *Bmp2* on the other means that either *Gli1* is repressed in the posterior limb mesenchyme at late stages even in the presence of Shh signal transduction, or alternatively, *Ptc1*, *Ptc2* and *Bmp2* expression continues even when the posterior cells stop receiving and interpreting the Shh signal. To differentiate between these possibilities, we removed Shh activity late in limb development using a highly specific antagonist of Shh signal transduction; cyclopamine (Cooper et al., 1998; Incardona et al., 1998). We reasoned that if *Ptc1* and *Ptc2* expression in the posterior limb are dependent on Shh signaling in the late limb bud then they would be down-regulated by cyclopamine treatment, whereas if their continued expression were independent of Shh signaling, they would be maintained immediately following

cyclopamine treatment. As previously described, in chick limb buds at stage 21 (E3.5), the Shh-responsive genes *Ptc1* (Figure 2B) (Marigo et al., 1996b), *Ptc2* (Figure 2C) (Pearse et al., 2001), and *Gli1* (Figure 2D) (Marigo et al., 1996a) are all expressed in the posterior chick limb bud, around the domain of *Shh* expression (Figure 2A). By stage 24, *Ptc1* (Figure 2F) and *Ptc2* (Figure 2G) are still expressed in the posterior limb mesenchyme around the domain *Shh* expression (Figure 2E), but *Gli1* expression is restricted from the mesenchyme of the posterior margin (Figure 2H) (Marigo et al., 1996a), as in the mouse (Ahn and Joyner, 2004). We find that four hours after cyclopamine treatment both *Ptc1* (Figure 2J) and *Ptc2* (Figure 2K) are completely down-regulated in the chick limb mesenchyme, including the posterior mesenchyme. *Ptc2* expression in the apical ectodermal ridge (AER), which is not Shh-dependent (Pearse et al., 2001), remains (Figure 2K), showing that the down-regulation is specific. These results demonstrate that the posterior limb mesenchyme is still Shh-responsive at late stages of limb development and that *Ptc1* and *Ptc2* expression are better indicators of the presence of Shh signaling than *Gli1* expression.

The dynamic pattern of *Gli1* expression in the limb bud, while not a direct read-out for the presence of Shh signaling, none-the-less represents a complex response to Shh. Like *Ptc1* and *Ptc2* in the posterior limb, the expression of *Gli1* in the central-distal limb bud at these stages is dependent on continued Shh signaling, as *Gli1* is repressed throughout the limb bud by cyclopamine treatment (Figure 2L). Moreover, the down-regulation of *Gli1* in the posterior limb bud appears to be a consequence of prolonged or high levels of Shh signaling as, when we implant beads soaked in high (1 mg/mL) concentrations of Shh protein, we indeed see down-regulation of *Gli1* near the bead (Figure 2M). Taken together, these results provide strong evidence that there is continued Shh signaling in the posterior digit primordium at late stages of limb patterning, consistent with the temporal model for posterior digit specification.

Effect of reducing the time of Shh exposure in the limb bud

To specifically examine the role of time of exposure to Shh in digit patterning, we used cyclopamine to inhibit Shh signaling at different times in chick limb development. Since limbs receive wildtype levels of endogenous Shh until they are treated with cyclopamine, this experiment only alters the time (and not the concentration) of exposure. First we determined the speed and efficiency with which cyclopamine inhibits Shh signal transduction in the context of the limb bud. Within two hours of treatment at stage 22 (E4), *Ptc1* expression is down-regulated (Figure 3B) compared to wildtype (Figure 3A), and by four hours, it is undetectable, or in one case was barely detectable (Figure 3C). Other genes downstream of Shh respond similarly, if somewhat more slowly. *Gli1* (Figure 3D) and *Bmp2* (Figure 3F) (Laufer et al., 1994) are downregulated within eight hours when compared to wildtype (Figure 3E,G). *Fgf4* is also downregulated, as expected, although *Fgf8* is not affected to an appreciable extent (data not shown). Within eight hours of cyclopamine treatment, *Gli2* (Figure 3I) and *Gli3* (data not shown) are upregulated in the posterior of the limb, where they are normally downregulated by Shh (Figure 3H) (Schweitzer et al., 2000). Thus, cyclopamine acts quickly and thoroughly to inhibit Shh signaling in the limb. This effect is apparently prolonged since we see no evidence for reinitiation of target genes such as *Ptc1* at least 24 hours after cyclopamine treatment (data not shown), consistent with previous reports that cyclopamine treatment is effective for at least 24 hours in mouse limb buds (Panman et al. 2006).

With the parameters of cyclopamine action defined, we investigated the effects of cyclopamine treatment at different timepoints on the limb skeletal pattern. At each stage, there were different phenotypes of varying severity, probably due to variation in the uptake of cyclopamine by the embryos and a few hours difference in staging. A summary of skeletal malformations observed is presented in Table 1. Control limbs treated with the carrier 2-hydropropyl-ß-cyclodextrin (HBC) and examined at Stage 35 (E9) were morphologically normal (15/15) (Figure 4A-B).

We first treated embryos with cyclopamine at stage 18 (E3), when Shh is initially expressed in the limb. The most severely affected pairs of wings (2/7) were completely disorganized, lacked an ulna, had a single cartilage mass for the carpus, and "digits" consisting of two cartilage rods that appear to be a single phalanx of digits 2 and 3 emerging from a cartilage mass (Figure 4C). We view these as likely representing a single phalanx of aborted attempts to produce digits 2 and 3, an interpretation consistent with the less severely affected treated wings, which have well formed digits 2 and 3, but lack digit 4 (data not shown (Table 1)). The most affected pairs of legs (4/7) had lost the fibula and digit 4 (Figure 4D). Digits 1 - 3 each had two phalanges, meaning that digits 2 and 3 were truncated since they usually have three and four phalanges respectively. Both wings and legs were smaller than wildtype, probably due to the early loss of the Shh-Fgf feedback loop. The less severely affected limbs showed digit 4 loss in the wing (5/7) and digit 4 reduction in the hindlimb (3/7).

It is worth noting that there are distinct differences between the phenotypes observed following cyclopamine administration at stage 18 and the chicken *Ozd* mutant that never produces Shh in the limb (Ros et al., 2005). In particular, there are more digits in the cyclopamine-treated limbs, presumably because of the short exposure to Shh in these limbs prior to cyclopamine application. In addition, there is less complete division of the wrist elements into distinct condensations and the proximal bones that do form are shorter and thicker than normal. These latter defects are likely due to residual cyclopamine interfering with Indian hedeghog (Ihh) activity during cartilage condensation and growth, although growth defects are also observed following cyclopamine application at later stages. The changes in digit pattern in embryos treated at a later time are morphologically more straightforward to interpret.

Embryos treated with cyclopamine at stage 20 (E3.5) had less dramatic phenotypes than those treated at stage 18. In the wing, the most severe phenotype was a loss of digit 4 (10/14) (Figure 4E) while others had a reduced digit 4 (4/14). The most severe leg phenotype was the loss of digit 4 with a reduction of digit 3 to only three phalanges (Figure 4F) instead of its usual four (4/14). Both wings and legs from embryos treated at E3.5 were smaller than wildtype, but larger than those treated at E3. Milder leg phenotypes included the loss of digit 4 with normal digit 3 patterning (6/14) and the reduction and fusion of digits 3 and 4 into a single phalanx (4/14).

Embryos treated with cyclopamine at stage 22/23 (E4) still had slightly smaller limbs than untreated embryos, but had more normal patterning. The most affected wings had a reduction of digit 4 (10/11) (Figure 4G). One major class of leg phenotype was a digit 4 that was formed normally but was reduced to four phalanges instead of its normal five (4/17) (Figure 4H). Another major class of leg phenotype was the reduction and fusion of digits 3 and 4 into a single phalanx as seen in some embryos treated at E3.5 (6/17). Minor phenotypes included the loss of digit 4 with (1/17) or without (1/17) a digit 3 reduction to three phalanges in the leg. One wing (1/11) and five legs (5/17) were morphologically normal. Most limbs treated at stage 24 (E4.5) or later are also normal in appearance (9/10). These results suggest that reducing the time of exposure to Shh signaling causes defects in digit patterning with posterior digits requiring longer Shh signaling to be specified and properly formed.

Effect of reducing the concentration of Shh for the normal duration of Shh signaling

Previous studies showed that Shh regulates its own expression in the limb (San-Ezquerro and Tickle, 2000). If a Shh-soaked bead is added to the posterior chick limb, *Shh* expression decreases, while if *Shh*-expressing cells are removed, the remaining expression domain of *Shh* expands. Similarly, we have found that the *Shh* expression domain expands eight hours after cyclopamine treatment (Figure 5A) compared to wildtype limbs (Figure 5B). This is due to limb cells reading the loss of Shh signal transduction as reduced levels of Shh, and therefore upregulating Shh expression. This phenomenon occurs before the Shh-Fgf feedback loop breaks down. Consistent with this, when Shh signaling is eliminated in the mouse in *Shhgfpcre/*

Shhgfpcre homozygotes (embryos expressing *cre* in place of *Shh* from the *Shh* promoter), a greater percentage of limb cells are marked as descendents of *Shh*-expressing cells (using the *R26R* reporter) at E12.5 (Figure 5C) compared to phenotypically wildtype *Shhgfpcre*/+ heterozygote limbs (Figure 5D) (roughly 43% marked in the mutant as opposed to 28% in wildtype, see Methods), presumably due to limb cells sensing the loss of Shh and trying to express it.

We reasoned that if a conditional allele of *Shh* were knocked out in *Shh*-expressing cells by the Shhgfpcre, surrounding cells would sense the lower levels of Shh and respond by upregulating *Shh*. However, the *Shhgfpcre* allele would also be activated in those neighboring cells. Shhgfpcre would therefore, quickly knock out the conditional allele of *Shh* in these cells, but further cells would upregulate it, and so on. As a result, a continuous, but low, level of Shh would be made in the limb. $Shh^{conditionalgfpcre}$ embryos were therefore generated, and indeed, analysis at E10.5 indicates a spatially restricted domain of low-level *Shh* expression in the *Shhgfpcre* conditional knockout forelimbs (Figure 5F) when compared to wildtype forelimbs (Figure 5E). Low level *Shh* expression persists until E11.5 in *Shhgfpcre* forelimbs at the very proximal autopod boundary (Figure 5H). Shh pathway activity, as monitored by *Ptc1* expression, is similarly found to be reduced in E10.5 *Shhgfpcre* forelimbs (Figure 5J) compared to wildtype (Figure 5I), with extremely faint expression persisting until E11.5 in the mutant forelimb (Figure 5L).

For comparative purposes, we also wanted to create embryos in which Shh signaling would be reduced early, but not maintained at later stages. To accomplish this, we utilized a second cre-driver to remove Shh throughout the entire limb mesenchyme ($Prx1Cre;Shh^{conditional/conditional}$), in a similar manner to previous reports (Lewis et al., 2001). As *Prx1Cre* is expressed throughout the mesenchyme (Logan et al., 2002), Shh cannot autoregulate its own expression in adjacent cells as described earlier. Low levels of *Shh* and *Ptc1* expression are observed in E10.5 *Prx1Cre* conditional knockout forelimbs, however in contrast to the Shhgfpcre conditional embryos, all *Shh* expression is abolished by E11 in the *Prx1Cre* conditional knockout forelimbs (Supplementary Info).

These mutant backgrounds allowed us to study the effects of reduced levels of Shh on digit patterning. The assignment of digit identity is based on both overall digit length (digit 3 being longer than either digit 2 or 4) as well as the length of the primary ossification centre of E18.5 metacarpal elements. At this stage of development, metacarpal 3 exhibits the longest ossification centre and a sequential decrease in ossification centre length is observed in the order 3-4-2-5 (Patton and Kaufman, 1994). Decreased levels of Shh signaling for a shorter amount of time in the *Prx1Cre* conditional knockout forelimbs leads to the loss of 2 digits (Figure 6C), which, given the relative length of metacarpal ossification centers appears to be loss of digits 4 and 5, consistent with digit identities assigned in a previous analysis of this phenotype (Lewis et al., 2001). When even lower levels of Shh is maintained for a longer period of time in the *Shhgfpcre* conditional knockout forelimbs, we observe the loss of only one digit (Figure 6B). In mutant limbs, the anterior most digit exhibits digit 1 identity as confirmed by phalangeal number. The pattern of ossification strongly suggests that the second most anterior digit has the identity of digit 3, indicating it is digit 2 which is lost in *Shhgfpcre* forelimbs. While digits 1-3-4-5 all form (n=15/16); there is a reproducible loss of one phalanx from digit 5 (n=16/16) and partial phalangeal fusion between digits 3 and 4 in a small number of cases (n=3/16). The consistent loss of a phalanx from digit 5 in the *Shhgfpcre* mutant is important and indicates that more cumulative Shh signaling is required for complete digit 5 formation than is present in these embryos. It also suggests that digit 5 has a higher concentration threshold requirement than digit 4. To confirm the assignment of digit identities made on morphological grounds, we took advantage of the fact that Shh descendants mark digits 3, 4, and 5 (Harfe et al., 2004). When we fate map descendants of *Shh*-expressing cells in the *Shhgfpcre* conditional

knockout forelimb (Figure 6F), we find that they are in a remarkably similar domain to wildtype (Figure 6G). The descendants of *Shh*-expressing cells make up part of digit 3 and all of digits 4 and 5. Consistent with a reduction in Shh signaling, *Fgf4* expression is reduced and posteriorly restricted in the conditional mutants (Figure 6D-E). These results suggest that high levels of Shh signaling are not required for the posterior-most digits to form as long as Shh signaling is maintained for its normal duration in the posterior digit primordia.

Discussion

Our data support a model where cumulative exposure to Shh determines digit identity, but that it is differences in length of exposure (a temporal gradient) of autocrine Shh signaling that is critical for differential patterning of posterior digits (Figure 7A). According to this view, when we block Shh signaling in the chick limb bud at progressively later stages using cyclopamine, posterior digits fail to form because the limb mesenchyme has not been exposed to Shh for a long enough period of time. A similar result is achieved when Shh activity is removed in the mouse at an early limb bud stage (Figure 7B). In contrast, when we decrease the level of Shh signaling, but allow it to proceed for the normal duration during limb development, digit 2 is missing while the posterior digits are present. This suggests that decreased levels of Shh are insufficient to induce digit 2 during the early time window when that digit is normally specified. In contrast, the long period of time when Shh is produced is sufficient, even at decreased levels, to specify the posterior digits (although the decrease in Shh levels does result in a missing phalanx from digit 5 (see below, (Figure 7C)).

It is important to note that other interpretations can fit the data presented here. For example, rather than the length of exposure to Shh being critical for specifying posterior digits, it is formally possible that posterior digit specification could be based on the precise time during development that the signal is received, with anterior digits forming due to an early signal and posterior ones forming due to a late signal. According to this hypothesis, blocking Shh signaling early will result in a failure to form posterior digits, while a reduction in Shh levels will have no effect as long as exposure to the signal is long enough. If our experiments were viewed in isolation, one would have to take such alternative interpretations very seriously. However, the model espoused here becomes more compelling when taken in the context of previous studies showing that time of exposure is critical in the formation of ectopic digits in the chick (Yang et al., 1977; Harfe et al., 2004), and suggesting a “promotion” mechanism where digit primordia are first specified to an anterior and subsequently more posterior identity (Yang et al., 1977).

Prolonged active Shh signaling in the posterior limb bud

The temporal gradient model for posterior digit specification requires that Shh continue to act on the posterior limb mesenchyme throughout the patterning phase of the early limb bud. Indeed, we have shown that the posterior limb continues to actively respond to Shh signaling in later limb development, since *Ptc1* expression is down-regulated in the posterior most mesenchyme at stage 24 after cyclopamine treatment, even in areas where *Gli1* is not expressed. A corollary of these results is that Gli1 does not provide an accurate representation of the domain of active Shh signaling in the limb bud at later stages. A potential mechanism by which prolonged exposure to Shh could lead to *Gli1* repression while, at the same time, continuing to induce the expression of other targets, is suggested by consideration of the transcription factors mediating Shh signaling. Gli3 appears to be the only transcription factor mediating Shh signal transduction in the limb bud (Litingtung et al., 2002; te Welscher et al., 2002). Gli3 exists in both transcriptional activator and repressor forms (Litingtung et al., 2002; Van Mering and Barker, 1999; Dai et al., 1999; Aza-Blanc et al., 2000; Bai et al., 2004; Tyurina et al., 2005; Reviewed in Ingham and McMahon, 2001). In the absence of Shh, Gli3 exists as a processed repressor (Litingtung et al., 2002). Shh acts both to stabilize Gli3 protein converting

it to an activator form (Litingtung et al., 2002) and also leads to the transcriptional down-regulation of Gli3 (Schweitzer et al., 2000). Together these produce a very sharp gradient of Gli3-repressor form from the anterior to the posterior of the limb bud (Wang et al., 2000). However the transcriptional down-regulation of *Gli3* also results in a decrease in Gli3 activator in the posterior limb bud. The expression patterns of *Ptc1* and *Ptc2* are consistent with their being activated purely by derepression in the absence of Gli3 repressor. In contrast, the lack of *Gli1* expression in the late posterior limb bud could be explained if its expression required threshold levels of Gli3 activator. Thus *Gli1* expression is lost either in the absence of Shh signaling (e.g. cyclopamine treatment) where no Gli3 activator is produced, or in the presence of very high levels of Shh, where Gli3 is down-regulated transcriptionally and very low levels of all Gli3 isoforms are present. In contrast, *Ptc1* and *Ptc2* are expressed under conditions of very high Shh signaling as both lack of processing of Gli3 and transcriptional down-regulation of Gli3 result in an absence of Gli3 repressor.

The effect of truncating Shh signaling during limb development

The above experiments showed that susceptibility to Shh signaling occurs over a longer period of time than previously suggested. Using cyclopamine in the chick limb, we have investigated the effects of reducing only the time of Shh signaling and shown that the less time a limb is exposed to Shh, the more posterior digits are lost or anteriorized. In the most affected legs treated at E3, only digit 1 is present, albeit malformed, as one would expect since it is Shh-independent in both the mouse Shh knockout (Chiang et al., 2001; Kraus et al., 2001) and the hindlimbs of chick oligozeugodactyly mutants which specifically lack Shh expression in the limb bud (Ros et al., 2003). In many E3.5 treated embryos, both digits 1 and 2 are normally patterned in the most affected specimens, but digit 3 is transformed into the digit 2 phalangeal formula. Finally at E4, digits 1 - 3 are all patterned normally, but digit 4 is transformed into a digit 3 phalangeal formula. This demonstrates that posterior digits require a longer exposure to Shh for correct patterning. The loss of posterior digits could in principle be ascribed to truncation of the Shh-Fgf feedback loop and loss of AER maintenance, which leads to smaller overall limb sizes and less mesenchyme in the autopod (Khokha et al., 2003; Michos et al., 2004), since reductions in limb mesenchyme lead to digit loss (Alberch and Gale, 1983), but the cases of anteriorization of digits 3 and 4 in embryos treated at E3.5 and E4 must be due to changes in the patterning of digit identity. Prolonged exposure to Shh is thus necessary to pattern skeletal elements, not just to ensure digit number. These examples, where reduced time of Shh exposure leads to formation of posterior digits, but with a more anterior character than in wildtype, also provide additional support for the promotion model (Tickle, 1995), where digits are first specified to have an anterior pattern and then are “promoted” to sequentially more posterior fates. Earlier data in support of this model came from experiments varying the length of time of exposure to ectopic Shh (Yang et al. 1997).

The effect of decreasing the level, but not time, of Shh exposure in the limb bud

The result of conditionally removing *Shh* only within the *Shh*-expressing cells, yielding continued low levels of Shh signaling in the mouse limb buds, demonstrate that the length of time of Shh signaling is more important than the absolute concentration of Shh in the limb for posterior digit patterning. It had been previously reported that removal of Shh-expressing cells from the posterior limb bud results in an activation of Shh gene activity in neighboring cells (Sanz-Ezquerro and Tickle 2000). We similarly observed an expansion of the Shh-expressing domain, following a loss of Shh signaling as a result of cyclopamine treatment, especially in the proximal half of the domain (Compare Figures 5A and B). The compensation seen in response to surgical removal of Shh-expressing cells was similarly biased proximally (Sanz-Ezquerro and Tickle 2000). This may reflect the shape of an underlying zone of competence for Shh-expression within the limb bud. Building on these observations, we designed a genetic situation where Shh would be continually activated and inactivated in ever-more peripheral

cells within this zone of Shh competence, by combining floxed Shh conditional allele within an allele where cre-recombinase is driven from the Shh promoter. Indeed, high levels of *Shh* or Shh target gene expression are never observed in these limbs, yet low levels are continually produced throughout the normal timeframe of Shh expression. As before, the activation of ectopic *Shh* was strongly biased towards the proximal extent of the normal ZPA.

The limbs developing under this condition of reduced levels of exposure exhibited a digit pattern 1-3-4-5. A distal phalanx is consistently missing from digit 5 in these limbs, indicating that the severely decreased level of Shh signaling prevents complete posterior patterning. However, it is striking that the posterior digits are retained, in contrast to the 1-2-3 digit pattern observed, when Shh activity is abolished at an early stage in animals carrying the *Prx1-cre* allele. Thus, greatly reduced levels of Shh signaling, relative to that found in wildtype limb buds, are sufficient for posterior digit patterning in the context of the normal length of time of exposure to Shh.

Assignment of digit identities

The interpretation of our experiments depends upon our having made a correct determination of digit identity, that indeed digit 2 is the digit that is lost and the three most posterior digits remain. Examining the skeletal phenotypes, including the articulation with the wrist elements, there does not appear to be any ambiguity regarding digit 5. Hence, we can have confidence in what is perhaps the most critical observation from these experiments that a nearly complete posterior-most digit forms in the absence of high levels of Shh signaling if the reduced levels are prolonged. Perhaps more open to question is whether it is digit 2 or digit 3, which is lost. Besides morphological considerations, we base our interpretation on fate mapping experiments which show that former Shh-expressing, lacZ-positive cells account for all of digit 5 and 4, and contribute significantly to digit 3, as in wildtype limbs. This contrasts with a recent analysis of a different mutant (in which the Fgfr1 gene is conditionally inactivated in the mouse limb), which has a digit pattern 1-2-4-5. In this mutant, the digit pattern was first assessed morphologically. (For example, the largest digit in these limbs is digit 4, rather than digit 3 as in the limbs examined here.) Significantly, when the former ZPA cells were fate mapped in these limbs, the lacZ staining encompassed only the most posterior two digits (Verheyden et al., 2005; compare their Figure 7 to our Figure 6). Thus the fate map of former Shh-expressing cells indeed provides a reflection of the identity of the posterior digits; substantiating our interpretation that indeed the three most posterior digits are formed in limbs analyzed here, expressing greatly reduced levels of Shh for the normal duration. Surprisingly, however, digit 2 is lost in the Shhgfpcre conditional forelimbs. The explanation for this may relate to the fact that digit 2 is the only digit that absolutely requires diffusible Shh (Harfe et al., 2004). It might actually require production of more Shh protein to achieve low level paracrine signaling after diffusion, than is required for achieving significant levels of Shh by more efficient autocrine signaling. The progenitor cells fated to give rise to the posterior three digits still produce Shh (albeit at low levels) and respond to it by an autocrine mechanism for an approximately normal duration and hence are patterned as digits 3-4-5, and so it is digit 2 that is lost.

Conclusions

Despite the insights our experiments provide in understanding how both concentration and time of Shh exposure instructs morphological output, remarkably little is known as to how this information is integrated at a molecular level. Novel insight has recently been provided through detailed temporal analysis of multiple Shh downstream target genes (Panman et al., 2006). Indeed, Shh was shown to not only initiate target gene expression with differential kinetics, but the temporal dependence on Shh is strikingly different for various target genes. In particular,

5′ Hoxd genes exhibit differential temporal dependence on Shh relative to their position within the cluster, *Hoxd13* requiring Shh for the longest amount of time (Panman et al, 2006).

Prior to the identification of Shh as the key signal establishing posterior digit identities in the limb, it was demonstrated that the Zone of Polarizing Activity (now redefined as the set of cells in the limb expressing Shh) acts in an dose-dependent manner to establish different digit identities (Tickle, 1981). It is now apparent that dose of polarizing Shh activity is integrated on the basis of both concentration and time of exposure. The experiments presented here indicate that for the patterning of the posterior digits, the time of exposure is the more significant parameter.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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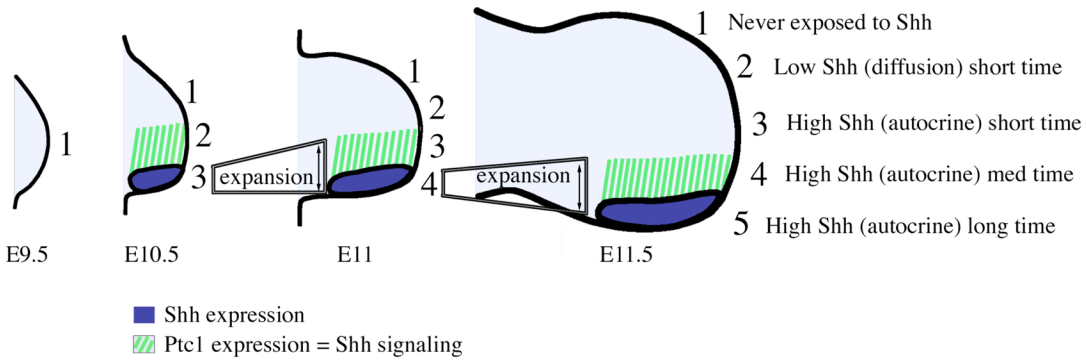

Figure 1.

Proposed model of digit specification. The proposed model is presented here in the context of mouse limb bud development. Digit 1 arises independent of Shh signaling while digit 2 is specified by early diffusion of Shh protein from the posterior margin. Cells fated to give rise to digits 3, 4 and 5 all receive high level autocrine Shh signaling, and differential specification is primarily dependent on the length of time cells are exposed to high level Shh signaling before proliferation/posterior mesenchymal expansion forces cells outside of the zone competent to express Shh.

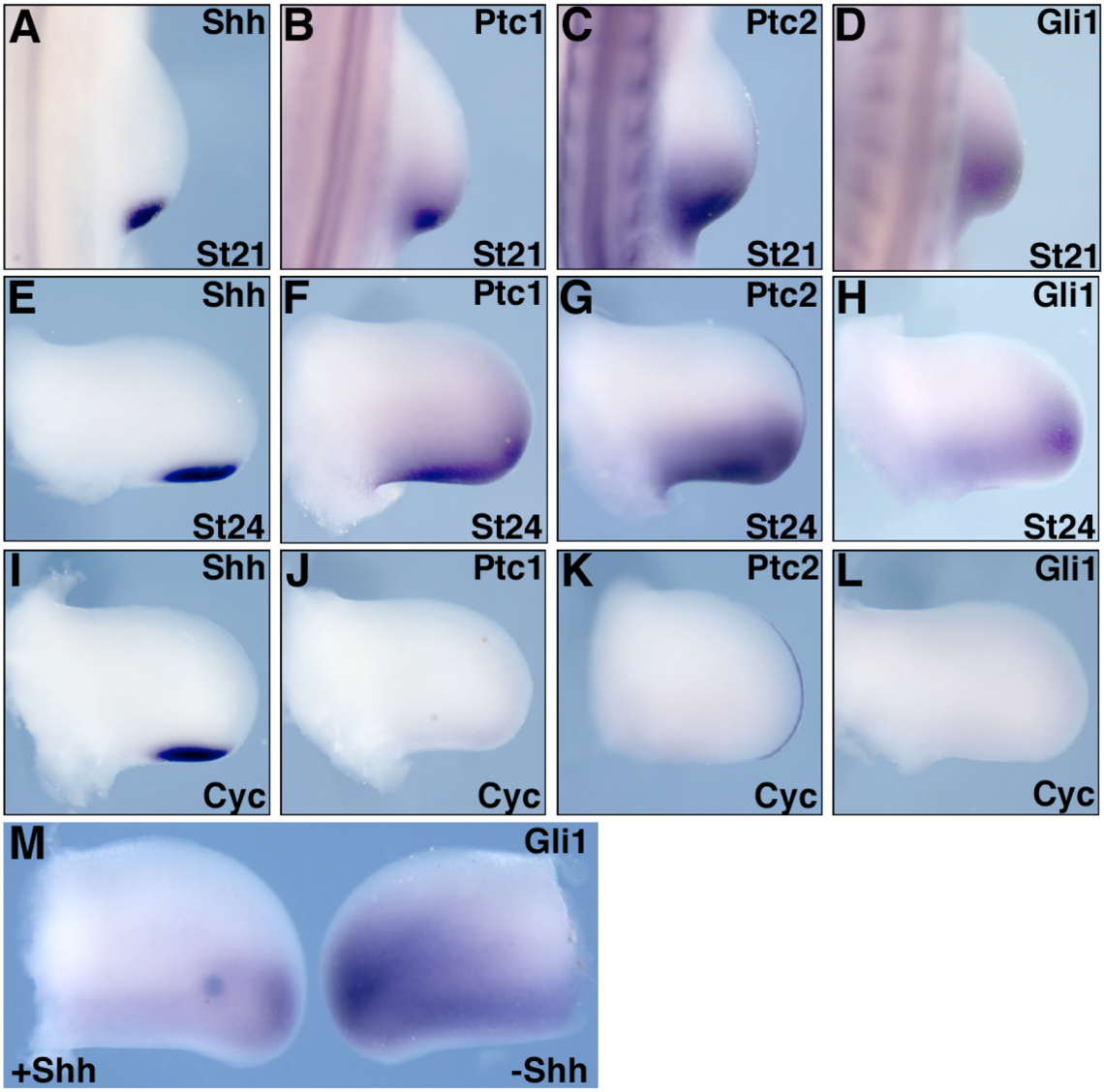

Figure 2.

The posterior forelimb continues to respond to Shh signaling at late stages. *Shh*, *Ptc1*, and *Ptc2* are all expressed in the posterior limb at stage 21 (A,B,C) and stage 24 (E,F,G). *Gli1*, though expressed in the very posterior limb at stage 21 (D), is downregulated in the posterior at stage 24 (H). This tissue is still sensitive to Shh signaling at late stages as shown by the downregulation of *Ptc1* and *Ptc2* 12 hours after cyclopamine treatment (J,K). *Gli1* is also downregulated (L). *Shh* continues to be expressed (I) as does the AER domain of *Ptc2* expression (K), showing that the effect is not nonspecific. Extended exposure to a Shh bead downregulates *Gli1* (M).

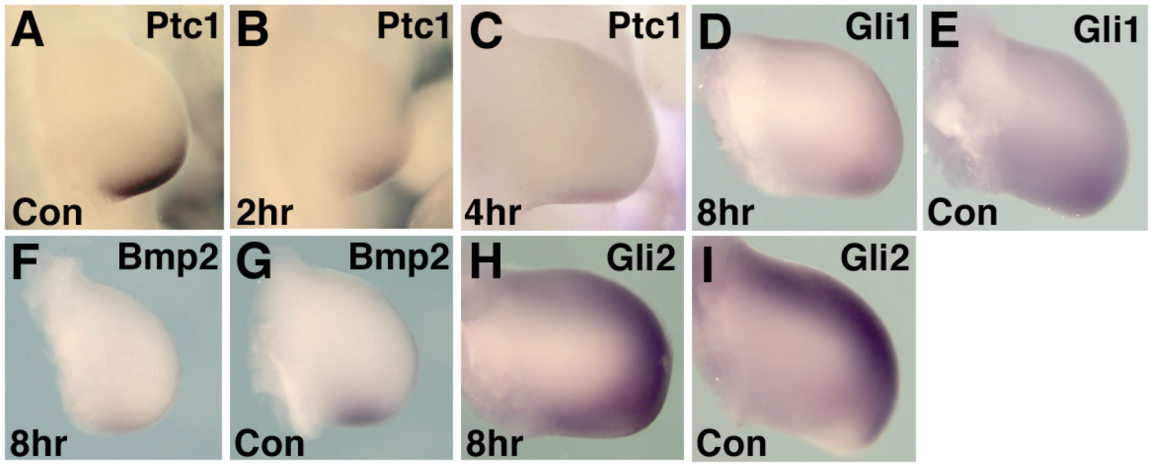

Figure 3.

Cyclopamine treatment leads to downregulation of Shh targets in the forelimb. Within two hours, *Ptc1* is downregulated in treated forelimbs (B) when compared to control forelimbs (A), and its expression ceases within four hours of cyclopamine treatment (C). *Gli1* and *Bmp2* are downregulated within eight hours (D,F) compared to control forelimbs (E,G). On the other hand, *Gli2* expression expands into the posterior forelimb within eight hours of treatment (H) when compared to controls (I).

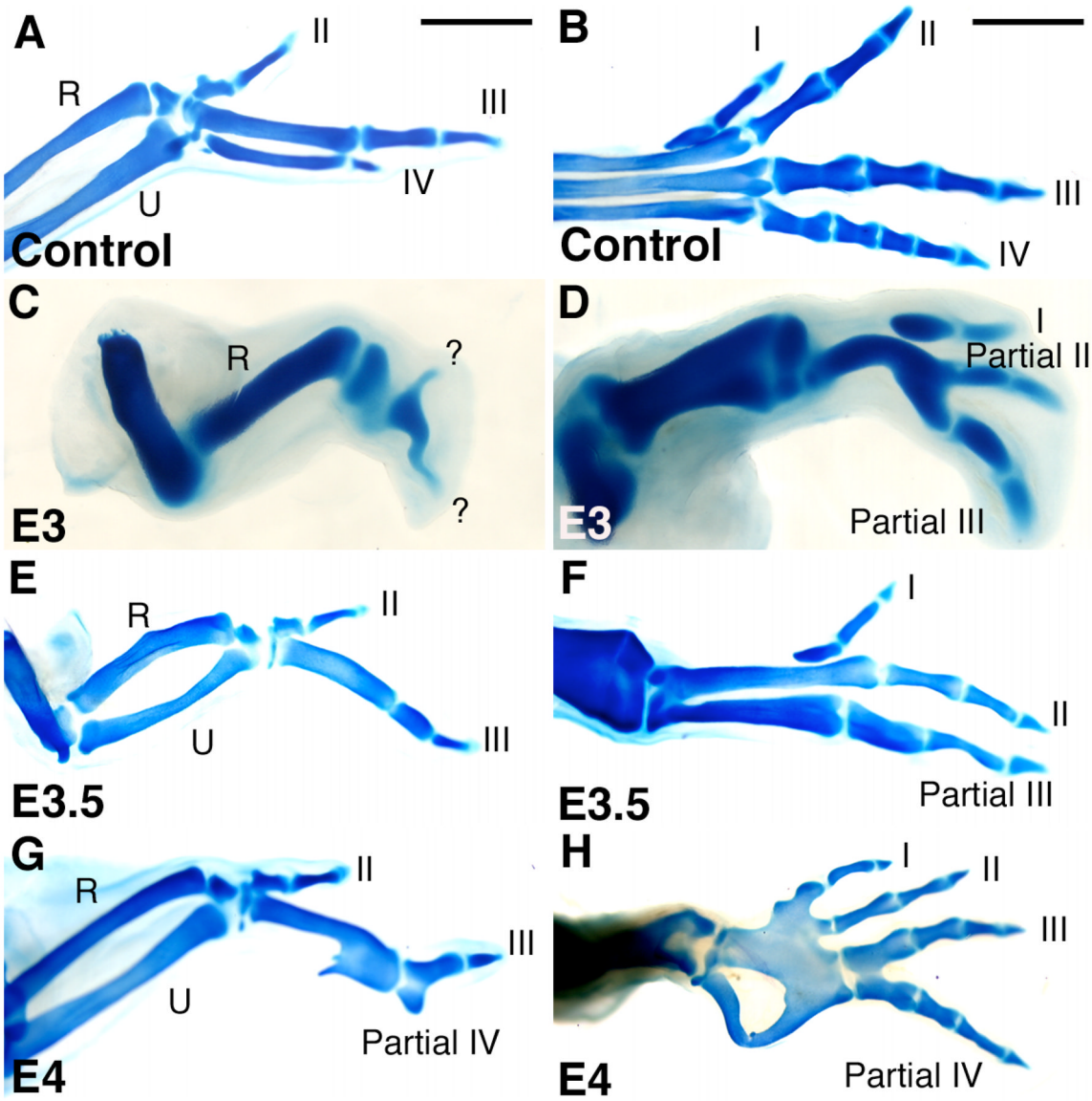

Figure 4.

Shorter exposure to Shh signaling causes the loss or anteriorization of posterior skeletal elements. Digit identities were determined by position and counting phalanges. Control forelimbs (A) and hindlimbs (B) treated with carrier are morphologically indistinguishable from wildtype. Treating with cyclopamine at E3 frequently leads to the loss of the ulna and the reduction of the autopod to carpus and two digit-like elements in the forelimb (C), and the loss of the fibula and digit 4 in the hindlimb (D). Digits 1 - 3 have only two phalanges each, meaning that digits 2 and 3 are anteriorized (D). Treating with cyclopamine at E3.5 frequently leads to the loss of digit 4 in the forelimb and hindlimb (E,F), as well as the reduction of digit 3 in the hindlimb to three phalanges (F). Treating with cyclopamine at E4 leads to a partial digit 4 in the forelimb (G), and to a hindlimb digit 4 with only four phalanges (H). All images were acquired at the same magnification and cropped to 1400×700 pixels. Scale bar represent 1 mm, and is approximated from an image of an identically staged limb taken under equivalent magnification.

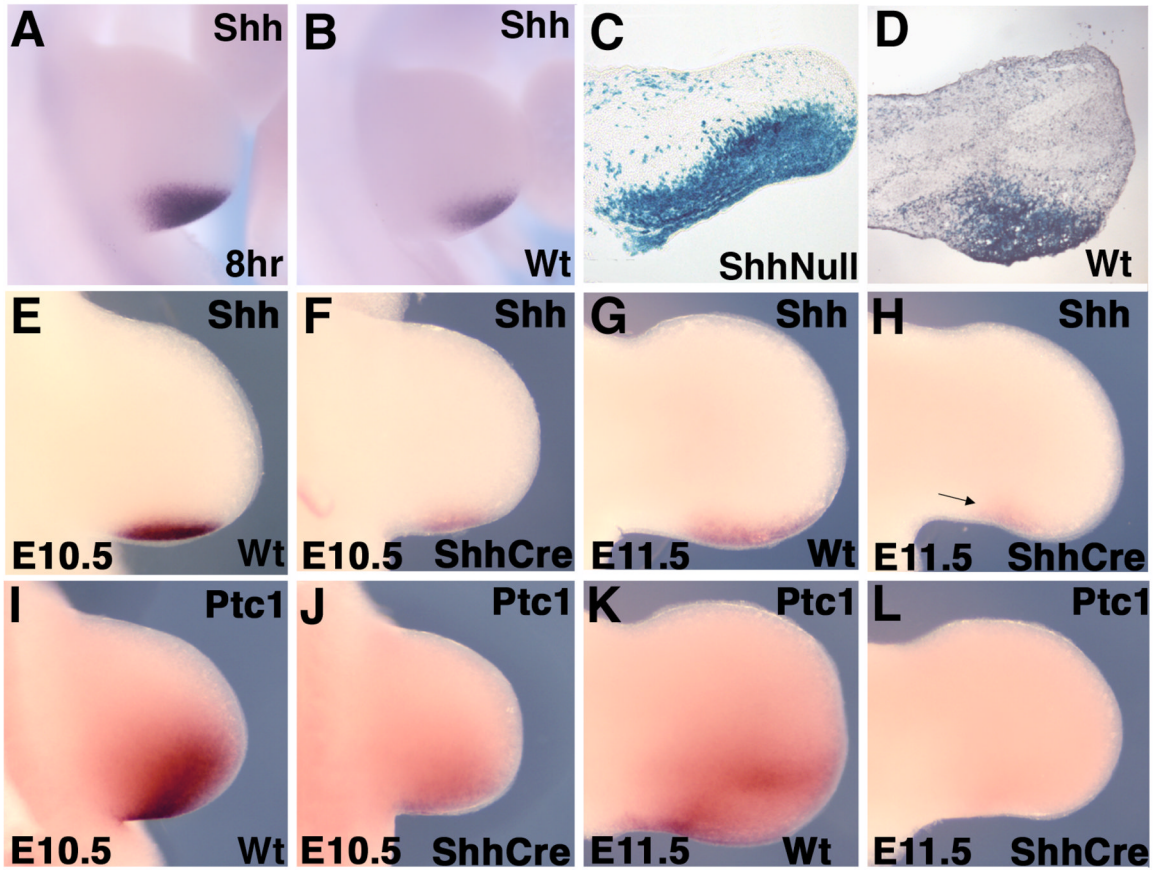

Figure 5.

A conditional knockout of *Shh* by the *Shhgfpcre* causes a continuous low level of Shh signaling throughout limb development. *Shh* expression expands after eight hours of cyclopamine treatment (A) compared to control forelimbs (B) due to Shh autoregulation. Likewise, a greater amount of the forelimb mesenchyme has attempted to upregulate *Shh* at E12.5 in *Shh* nulls (C) than in wildtype (D). At E10.5, *Shhgfpcre/Shh*^{c} forelimbs (F) have reduced levels of *Shh*, compared to wildtype forelimbs (E). At E11.5 *Shh* is still expressed, albeit at low levels (arrowhead), in *Shhgfpcre/Shh*^{c} forelimbs (H) in a domain proximal and anterior to the *Shh* domain in wildtype forelimbs (G). Shh signaling, as indicated by *Ptc1* expression, is significantly reduced in E10.5 *Shhgfpcre/Shh*^{c} forelimbs (J) compared to wildtype forelimbs (I). At E11.5, only extremely faint *Ptc* expression can be observed in E11.5 *Shhgfpcre/Shh*^{c} forelimbs (K) compared to wildtype forelimbs (L).

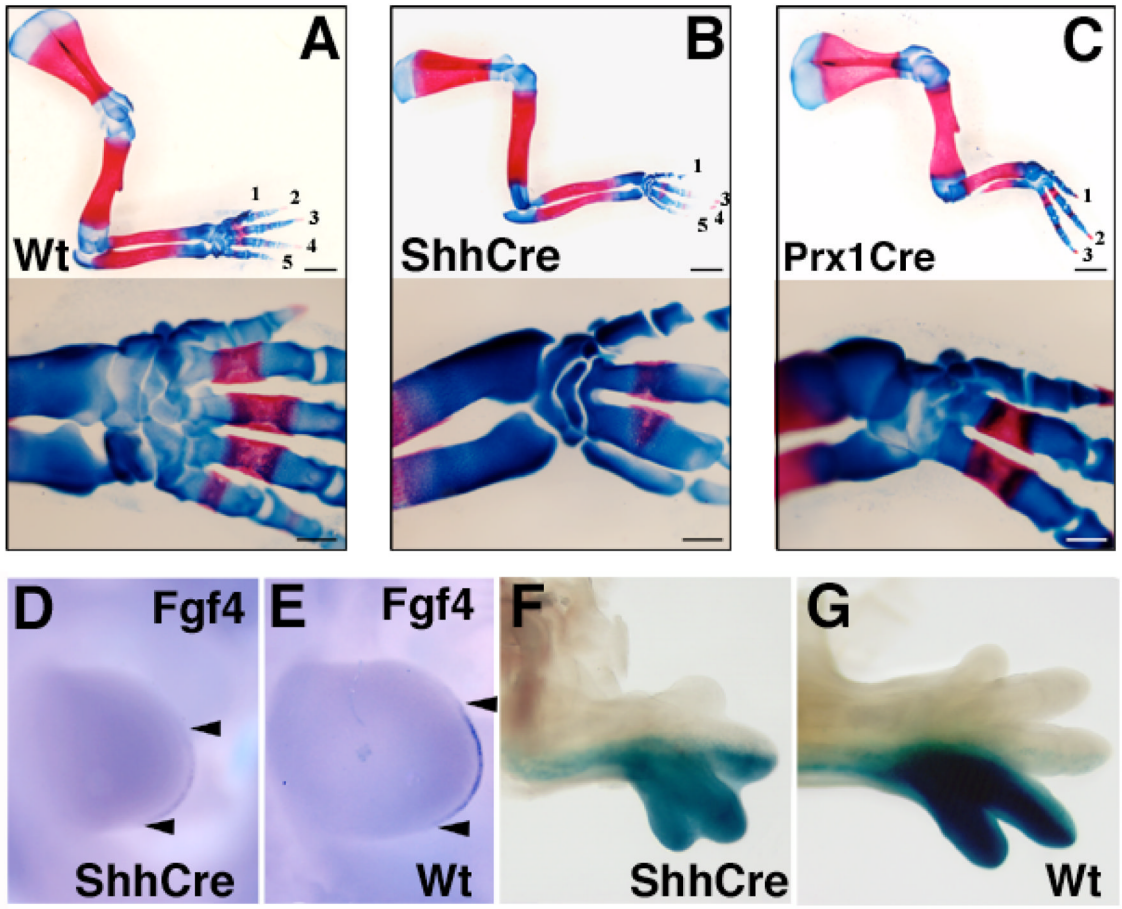

Figure 6.

Extended exposure to low levels of Shh leads to posterior digit specification in *Shhgfpcre/Shh^{c}* mice. A-C upper panel whole limbs (scale bar represents 1 mm); A-C lower panel (scale bar represents 250 μm) allows analysis of carpal/metacarpal element morphology. Whereas E18.5 wildtype forelimbs have all five digits (A), *Shhgfpcre/Shh^{c}* mice only have digits 1-3-4-5 (B), and *Prx1Cre;Shh$^{c/c}$* mice only have digits 1-2-3 (C). *Fgf4* expression becomes posteriorly restricted in *Shhgfpcre/Shh^{c}* forelimbs (D) when compared to wildtype forelimbs (E). The Shh descendents still make up digits 4 and 5, and part of digit 3 in the *Shhgfpcre/Shh^{c}* mice (F) as in wildtype mice (G).

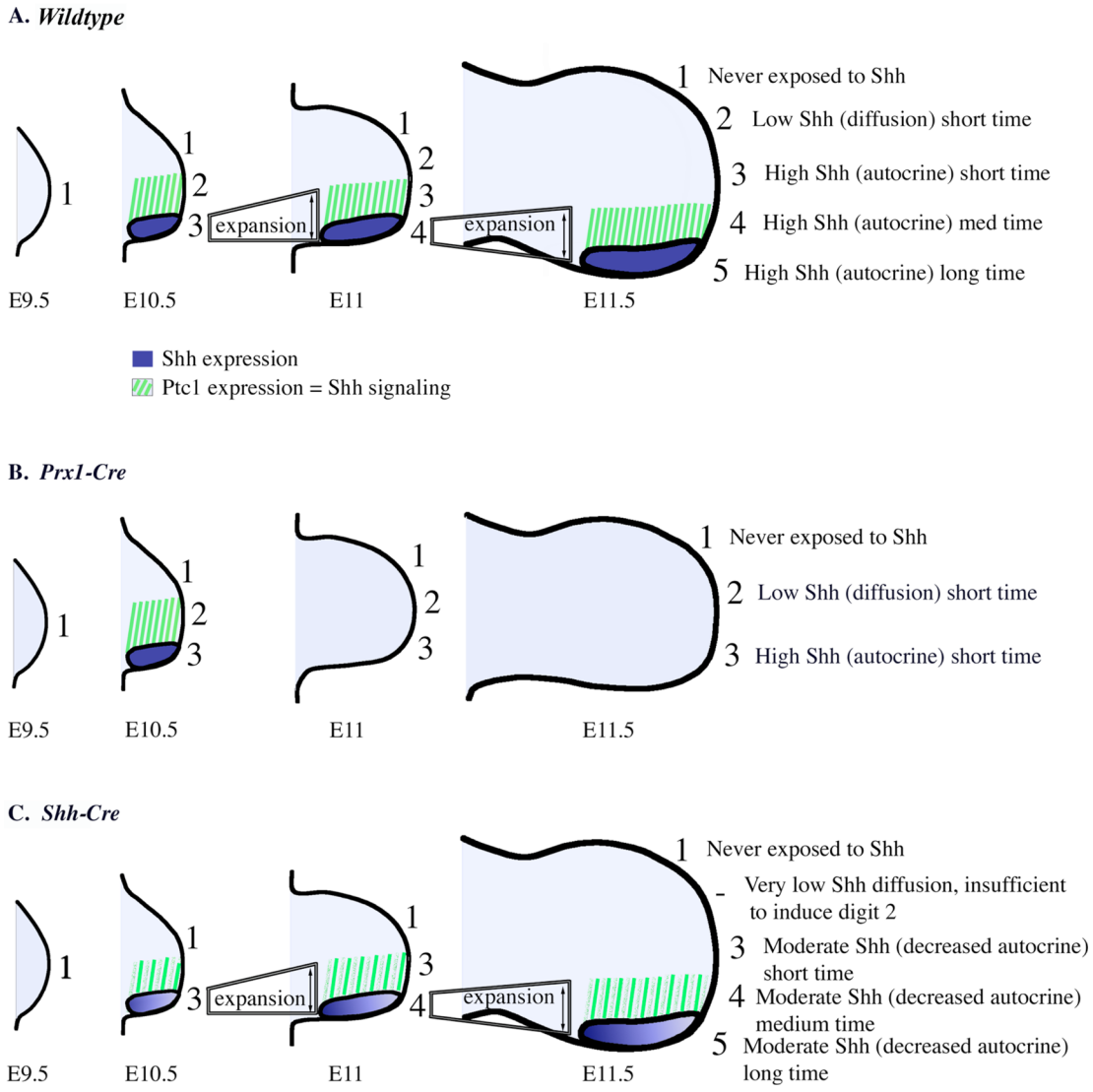

Figure 7.

Proposed model of digit specification incorporating mouse mutant analysis. Digit specification in wildtype (A) mouse limb buds as previously detailed (see Figure 1). In *Prx1-Cre* mutant limb buds (B), early diffusion of Shh allows specification of digit 2 while in the posterior, only digit 3 can be specified before Cre activity abolishes all Shh signaling by E11.0. In contrast, continuous but low level Shh signaling observed in *Shh-Cre* limb buds (C) is not sufficient to specify digit 2; Posterior digits are however specified with time despite significant reduction of Shh levels. Together, mouse mutant analysis supports the proposed model of digit specification whereby digit 2 is most sensitive to the concentration of Shh while for posterior digit specification, the length of Shh exposure is the more critical parameter.

Table 1

Spectrum of limb malformations observed following treatment of chick limb buds with cyclopamine

The chick wing has 3 digits, denoted digits II-III-IV from anterior to posterior, while the chick leg has 4 digits, denoted digits I-II-III-IV. Cyclopamine was added at a range of embryonic stages as indicated. The number of embryos exhibiting a given phenotype is represented relative to the total number of embryos treated at the same stage.

Stage treatment	Number affected	Phenotype
Wing (II-III-IV)		
S18	2/7	Severely disorganized elements. Shortened humerus, loss of ulna, single fused carpus, 2 digits each with a single phalange
	5/7	Digit IV loss
S20	10/14	Digit IV loss
	4/14	Digit IV reduction
S22/23	10/11	Digit IV reduction
	1/11	Morphologically normal digits
S24	9/10	Morphologically normal digits
Leg (I-II-III-IV)		
S18	4/7	Loss of fibular and digit IV loss, digits I-II-III each have 2 phalanges.
	3/7	Digit IV reduction
S20	4/14	Digit IV loss, digit III reduction
	6/14	Digit IV loss
	4/14	Reduction and fusion of digits III and IV into a single phalange
S22/23	4/17	Digit IV reduction
	6/17	Reduction and fusion of digits III and IV into a single phalange
	1/17	Digit IV loss with digit III reduction
	1/17	Digit IV loss
	5/17	Morphologically normal digits