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Paraxial T-box genes, *Tbx6* and *Tbx1*, are required for cranial chondrogenesis and myogenesis

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ABSTRACT

We previously reported that *Tbx6*, a T-box transcription factor, is required for the differentiation of ventral body wall muscle and for segment formation and somitic muscle differentiation. Here, we show that *Tbx6* is also involved, at later stages, in cartilage differentiation from the cranial neural crest and head muscle development. In *Tbx6* knockdown embryos, the cranial neural crest was shown to be correctly induced at the border of the neural plate and migrated in a slightly delayed manner, but finally reached positions in the pharyngeal arches nearly similar to those in the normal embryos as revealed by *in situ* hybridization and the neural crest-transplantation experiments. However, the neural crest cells failed to maintain *Sox9* expression. *Tbx6* knockdown also reduced the expression of *Tbx1*, another T-box gene expressed in more anterior paraxial structures. *Tbx1* knockdown caused phenotypes milder but similar to those of *Tbx6* morphants, including reduced formation of head muscles and cartilages, and attenuated *Sox9* expression. Furthermore, the phenotypes caused by *Tbx6* knockdown were partially rescued by *Tbx1* plasmid injection. These results suggest that *Tbx6* is involved in the cranial cartilage and head muscle development by regulating anterior paraxial genes such as *Tbx1* and *Sox9*.

Introduction

In vertebrates, craniofacial structures are formed by the orchestrated integration of multiple tissue interactions. The cranial bones, cartilages, and attached muscles play important roles in determining the shape and motor functions of the head.

The cranial neural crest is the source of nerves such as the cranial ganglia and sympathetic neurons, a lot of the cranial bones and cartilages, and some of the musculature in mice, chicks, and fish (Noden, 1991; Le Douarin and Kalcheim, 1999). During embryogenesis, the neural crest cells develop through a course of three key events: induction, migration, and differentiation. Neural crest induction requires partial attenuation of BMP signaling by BMP antagonists (Marchant et al., 1998; Nguyen et al., 1998; Tribulo et al., 2003), canonical Wnt signaling (Wu et al., 2003; Heeg-Truesdell and LaBonne, 2006; Steventon et al., 2009), and Fgf signaling (Kengaku and Okamoto, 1993; Mayor et al., 1995, 1997; Monsoro-Burq et al., 2003; Hong et al., 2008). In *Xenopus*, the cranial neural crest cells migrate from the hindbrain in three streams and populate the pharyngeal arches (Sadaghiani and Thiebaud, 1987). It has been suggested that neural crest migration requires noncanonical Wnt signaling and is controlled by contact inhibition of locomotion (De Calisto et al., 2005; Matthews et al., 2008; Carmona-Fontaine et al., 2008). Neural crest differentiation relies on intrinsic programs and extrinsic cues. *Sry*-related transcription factor *Sox9* is required for cartilaginous, testicular, neural, and cardiac development (Akiyama et al., 2002; Stolt et al., 2003; Akiyama et al., 2004; Chaboissier et al., 2004). *Sox9* is expressed in the neural crest and is required for cranial chondrogenesis in mice (Wright et al., 1995; Zhao et al., 1997; Ng et al., 1997) and *Xenopus* (Spokony et al., 2002).

The contribution of head mesoderm to muscle development has been well studied in mice and chicks (Trainor et al., 1994; Noden and Francis-West, 2006). All muscle genes reflecting early steps of the myogenic program including *Capsulin*, *Tbx1*, *MyoR*, *Myf-5*, *MyoD*, and *desmin* are expressed in the branchial arches following neural crest ablation in chick embryos. However, in the absence of neural crest cells, jaw muscles were severely reduced, indicating that the neural crest cells are required for normal muscle differentiation after the onset of muscle specification (Tzahor et al., 2003; von Scheven et al., 2006; Rinon et al., 2007). Head muscle differentiation in *Xenopus* has not been studied well, but the morphogenesis and histological differentiation of the cranial muscles have been documented (Ziermann and Olsson, 2007).

The T-box transcription factor family plays a crucial role in embryonic development (Papaioannou and Silver, 1998; Showell et al., 2004). A member of this family, *Tbx1*, has been indicated by genetic analysis to be the major gene responsible for 22q11 deletion/DiGeorge syndrome, in human and mouse (Merscher et al., 2001; Meechan et al., 2009; Ryckebüsch et al., 2010). *Tbx1* null mutant mice have craniofacial and cardiovascular defects associated with

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pharyngeal hypoplasia (Baldini, 2005). In *Xenopus*, an injection of a repressor form of *Tbx1* caused cranial cartilage disorganization, reduction or absence (Ataliotis et al., 2005). Another member of this family, *Tbx6*, is expressed in the primitive streak, paraxial mesoderm, muscle lineage blastomeres, and tailbuds in various species (Chapman et al., 1996; Yasuo et al., 1996; Uchiyama et al., 2001). *Tbx6* is required for somite segmentation and differentiation and for ventral body wall muscle differentiation (Chapman and Papaioannou, 1998; Tazumi et al., 2008).

Here, we show that *Tbx6* is also involved in differentiation of more anterior structures such as cranial cartilages and head muscles by regulating more anterior genes, including *Tbx1* and *Sox9*.

Materials and methods

Plasmid constructs

Tbx6/pCS2+ and β-*gal*/pBluescript II were described previously (Tazumi et al., 2008). cDNA clones including the full open reading frame of *PCNS*, *Wnt11*, and *Fz7* (GenBank accession numbers: BG020773, BC042228, and BC084745; each inserted in pCMV-SPORT6 vector) were obtained from Open Biosystems Inc. (Huntsville, AL, USA). *Tbx1*, full-length cDNA clone, was obtained from a Lambda ZAP II cDNA library derived from embryos at stages 22–25 with a PCR product as a probe, according to the screening procedures as previously described (Uchiyama et al., 2001). To generate *Wnt8*/pBluescript II, PCR products were amplified from *Xwnt-8*/pSP64T (Christian et al., 1991) and ligated into the T-vector made from pBluescript II (Uchiyama et al., 2001). To produce *Fgf8b*/pBluescript II, *Snail2*/pBluescript II, and *Sox9*/pBluescript II, PCR fragments were amplified from whole embryonic cDNA and ligated into the T-vector. Forward (F) and reverse (R) PCR primers for *Tbx1*, *Wnt8*, *Fgf8b*, *Snail2*, and *Sox9* were as follows: *Tbx1* (F: 5′-GTGTCAATCATCTGTGTA-GAGCTGCG-3′; R: 5′-CAACACTGGAGGAACAGCATTAATGC-3′), *Wnt8* (F: 5′-CCTTCATCATGCAAAACACC-3′; R: 5′-TCTGGAATGCCGTCATCTCC-3′), *Fgf8b* (F: 5′-CTGAGCAACATGAACTACATCACC-3′; R: 5′-CCTACCGA-GAACTTGAATATCGAG-3′), *Snail2* (F: 5′-GACCGTTACTTGTGCGTCC-3′; R: 5′-GCAGCTACACACTGCTTCTCC-3′), and *Sox9* (F: 5′-CGCATGAATCTCTTG-GATCC-3′; R: 5′-CTAGGGTCTTGTGAGCTGTGTGTAC-3′).

A partial *protocadherin-18 (Pcdh18)* sequence was identified from two nonoverlapping EST clones of *Xenopus laevis* (BX846839 and BQ732394). Using primers derived from these clones (F: 5′-GGAGCA-GATGGTCGGTACCG-3′; R: 5′-CCAAGAGCACAGCACATATGGC-3′), we obtained a PCR fragment of 2030 bp that encodes 676 amino acids of

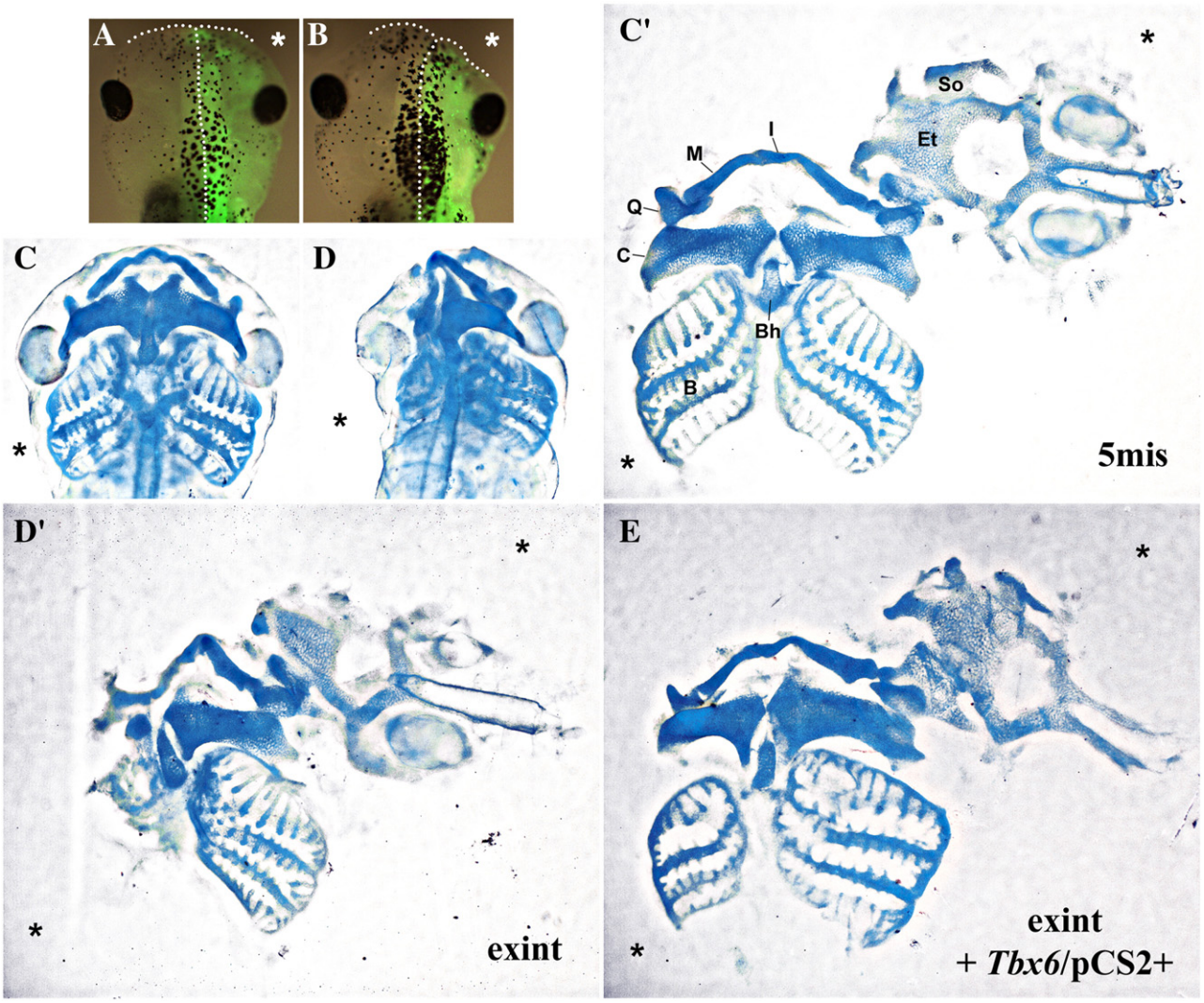

Fig. 1. *Tbx6* knockdown causes defects in cranial cartilages. The heads of stage 47 larvae injected with *Tbx6* 5mis-MO (A) or exint-MO (B) plus ODA and viewed from the ventral side (C–E). Dotted lines indicate the dorsal midline and rostral border of the larvae. MO-injected sides are marked with *. Alcian blue-stained cartilages from stage 47 larvae injected with *Tbx6* 5mis-MO or *Tbx6* exint-MO before (C, D) and after (C′, D′) dissection. So, subocular; Et, ethmoid-trabecular; I, infrarostral; M, Meckel's cartilage; Q, quadrate; C, ceratohyal; Bh, basihyal; B, branchial. (E) Alcian blue-stained cartilage from stage 47 larvae injected with *Tbx6*/pCS2+ (10 pg) together with *Tbx6* exint-MO.