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Shh signaling influences the phenotype of *Pitx1*-/- hindlimbs

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ABSTRACT

Forelimbs (FLs) and hindlimbs (HLs) develop under the instructive and integrated guidance of signaling centers and transcription factor (TF) action. The development of structures specific to each limb type depends on the limb-specific modulation of these integrated components. *Pitx1* is a transcription factor gene expressed in HL, absent in FL, and required for HL-specific patterning and development, in particular for formation of anterior HL skeletal elements. *Pitx1* achieves this function by direct TF action on the core limb program, which is largely shared between FL and HL. Shh signaling plays a crucial role in anterior-posterior (AP) patterning in both FL and HL. The present work assessed the relationship between Shh signaling and Pitx1 action for AP patterning. We found that reducing the gene dosage of *Shh* in the context of the *Pitx1*-/- HL decreases the severity of the *Pitx1*-/- phenotype, in particular, the loss of anterior limb structures and the shortening of femur length. However, this did not rescue HL-specific patterning features. Thus, Pitx1 action integrates Shh signaling but not for limb-type-specific patterning.

1. Introduction

Forelimbs (FLs) and hindlimbs (HLs) share an interrelated evolutionary history and a conserved anatomical organization despite the widespread and divergent forms these limbs take in different species. The components of the FL and HL developmental programs that manifest the differences between the limbs, and how these components are interconnected with the mechanisms controlling anterioposterior (AP) polarity in both limbs, is a central question in limb development.

Pitx1 is a transcription factor expressed exclusively in HL and absent from FL (Lanctôt et al., 1997). The *Pitx1 gene* is necessary for development of HL, especially the anterior features of HL such as the ilium, patella, and related knee structures (Lanctôt et al., 1999; Szeto et al., 1999). Some, but not all, *Pitx1*-directed HL patterning occurs through Pitx1 regulation of *Tbx4* expression: rescuing *Tbx4* expression in the *Pitx1*-/- HL restores the length of the femur and certain HL muscle pattern characteristics, but major HL pattern characteristics are not restored (Ouimette et al., 2010). Genomic studies of Pitx1 in the context of FL vs. HL development showed that Pitx1 broadly modulates the core limb development program, which is to say Pitx1 influences HL development by its direct action on a limb program that is largely conserved between FL and HL (Nemec et al., 2017).

Further, *Pitx1* expression in FL confers HL-like anatomical features to FL (DeLaurier et al., 2006; Spielmann et al., 2012), and this ectopic expression results in downregulation of posterior signaling centers, as indicated by decreased expression of *Shh* (DeLaurier et al., 2006). *Shh* is the gene responsible for patterning influence of the zone of polarizing activity (ZPA) (Riddle et al., 1993) and *Shh* expression in both FL and HL is necessary for AP patterning: loss of *Shh* signaling leads to truncation of the AP-axis of the limb, with especially drastic phenotypes in the distal limb (Chiang et al., 2001). These defects can be traced to the prevalence of Gli3 activity in the anterior limb bud mesenchyme and in absence of *Shh*, as the two genes have an antagonistic relationship with respect to AP polarity (Litingtung et al., 2002; Wang et al., 2000).

The *Pitx1*-/- HLs lose anterior identity mesenchyme (Marcil et al., 2003) and a recent study shows that *Gli3* mRNA and other anterior markers are decreased in *Pitx1*-/- HL (Nemec et al., 2017), suggesting that increased posteriorization signal from the ZPA may be responsible for the loss of anterior features in the *Pitx1*-/- HL. Interestingly, loss of *Irx3* and *Irx5* also leads to developmental defects in anterior HL (but not in FL despite the common expression of these genes in both limbs); *Irx3*-/-;*Irx5*-/- HL lack both the tibia and digit one (Li et al., 2014). Loss of *Irx3/5* expression leads to an expansion of the anterior territory of *Shh* expression; decreasing *Shh* gene dosage then leads to a normal, restored HL in *Irx3*-/-;*Irx5*-/-;*Shh*+/- mice (Li et al., 2014).

Hence, we hypothesized that the anterior mesenchyme of *Pitx1*-/- HL buds may also be posteriorized and that reducing *Shh* gene dosage might reverse this and attenuate the phenotype (Fig. 1A). Indeed, we found that lowering the dose of *Shh* reduces the severity of the *Pitx1*-/-

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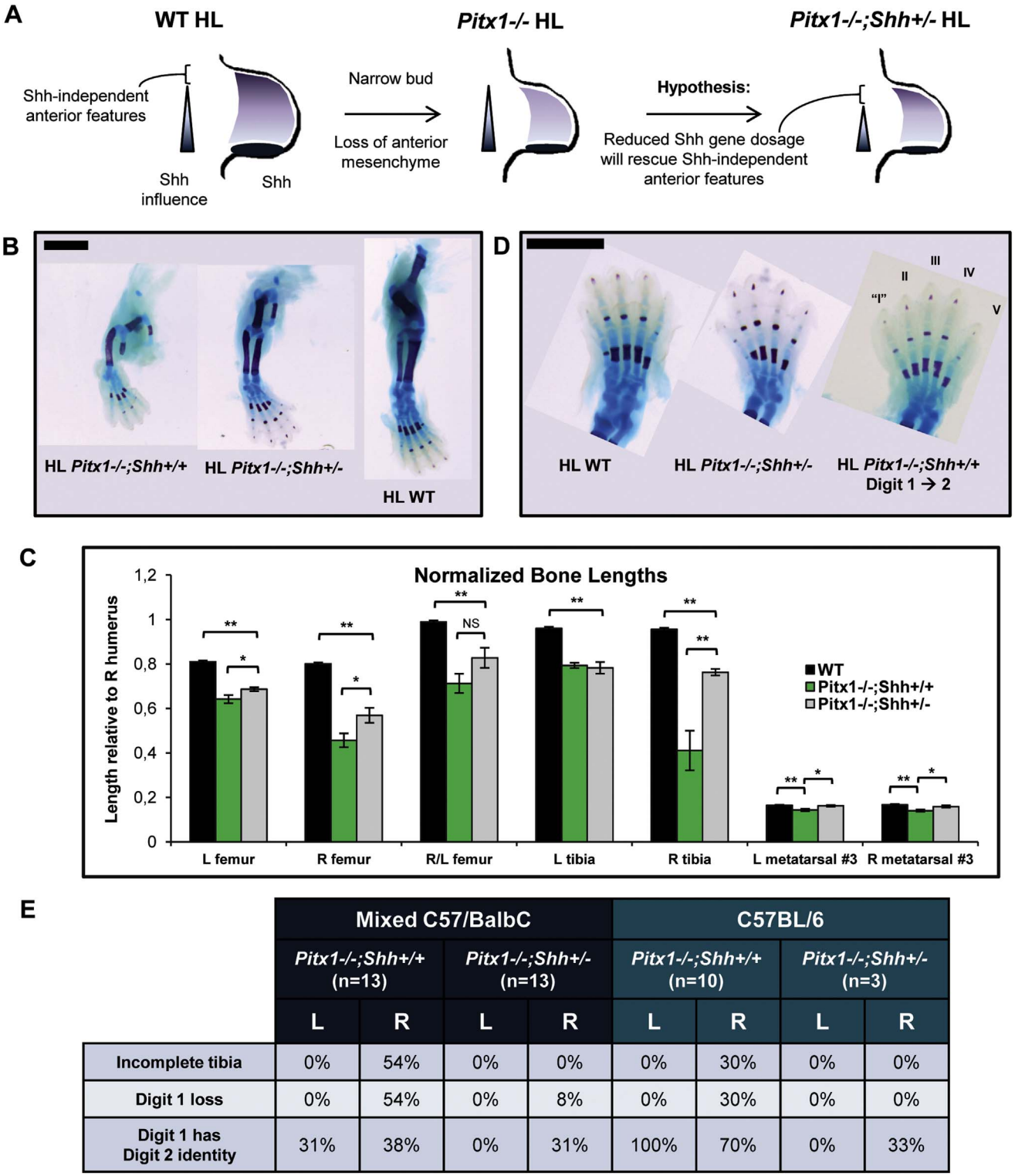

E

	Mixed C57/BalbC				C57BL/6			
	Pitx1-/-;Shh+/+ (n=13)		*Pitx1-/-;Shh+/-* (n=13)		*Pitx1-/-;Shh+/+* (n=10)		*Pitx1-/-;Shh+/-* (n=3)	
	L	R	L	R	L	R	L	R
Incomplete tibia	0%	54%	0%	0%	0%	30%	0%	0%
Digit 1 loss	0%	54%	0%	8%	0%	30%	0%	0%
Digit 1 has Digit 2 identity	31%	38%	0%	31%	100%	70%	0%	33%

Fig. 1. Partial rescue of *Pitx1-/-* hindlimb phenotype by reduced *Shh* gene dosage. (A) Model for loss of anterior features in *Pitx1 -/-* HL; these are hypothesized to derive from Shh-independent bud mesenchyme. Deletion of one *Shh* allele should decrease Shh signaling from ZPA and may lead to restoration of Shh-independent anterior mesenchyme and rescue of *Pitx1-/-* features. (B) Representative alizarin red and alcian blue bone and cartilage staining of *Pitx1-/-;Shh+/+*, *Pitx1-/-;Shh+/-*, and WT HL in mixed BALBc/C57 background. All fetuses are E17.5. Scale bar is 2 mm. (C) HL bone measurements (n = 13 for each group) normalized to the length of the right humerus for each skeletal preparation in order to control for fetus size. The length ratios of right/left femurs were then calculated for each fetus. All ** indicate *p*-value less than 0.001, while * indicates there is still significance at an α-level of 0.05 after correcting for multiple comparisons. NS indicates no significance. (D) Right *Pitx1-/-;Shh+/+ HL* autopods showing digit 1 to digit 2 transformation in skeletal preparations of E17.5 autopods. Scale bar is 2 mm. (E) Table of qualitative characteristics in each *Pitx1-/-* genotype and genetic background.

phenotype independently of genetic backgrounds that influence phenotype penetrance. Thus, heterozygosity for *Shh* (*Shh+/-*) buffers the decrease of femur size in *Pitx1-/-* HL and the loss of the tibia and digit 1, but without restoring HL pattern. In sum, sensitivity to *Shh* signaling contributes to the strain-sensitive loss of anterior features in the *Pitx1-/-* HL.

2. Results

The *Pitx1-/-;Shh+/+* HL display a shortened femur and tibia relative to wild type (WT) HL (Fig. 1B) and the right HL is more severely affected than the left HL, in accordance with previous studies that show *Pitx2* expression in the left lateral plate mesoderm compensates for loss of *Pitx1* in the presumptive limb field (Marcil et al., 2003). Deletion of one *Shh* allele rescued these features significantly (Fig. 1B).

Measurements of bone size reveal a considerable range of bone lengths in *Pitx1-/-;Shh+/+* HL, especially the length of the right femur and right tibia (Fig. S1A). In contrast, *Pitx1-/-;Shh+/-* HL do not show the same spread of phenotypes: the bone measurements of these samples cluster together neatly, indicating a more consistent phenotype that does not include the extreme shortening of the right femur and tibia. In order to control for the size of the fetus as well as any deviation in developmental stage, we normalized the bone lengths relative to the length of the right humerus (which is not affected by *Pitx1* loss (Lanctôt et al., 1999; Marcil et al., 2003)). Analysis of these normalized bone lengths reveals significant differences between the bone size of *Pitx1-/-;Shh+/+* and *Pitx1-/-;Shh+/-* HL for both femurs, the right tibia, as well as the third metatarsal of both right and left feet, an indication of reduced digit length (Fig. 1C). Despite the rescue by *Shh* dosage, the asymmetry between the length of the left and right femurs remains. However, the *Shh* rescue of bone length is incomplete and salient *Pitx1*-dependent HL features such as patella are not rescued by decreased *Shh* dosage.

The greatest variability in the *Pitx1-/-;Shh+/+* HL is in the tibia and digit one: the most extremely affected *Pitx1-/-;Shh+/+* fetuses are missing digit one whereas in many fetuses that retain five digits, digit one has adopted a digit two identity. These have three phalanges that extend to a distance roughly equivalent to digit two whereas digit one is usually much smaller than digit two and has only two phalanges (Fig. 1D). These transformations were not described in previous *Pitx1-/-* HL studies (Lanctôt et al., 1999; Szeto et al., 1999) that were performed in genetic backgrounds of Sv129/BALBc and C57BL/6, respectively. The spectrum of phenotypes in our results, which were performed in a mixed BALBc/C57 background, suggest a strain-sensitivity to loss of *Pitx1*. Of 13 *Pitx1-/-;Shh+/+* fetuses studied, seven of them have an incomplete tibia and lack digit one, and five of the remaining six present digit one with the identity of digit two (Fig. 1E). These digit one transformations occurred on the left side, as well, affecting four of 13 fetuses. Reducing *Shh* gene dosage greatly reduced the incidence of these phenotypes: none of the left HL in the *Pitx1-/-;Shh+/-* show digit one transformation, while none of the right HL in either mixed or C57BL/6 backgrounds have an incomplete tibia (Fig. 1E).

Additional backcrossing into C57BL/6 did not increase the quantitative effect of the *Pitx1-/-;Shh+/+* phenotypes, as the distribution of bone length measurements is similar after a total of 4 backcrosses into C57BL/6 (Supplementary Fig. S1B, C). However, *Pitx1-/-;Shh+/+* C57BL/6 mice were more sensitive to digit one transformations, especially on the left side. In summary, strain differences change phenotype penetrance but not its nature.

3. Discussion

Models of Pitx1 action combine an instructive role in chondrogenic determination with an upstream role in regulation of AP limb patterning genes. In other words, loss of *Pitx1* function leads to decreased chondrogenesis and deficient *Sox9* expression, but it also leads to reduced expression of anterior patterning genes such as *Gli3* (Marcil et al., 2003; Nemec et al., 2017). Gain of *Pitx1* function in FL leads to downregulation of *Shh* signaling (DeLaurier et al., 2006) while also increasing the capacity of FL cells to form cartilaginous nodule in micromass cultures (Butterfield et al., 2017). The goal of this study was to determine the extent to which overexposure to Shh signaling, as indicated by decreased anterior identity mesenchyme in the *Pitx1-/-* HL, is responsible for the loss of HL identity in *Pitx1-/-* HL.

Our results confirm that Shh signaling has a significant effect on development of the *Pitx1-/-* HL phenotype. *Pitx1-/-;Shh+/-* fetuses are buffered from the most extreme phenotypes observed in the *Pitx-/-;Shh+/+* HL, irrespective of mouse strain sensitivity to *Pitx1* loss. In light of the HL features not rescued in the *Pitx1-/-;Shh+/-* genotype, such as the patella, these results suggest that the multiple inputs of *Pitx1* action cannot be neatly separated to identify a single root cause. This result is a contrast to the *Irx3/Irx5* double mutant, in which *Shh* null heterozygosity is sufficient to rescue the anterior, HL-specific defects of the mutant mouse (Li et al., 2014). It is thus likely that in the context of reduced Shh signaling, *Pitx1* instruction is still necessary for the complete development of anterior HL features and the installation of HL identity.

The interplay between *Pitx1* and *Shh* is interesting: the appearance of a digit 1 to digit 2 transformation producing triphalangeal thumbs following crossing of the *Pitx1-/-* alleles into the C57Bl/6 background further highlights the similarity of the *Pitx1-/-* phenotypes with those of *Shh* overexpression mutants caused by ZRS mutations (Lettice et al., 2017, 2014). Indeed, various ZRS mutations are associated with limb malformations that include triphalangeal thumbs and tibial dysplasias (Wieczorek et al., 2010). In particular, digit 1 identity was associated with prevalence of the repressor form of Gli3, an alternate form of the Gli3 activator induced by Shh (Hill et al., 2009) and the absence of Shh signaling together with strong Gli3 repressor expression (*Shh-/-;Gli3+/-* mice) results in biphalangeal hindlimb digits (Litingtung et al., 2002; te Welscher et al., 2002). In summary, the loss of anterior HL bud mesenchyme in *Pitx1-/-* mice may not be balanced by the associated reduction of Gli3 expression (Marcil et al., 2003) such that a phenotype similar to a moderate *Shh* gain-of-function, the digit 1 to digit 2 transformation, can be observed in some genetic backgrounds.

4. Conclusions

Reducing the gene dosage of *Shh* in the *Pitx1-/-* HL lessens the severity of the *Pitx1* null phenotype. Heterozygosity for *Shh* null does not completely rescue *Pitx1*-dependent, HL-specific patterning features, but *Pitx1-/-;Shh+/-* HL are less sensitive to the shortening of the tibia, loss of digit one, and the shortening of femur length associated with loss of *Pitx1*.

5. Materials and methods

Fetuses were generated by crossing a BALB/c *Pitx1* null line into a C57BL/6 *Shh* null line and then directly mating the progeny of these mixed background crosses. Where C57BL/6 is indicated as the genetic background, fetuses are the result of four generations of backcrosses from BALB/c into C57BL/6, i.e. 15/16 C57BL/6. All fetuses labeled WT in figures are a mixture of *Pitx1+/+;Shh+/+*, *Pitx1+/-;Shh+/+*, *Pitx1+/+;Shh+/-*, and *Pitx1+/-;Shh+/-*, which are demonstrated to be phenotypically equivalent (Supplementary Fig. S2). All animal experimentation was approved by the IRCM Animal Ethics Review Board and followed Canadian guidelines.

All fetuses were dissected at E17.5. Skeletal preps were performed by differential staining of bone and cartilage with alizarin red and alcian blue 8GX as in (McLeod, 1980). Bone measurements were made with ImageJ software, normalizing line segment measurements to a 300 pixel, 2 mm scale bar. All measurements are of the ossified portion

of the bone stained by alizarin red as the extent of ossification was not affected in any mutant genotypes compared to WT. Statistical comparisons were made using Student's *t*-test and all comparisons indicated to be significant in figures remain so after adjusting for multiple comparisons with the Benjamini-Hochberg procedure (Benjamini, 1995).

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Author contributions

Conceptualization, S.N. and J.D.; Methodology, S.N.; Formal Analysis, S.N, A.H.S.; Investigation, S.N., A.H.S.; Writing – Original Draft, S.N.; Writing – Reviewing and Editing, S.N., A.H.S., J.D.; Visualization, S.N., A.H.S.; Funding Acquisition, J.D.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.04.024.

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