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Regionalized *Twist1* activity in the forelimb bud drives the morphogenesis of the proximal and preaxial skeleton

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ABSTRACT

Development of the mouse forelimb bud depends on normal *Twist1* activity. Global loss of *Twist1* function before limb bud formation stops limb development and loss of *Twist1* throughout the mesenchyme after limb bud initiation leads to polydactyly, the ulnarization or loss of the radius and malformations and reductions of the shoulder girdle. Here we show that conditional deletion of *Twist1* by *Mesp1*-Cre in the mesoderm that migrates into the anterior-proximal part of the forelimb bud results in the development of supernumerary digits and carpals, the acquisition of ulna-like characteristics by the radius and malformations of the humerus and scapula. The mirror-like duplications and posteriorization of pre-axial tissues are preceded by disruptions to anterior–posterior Shh, Bmp and Fgf signaling gradients and dysregulation of transcription factors that regulate anterior–posterior limb patterning.

Introduction

Limb morphogenesis in the mouse embryo begins with the formation of a bud, followed by outgrowth, the shaping of the autopod and zeugopod segments, and the specification and formation of digits. Proximal–distal growth and patterning are controlled by Fgf10 and Bmp signaling in the mesenchyme and Fgf4/8 signals from the apical ectodermal ridge (AER) (Bénazet and Zeller, 2009). Specification of the number and identity of digits is controlled by their position in the gradient of Shh signaling from the zone of polarizing activity (ZPA) and the duration of exposure to the signal. Shh signaling is in turn regulated by feedback loops involving Fgf signals from the AER and Bmp in the mesenchyme (Robert, 2007; Sun et al., 2000). The characteristics of skeletal elements are specified by transcription factors including members of the Hoxd, Alx and Msx families that are subject to regulation by the Fgf, Shh and Bmp signaling pathways (Bensoussan-Trigano et al., 2011; Kuijper et al., 2005; Zakany et al., 2007).

Twist1, a basic helix–loop–helix transcription factor, plays a key role in integrating the actions of signaling pathways and transcription factors during limb development (O'Rourke and Tam, 2002; Zuniga et al., 2002). In *Twist1*$^{-/-}$ mouse embryo outgrowth of the forelimb buds is impaired and they are smaller than wild-type limbs by E10 (Chen and Behringer, 1995). Poor forelimb outgrowth in *Twist1*$^{-/-}$ embryos is accompanied by reduced expression of *Fgf* genes in the AER, and weaker Bmp and Shh signaling in the mesenchyme (O'Rourke et al., 2002; Zuniga et al., 2002). Outgrowth of the hindlimb buds is affected to a lesser degree, and the disruptions to Fgf, Bmp and Shh signaling are less severe (O'Rourke et al., 2002). Further investigation into the morphogenetic outcome of these defects has been prevented by the death of *Twist1*$^{-/-}$ embryos at approximately E11.5, not long after the limb pattern emerges.

In contrast, partial reductions in *Twist1* activity affect anterior–posterior patterning of the limb buds. In *Twist1*-heterozygous mice, this manifests as pre-axial polydactyly of the hindlimbs (O'Rourke et al., 2002). Homozygosity for a hypomorphic *Twist1* point mutation affecting protein–protein interactions (*Ska10*, also known as *Charlie Chaplin*, *CC*) or a combination of Ska10 and null alleles results in loss of digits, and abnormal or missing long bones of the forelimb (Krawchuk et al., 2010). However, conditional loss of *Twist1* function widely in the mesenchyme after the limb bud has begun to develop, driven by *Prx1*-Cre, leads to a disruption of A–P patterning that causes polydactyly and ulnarization of the radius, as well as abnormalities of the humerus and scapula, (Krawchuk et al., 2010; Zhang et al., 2010). Genetic and biochemical studies suggest that Twist1 influences anterior–posterior patterning of the limbs through interactions with ETV-family proteins (Zhang et al., 2010) that influence Shh signaling.

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In addition, *Twist1* interacts genetically with *Gli3*, which encodes a Shh signaling repressor (O'Rourke et al., 2002). These findings suggest that *Twist1* function is critical for the acquisition of anterior limb characteristics and the formation of pre-axial limb structures. Whether *Twist1* is required locally in the anterior tissues of the limb bud is not yet known.

Precursors of limb bud tissues are derived from the lateral plate mesoderm, which forms the skeletal elements (Koussoulakos, 2004) and the hypaxial dermomyotome, which contributes to the muscles (Buckingham et al., 2003). We have discovered that Cre recombinase expressed from a knock-in allele at the *Mesp1* locus (Saga et al., 1999) leads to the loss of *Twist1* early in the mesoderm that eventually populates the mesenchyme in the anterior and proximal regions of the forelimb bud, providing a unique experimental system in which to investigate the contribution of *Twist1* in a specific region to the anterior–posterior patterning of the limb. We show that loss of *Twist1* function in this restricted domain within the forelimb bud mesenchyme results in anterior–posterior patterning defects reminiscent of those that result from deletion of *Twist1* in the entire limb bud mesenchyme (Krawchuk et al., 2010; Zhang et al., 2010), but less severe defects in the humerus and scapula. The patterning defects are likely to be consequences of reduced *Gli3* and *Alx4* expression and disruptions to Fgf, Bmp and Shh signaling during a critical phase of limb patterning.

Material and methods

Mouse strains and genotyping

Twist1$^{3loxPneo/3loxPneo}$ and *Twist1*$^{del/+}$ mice were maintained and genotyped as previously described (Bildsoe et al., 2009; Chen et al., 2007). *Mesp1*-Cre mice (Saga et al., 1999) were maintained on a C57Bl/6 background and genotyped by PCR with the following primers:

5′-CTGACCGTACACCAAAATTTGCCTG-3′ (CreF),
5′-GATAATCGCGAACATCTTCAGGTTC-3′ (CreR).

We first crossed *Mesp1*-Cre mice (Saga et al., 1999) to Twist1$^{del/+}$ mice to generate *Twist1*$^{del/+}$; *Mesp1*$^{cre/+}$ mice. To generate embryos with a mesoderm-specific *Twist1* deficiency, *Twist1*$^{del/+}$; *Mesp1*$^{cre/+}$ mice were crossed with *Twist1*$^{3loxPneo/3loxPneo}$ mice. Conditional knockout (CKO) embryos of Twist1$^{3loxPneo/del}$;Mesp1$^{Cre/+}$ genotype were compared with Twist1$^{3loxPneo/+}$, *Mesp1*$^{+/+}$ (WT) embryos and *Twist1*$^{3loxPneo/del}$;*Mesp1*$^{+/+}$ (heterozygous) embryos.

To trace the distribution of the descendants of *Mesp1*-Cre expressing cells, we initially crossed the Rosa26R line (Soriano, 1999) to the *Twist1*$^{3loxPneo/3loxPneo}$ line generating *Twist1* $^{3loxPneo/+}$; Gt(ROSA)26Sor$^{tm1Sor/+}$ offspring which were to intercrossed generate *Twist1*$^{3loxPneo/3loxPneo}$; Gt(ROSA)26Sor$^{tm1Sor/tm1Sor}$ offspring. These were crossed with *Twist1*$^{del/+}$; *Mesp1*$^{cre/+}$ mice to generate β-galactosidase positive CKO and WT embryos for analysis. The Rosa26R allele was detected by PCR for *lacZ* with the following primers:

LacZ-F: 5′ TTATCGATGAGCGTGGTGGTTATGC 3′
LacZ-R: 5′GCGCGTACATCGGGCAAATAATATC 3′

Bone and cartilage staining

Embryos were collected at E16.5 and E17.5 in PB1 (Kinder et al., 2000) and rinsed in cold PBS (without calcium and magnesium). Bone and cartilage were stained with alizarin red and alcian blue (Hogan et al., 1994). Stained specimens were washed, stored and photographed in aqueous 20% ethanol: 20% glycerol. Images were captured using a SPOT camera and Leica microscope.

ß-galactosidase reporter staining

For whole mount staining to detect ß-galactosidase activity, embryos were fixed for at least 2 h in glutaraldehyde solution (Igepal CA630 0.02%, Sodium Deoxycholate 0.01%, Glutaraldehyde, 0.2%, EGTA 5 mM, MgCl2 2 mM), washed in X-gal washing buffer briefly before being incubated in X-gal staining solution at 37 °C for 2–3 h for color development (Watson et al., 2008). The embryos were then washed twice in X-gal washing buffer and fixed in 4% PFA.

For β-galactosidase staining of cryosections, limbs were dissected from E13.5 embryos in PB1 medium, rinsed in PBS and fixed in 4% PFA overnight at 4 °C. The limbs were then infiltrated with 15% sucrose/PBS overnight, followed by 25% sucrose/PBS overnight at 4 °C. Tissues were embedded in equal volumes of 25% sucrose/PBS and OCT embedding medium (Tissue Tek). Sections were cut at 5–8 μm on a cryostat (M1900, Leica), collected on Superfrost Plus slides (Menzel–Glaser) and stored at −80 °C until needed. Sections were thawed at room temperature, rinsed in Lac Z buffer 3 times and then incubated in X-gal staining solution at 37 °C overnight for color development followed by washing in X-gal washing buffer and water, counter-stained with nuclear fast red and mounted in Ultra-mount No.4 (Fronine).

Immunofluorescence

Embryos between E9.5–10.5 were dissected in PB1 and rinsed in cold PBS. The limb buds were fixed in 4% PFA overnight at 4 °C and cryoembedded as previously described (Bildsoe et al., 2009). Cryosections were stored at −20 °C until use. Prior to staining, slides were allowed to reach room temperature, washed three times for 3 min. in washing solution (PBS plus 0.1% BSA, 0.02% triton X-100) and blocked with 3% Donkey serum, 0.1% BSA 0.02% triton in PBS for 1 h at room temperature. Slides were incubated overnight at 4 °C with mouse anti-Twist1 (Abcam, 1/50 dilution) and rabbit anti-Ki67 (Abcam, 1/100 dilution) blocking solution and washed three times in washing solution for 5 min at room temperature. Secondary antibodies (AlexaFluor-488 Donkey anti-mouse and AlexaFluor-594 goat anti-rabbit, Invitrogen) diluted 1/500 in PBS plus 0.2% triton, 1% donkey serum were added in washing solution for 1–2 h at room temperature. Slides were then washed three times for 5 min in washing solution, stained with DAPI for 10 min, washed again for 5 min in PBS before mounting in 70% glycerol/PBS. Ki67 positive and DAPI stained nuclei were counted in identically sized and shaped regions within the *Twist1*-deleted zone of CKO limb buds and equivalent regions of wild-type limb buds.

In situ hybridization

Riboprobes for whole mount in situ hybridization to E10.5 or E11.5 mouse embryos were made from plasmid clones containing fragments of the following cDNAs: *Alx1*, *Alx4*, *Emx2*, *Fgf4*, *Fgf8*, *Fgf10*, *Gli3*, *Grem1*, *Gsc*, *Hand2*, *Hoxd13*, *Msx1*, *Pbx1*, *Ptch1*, *Shh* and *Twist1*. To generate a riboprobe to detect *Cre* transcript, a fragment of the open reading from was amplified using the following primers:

Cre-F: 5′ CCGTACACCAAAATTTGCCTGCATT 3′
Cre-R: 5′ ACCATTGCCCCTGTTTCACTATCCA 3′

The amplified fragments were gel purified and re-amplified using primers identical to the first round of amplification, except that the reverse primer contained a T7 promoter sequence at the 5′ end. This product was gel purified and used for riboprobe generation. Digoxigenin-labeled riboprobes were synthesized using Ampliscribe (Epicentre Technologies). Automated whole mount in situ hybridization was carried out using an InsituPro machine (Intavis AG) as described previously (Bildsoe et al., 2009; Loebel et al.,