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The mesenchymal architecture of the cranial mesoderm of mouse embryos is disrupted by the loss of Twist1 function

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ABSTRACT

The basic helix–loop–helix transcription factor Twist1 is a key regulator of craniofacial development. *Twist1*-null mouse embryos exhibit failure of cephalic neural tube closure and abnormal head development and die at E11.0. To dissect the function of *Twist1* in the cranial mesoderm beyond mid-gestation, we used *Mesp1*-Cre to delete *Twist1* in the anterior mesoderm, which includes the progenitors of the cranial mesoderm. Deletion of *Twist1* in mesoderm cells resulted in loss and malformations of the cranial mesoderm-derived skeleton. Loss of *Twist1* in the mesoderm also resulted in a failure to fully segregate the mesoderm and the neural crest cells, and the malformation of some cranial neural crest-derived tissues. The development of extraocular muscles was compromised whereas the differentiation of branchial arch muscles was not affected, indicating a differential requirement for *Twist1* in these two types of craniofacial muscle. A striking effect of the loss of *Twist*1 was the inability of the mesodermal cells to maintain their mesenchymal characteristics, and the acquisition of an epithelial-like morphology. Our findings point to a role of *Twist1* in maintaining the mesenchyme architecture and the progenitor state of the mesoderm, as well as mediating mesoderm–neural crest interactions in craniofacial development.

Introduction

Craniofacial structures are derived from the cranial neural crest (CNC) and the cranial mesoderm (CM) (Bronner and Ledouarin, 2012; Couly et al., 1992; Kontges and Lumsden, 1996; Minoux and Rijli, 2010; Noden and Francis-West, 2006; Sauka-Spengler and Bronner-Fraser, 2006). The CNC cells, a population of migratory cells arising from the lateral region of the neural folds, contribute to all the elements of the viscerocranium (comprised of the lower jaw, upper jaw and snout), the frontal bones of the skull and the anterior skull base, as well as the connective tissues, cranial ganglia and the smooth muscle of the blood vessels in the head. The CM, which is part of the mesoderm layer formed during gastrulation, contributes to the muscles of the face and neck, endothelial cells of the blood vessels, and bones of the neurocranium and the posterior skull base (Noden and Francis-West, 2006; Saga et al., 1999; Sambasivan et al., 2011; Yoshida et al., 2008). The CM can be subdivided topographically into the prechordal, cranial paraxial and cranial lateral populations, all of which contribute to the craniofacial muscles. Cell transplantation and lineage tracing experiments performed on avian embryos show that the prechordal and rostral parts of the cranial paraxial mesoderm contain the precursors of the extraocular muscles (Couly et al., 1992) while the lateral part of the cranial mesoderm contributes to the muscles of the branchial arches. However, genetic lineage tracing studies in mouse embryos have revealed a clonal relationship between some cells of the extraocular muscles and the mandibular arch-derived muscles, suggesting that they are not derived from entirely separate lineages (Lescroart et al., 2010).

Twist1 encodes a basic helix–loop–helix transcription factor that is expressed in both the CNC and the CM during craniofacial development (Fuchtbauer, 1995; Stoetzel et al., 1995). In humans, haploinsufficiency of *TWIST1* is associated with the autosomal dominant Saethre–Chotzen syndrome (SCS) (El Ghouzzi et al., 1997; Qin et al., 2012; Rose and Malcolm, 1997), characterized by a varied pattern of craniofacial defects including craniosynostosis, ptosis and facial asymmetry. In *Drosophila,* loss of *twist* leads to abnormal gastrulation and failure of mesoderm formation (Thisse et al., 1987), pointing to a function in eliciting epithelial–mesenchymal transition (EMT).

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In the mouse, loss of *Twist1* is associated with major craniofacial defects including a failure to close the cranial neural tube, branchial arch hypoplasia, poor vascular development and the demise of the embryo at around embryonic day (E) 11 (Chen and Behringer, 1995).

The embryonic lethality of the *Twist1*-null mutation does not allow analysis of *Twist1* function in craniofacial development beyond mid-gestation. This obstacle can be overcome by conditional ablation of *Twist1* in specific tissues, which minimizes the impact of loss of gene function on the viability of the embryo (Chen et al., 2007). Using this approach we have shown that loss of *Twist1* in the CNC cells leads to reduced cell viability and impaired differentiation, loss or malformation of CNC-derived skeleton, poor frontonasal development and mandibular hypoplasia (Bildsoe et al., 2009). In addition, the formation the parietal and interparietal bones, which are derived from the CM, is also affected, suggesting that the CNC-derived tissues may play a supportive role in the development of some mesodermal bones.

To investigate the role of *Twist1* in the cranial mesoderm, a similar conditional ablation strategy was employed to eliminate *Twist1* activity by expressing Cre-recombinase in the nascent mesoderm during gastrulation, via a Cre transgene insertion into the *Mesp1* locus (Loebel et al., 2012; Saga et al., 1999). In this study, *Twist1* activity in the cranial mesoderm is shown to be essential for normal development of the mesoderm-derived components of the neurocranium and the formation of the extraocular muscles. We have also uncovered an unexpected effect of the loss of *Twist1* on the ability of the CM cells to maintain their mesenchymal characteristics, resulting in the acquisition of an epithelial phenotype.

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.

Crosses were performed as previously described (Loebel et al., 2012). We first crossed *Mesp1*-Cre mice (Saga et al., 1999) to $Twist1^{del/+}$ mice to generate $Twist1^{del/+}$; $Mesp^{Cre/+}$ mice. To generate embryos with a mesoderm-specific *Twist1* deficiency, $Twist1^{del/+}$; $Mesp1^{Cre/+}$ mice were crossed with $Twist1^{3loxPneo/3loxPneo}$ mice. Conditional knockout (CM–CKO) embryos of $Twist1^{3loxPneo/del}$;$Mesp1^{Cre/+}$ genotype, wild-type (WT) $Twist1^{3loxPneo/+}$, $Mesp1^{+/+}$, and heterozygous $Twist1^{3loxPneo/del}$; $Mesp1^{+/+}$ or $Twist1^{3loxPneo/del}$; $Mesp1^{+/Cre}$ embryos were collected for phenotypic analysis.

To trace the distribution of the descendents of *Mesp1*-Cre expressing cells, mice carrying the *Rosa26R* allele (Soriano, 1999) the $Twist1^{3loxPneo/3loxPneo}$ (Loebel et al., 2012) and $Mesp1^{Cre/+}$ alleles were crossed to generate embryos in which *Mesp1*-Cre activity will activate the lacZ reporter in the wild type cells and also excise the floxed *Twist1* allele such that *Twist1*-CKO cells can be tracked by lacZ expression.

Trp53 mutant mice (#002101), obtained from the Jackson Laboratory (Jacks et al., 1994), were crossed with *Twist1*-del mice (Bildsoe et al., 2009) to generate double heterozygous mice. These mice were crossed together and embryos with various combinations of *Twist1* and *Trp53* alleles collected at E9.5-E10.5. Mice and embryos were genotyped for *Twist1* and *Trp53* as described (Bildsoe et al., 2009; Chen et al., 2007; Jacks et al., 1994).

Bone and cartilage staining

Embryos were collected between E15.5 and E17.5 in PB1 and rinsed in cold PBS. Bone and cartilage was stained with alizarin red and alcian blue as previously described (Bildsoe et al., 2009; Hogan et al., 1994; Loebel et al., 2012). Stained specimens were washed, stored and photographed in aqueous 20% ethanol: 20% glycerol. Images were captured using a SPOT camera (SciTech) and Leica microscope.

β-galactosidase reporter staining

Embryos between E 7.5–11.5 were stained as whole mount specimens as described (Loebel et al., 2012;Watson et al., 2008). Briefly embryos were collected in PB1 media and rinsed in cold calcium- and magnesium-free PBS before fixing for a minimum of 2 h at 4 °C in glutaraldehyde fixative. After fixation the embryos were rinsed in X-gal washing buffer briefly and incubated in the X-gal staining solution at 37 °C for 2–6 h for color development. The embryos were washed twice in X-gal washing buffer, then in PBS and fixed in 4% PFA.

For staining of cryosections, heads of E13.5 and E15.5 embryos were dissected in PB1 medium, rinsed in calcium–magnesium free PBS and fixed on ice (Mani et al., 2010;Rivera-Perez et al., 1999). The heads were dehydrated in 10% sucrose/PBS overnight at 4 °C, followed by 25% sucrose/PBS overnight at 4 °C. Tissues were embedded in 25% sucrose/PBS:OCT (Tissue Tek) embedding medium (2:1). Sections were cut at 5–9 μm on a cryostat (M1900, Lexica), collected on Super frost Plus slides (Mensal-Glaser) and stored at −80 °C until needed. Sections were thawed at room temperature, rinsed in X-gal washing buffer three times and then incubated in the X-gal staining solution at 37 °C overnight for color development (Watson et al., 2008). Sections were washed in the X-gal washing buffer and water, and counterstained with nuclear fast red and mounted in Ultra-mount No.4 (Fronine).

In situ hybridization

Embryos from E7.5–11.5 were dissected in PB1, rinsed in cold PBS and fixed in 4% PFA. Automated whole mount in situ hybridization was carried out using an InsituPro machine (Intavis AG) (Bildsoe et al., 2009;Loebel et al., 2004). Stained embryos were washed in 0.1% Tween20 in H_2O, fixed in 4% PFA and photographed. Probes to detect the following transcripts were used: *Cre* (Loebel et al., 2012), *Myf5*, *Pitx2*, *Sox10*, *Tbx1* and *Twist1*. Embryos were imaged using a Leica dissecting microscope and SPOT digital camera (SciTech).

Cell death analysis

Apoptotic cells were detected by whole mount TUNEL staining with the ApopTag Plus Peroxidase In situ Apoptosis kit (Millipore) as previously described (Martinez-Barbera et al., 2002). Embryos were dissected in PB1, rinsed in cold PBS and fixed in 4% PFA overnight at 4 °C, and incubated in blocking buffer containing 2% blocking reagent (Roche) and 20% heat inactivated fetal calf serum in PBS/0.1% Tween. After antibody labeling, embryos were washed overnight at 4 °C in 2 mg/ml BSA in PBS, 0.1% Tween with at least four changes of buffer. After treatment, embryos were fixed in 4% PFA and the embryos were imaged using a Leica microscope and SPOT digital camera (SciTech).

Histology

Embryos at E8.5–E15.5 were collected in PB1 and rinsed in cold PBS, followed by fixation in 4% PFA. For wax embedding, the embryos were dehydrated stepwise through an ethanol series (30%, 50%, 70%, 80%, 90% and 100%), cleared in xylene and embedded in paraffin wax. Serial sections cut at 5–10 μm were stained with haematoxylin and eosin.