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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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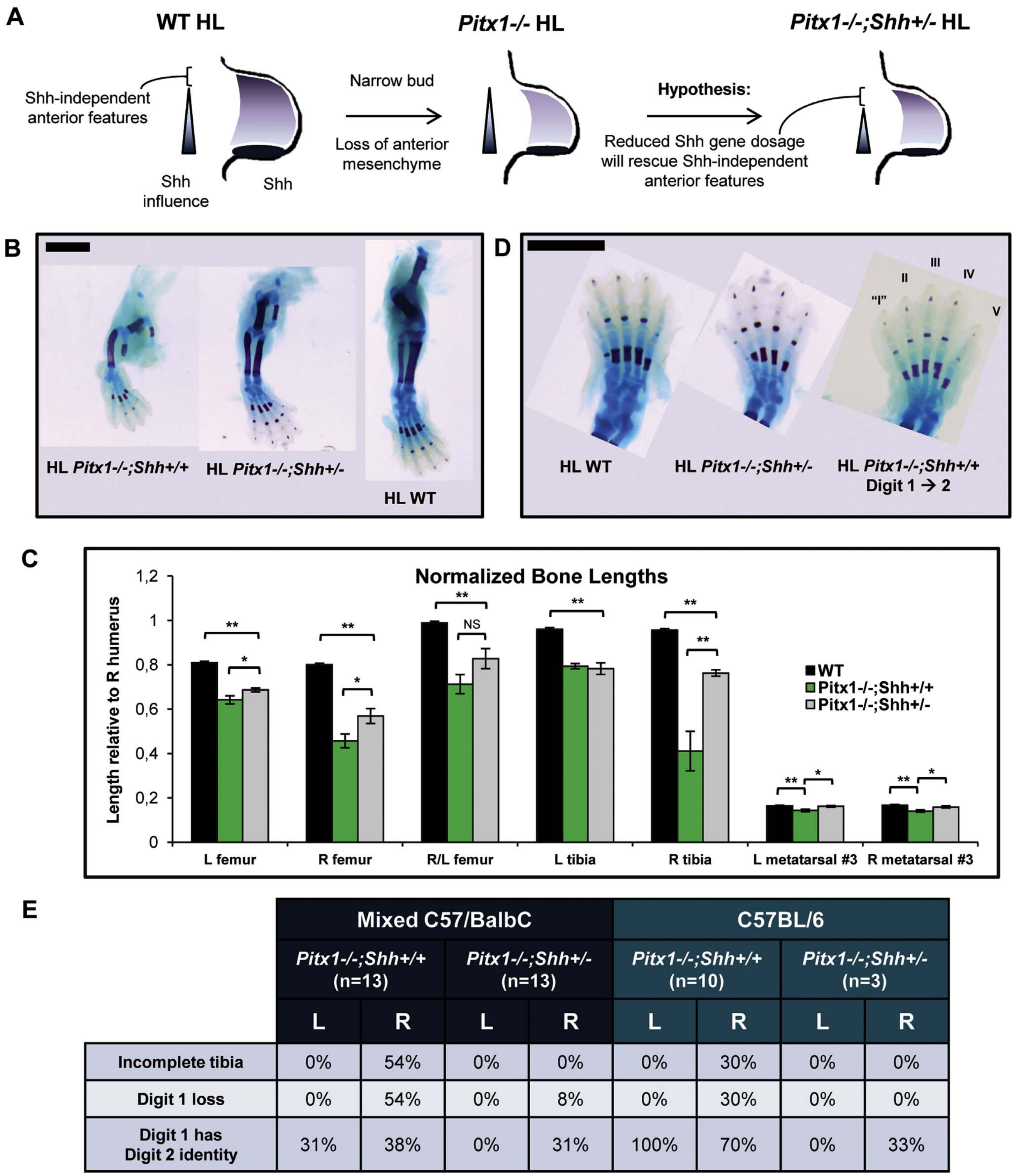

	Mixed C57/BalbC				C57BL/6			
	Pitx1-/-;Shh+/+ (n=13)		*Pitx1-/-;Shh+/-* (n=13)		*Pitx1-/-;Shh+/+* (n=10)		*Pitx1-/-;Shh+/-* (n=3)	
	L	R	L	R	L	R	L	R
Incomplete tibia	0%	54%	0%	0%	0%	30%	0%	0%
Digit 1 loss	0%	54%	0%	8%	0%	30%	0%	0%
Digit 1 has Digit 2 identity	31%	38%	0%	31%	100%	70%	0%	33%

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.