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Tbx18 and *Tbx15* null-like phenotypes in mouse embryos expressing Tbx6 in somitic and lateral plate mesoderm

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ABSTRACT

Members of the T-box family of transcription factors play essential roles in cell type specification, differentiation, and proliferation during embryonic development. All T-box family members share a common DNA binding domain – the T-domain – and can therefore recognize similar sequences. Consequently, T-box proteins that are co-expressed during development have the potential to compete for binding at downstream targets. In the mouse, *Tbx6* is expressed in the primitive streak and presomitic mesoderm, and is sharply down-regulated upon segmentation of the paraxial mesoderm. We sought to determine the phenotypic and molecular consequences of ectopically expressing *Tbx6* within the segmented paraxial mesoderm and its derivatives using a 3-component transgenic system. The vertebral column, ribs, and appendicular skeleton were all affected in these embryos, which resembled *Tbx18* and *Tbx15* null embryos. We hypothesize that these phenotypes result from competition between the ectopically expressed Tbx6 and the endogenously expressed Tbx18 and Tbx15 at the binding sites of target genes. *In vitro* luciferase transcriptional assays provide further support for this hypothesis.

Introduction

One hallmark of the vertebrate embryo is segmentation of the paraxial mesoderm (PAM) into reiterative somites flanking the central neural tube. PAM arising from the primitive streak (PS) forms two strips of mesenchymal tissue lateral to the axial mesoderm thus establishing the presomitic mesoderm (PSM) (Tam and Beddington, 1987). Somites arise through segmentation of the anterior region of the PSM via a mesenchymal-to-epithelial transition. Somites later undergo differentiation to form the sclerotome, dermatome, and myotome, which will become the ribs and vertebrae, dermis, and skeletal muscle of the adult animal, respectively (Christ et al., 2000). The sclerotome region of each somite is further divided into a rostral (R) and caudal (C) half, each with different molecular and physical characteristics. Proper R–C specification is essential for the process of re-segmentation, which joins the caudal sclerotome portion of one somite to the rostral portion of the next more posterior somite. Re-segmentation ultimately results in the formation of a vertebral unit (Huang et al., 1996).

The activity of tissue-specific transcription factors drives the establishment of specific cell types. Genetic and molecular data demonstrate the important roles that the T-box family of transcription factors play in cell type specification, differentiation and proliferation during embryonic development (Naiche et al., 2005). Mutations in human *TBX1*, *TBX3*, *TBX4*, *TBX5*, and *TBX22* result in the human syndromes DiGeorge, ulna-mammary, Small Patella, Holt–Oram, and X-linked cleft palate with ankyloglossia, respectively (Baldini, 2003; Bamshad et al., 1997; Basson et al., 1997; Bongers et al., 2004; Braybrook et al., 2001). A conserved DNA binding domain known as the T-domain defines the T-box family. Outside of this domain, family members share very little similarity. Conservation of the T-box DNA binding domain allows for all T-box transcription factors thus far examined to bind a core 5′-AGGTGT-3′ (Naiche et al., 2005). T-box transcription factors can serve as transcriptional activators or repressors, with some factors serving as both depending on their interacting partners (Kawamura et al., 2008). Functional specificity is in part determined by preferences for orientation of the half-sites and for bases flanking the conserved core, and physical interactions with different co-factors (Conlon et al., 2001). Additionally, when T-box proteins are endogenously co-expressed there appears to be competition for binding sites within the enhancers of common target genes. For example, during mouse heart development, Tbx2, a transcriptional repressor, competes with Tbx5, an activator, for binding at the *ANF* enhancer (Habets et al., 2002). Competition has also been observed between ectopically and endogenously expressed T-box transcription factors during zebrafish mesoderm formation (Goering et al., 2003; Habets et al., 2002).

Mouse *Tbx6* is expressed in the PS and PSM beginning at embryonic day (e) 7.5, and is critical for the proper specification

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and patterning of the posterior PAM (Chapman et al., 1996; Chapman and Papaioannou, 1998; White et al., 2003). Tbx6 mRNA and protein are rapidly down-regulated as the PSM segments to form the somite. *Tbx6*−/− embryos form only 7–9 abnormal anterior somites; posterior to the forelimb bud ectopic neural tubes form in place of the somites. Genetic and transcriptional studies show that Tbx6 cooperates with other signaling pathways, including Notch and Wnt signaling, to activate transcription of known downstream targets — *Ripply2*, *Mesp2*, *Dll1*, and *Msgn1* (Hitachi et al., 2008; Hofmann et al., 2004; Wittler et al., 2007; Yasuhiko et al., 2006). We sought to determine the phenotypic and molecular consequences of ectopic *Tbx6* expression within the segmented PAM and its derivatives, where it is normally not expressed. Using a 3-component transgenic system we drove ectopic Tbx6 expression in the formed somites and limb buds and show that forced Tbx6 expression within the segmented PAM results in phenotypes that resemble that of *Tbx18* null embryos, while expression of Tbx6 in the lateral plate mesoderm (LPM) results in *Tbx15* null-like phenotypes. We propose that these phenotypes arise due to competition between the ectopically expressed Tbx6 with endogenously expressed Tbx18 and Tbx15 at the binding sites of target genes.

Materials and methods

Generation of TRE:myc-Tbx6 transgenic mice

The TRE:myc-Tbx6 construct was generated by cloning a 5× Myc tag in-frame with the full-length *Tbx6* cDNA into the pTRE2-hyg vector (Clontech). The insert was released from the vector and gel purified. Transgenic mice were generated by pronuclear injection into fertilized FVB/N eggs (Transgenic Core Facility, University of Pittsburgh Medical Center). Three founder lines were generated. Mice were genotyped for the presence of the transgene by PCR using primers specific to the TRE and Tbx6 (forward 5′-GCCATCCACGCTGTTTTGAC-3′; reverse 5′-CCAGAGAGGAAGCAATCCAGTTTAG-3′), which generates a 453 bp product.

Mice

The *Dll1-msd:Cre Tg33* line has previously been described (Wehn et al., 2009). *R26-rtTA B6.Cg-Gt(ROSA)26*^{Sortm1}*(rtTA,EGFP)Nagy/J* mice were obtained from Jackson Laboratories (Belteki et al., 2005). Animals were mated and checked daily for the presence of a copulation plug. Noon on the day of the plug was considered e0.5. Doxycycline (DOX, 1.6 mg/ml) plus sucrose (50 mg/ml) was added to the drinking water of pregnant females at the designated times. Females were sacrificed and embryos dissected from e9.5 to e13.5. Control embryos were hemizygous for both the *R26-rtTA* and *Dll1-msd:Cre* transgenes. All animal work was performed in accordance with the guidelines established by the University of Pittsburgh's Institutional Animal Care and Use Committee.

Fibroblasts

Fibroblasts were derived from 3-component embryos according to standard protocols (Abbondanzo et al., 1993). Briefly, e13.5 embryos were dissected free of extraembryonic tissue and the head and liver were removed. Isolated tissue was macerated by passing it through an 18-gauge needle and then cultured in DMEM supplemented with 10% fetal calf serum. Fibroblasts were expanded and at the fourth passage plated onto coverslips for immunofluorescence or Western blotting. DOX was added to the tissue culture media at a final concentration of 1 μg/mL for 36 h before processing.

Immunofluorescence

Fibroblasts on coverslips were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 in PBS, and blocked in 5% goat serum in PBS. Coverslips were incubated with mouse anti-myc (9E10, Sigma, 1:250 dilution in blocking buffer) and rabbit anti-GFP antibody (Torrey Pines Biolabs, 1:1000 dilution in blocking buffer), washed and then incubated with goat anti-mouse 568 and goat anti-rabbit 488-secondary antibody (Molecular Probes, 1:500 dilution in blocking buffer) and TO-PRO 3 nuclear stain (Molecular Probes, 1:1000 dilution in blocking buffer). Cells were mounted on slides using Vectashield mounting medium and optical sections were visualized on a BioRad scanning laser confocal microscope. Photoshop was used to merge the different channel images. The number of EGFP or myc-Tbx6 positive nuclei in each frame was divided by the total number of nuclei to determine the percentage of EGFP- and Tbx6-positive cells. Two images derived from two separate coverslips were counted to obtain the average percentage of fibroblasts that were EGFP- and Tbx6-positive.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as previously described (Wilkinson, 1992) using antisense riboprobes for *Dll1*, *Fgf8*, *mesp2*, *myogenin*, *Ripply2*, *Tbx6*, *Tbx18*, *Tbx15* and *uncx4.1*. Hybridizations and washes were performed at 63 °C.

Immunocytochemistry

Immunocytochemistry was performed as described in Nagy et al. (2003). The Tbx6 N-terminal affinity purified antibody was used at a 1:500 dilution (White and Chapman, 2005). Goat anti-rabbit:HRP-conjugated secondary antibody (Jackson ImmunoResearch) was used at a 1:500 dilution and staining was performed in the presence of DAB, hydrogen peroxide and nickel chloride.

Western blotting

HEK293T cells transfected with the indicated expression plasmids or tissue dissected from approximately ten e10.5 embryos were homogenized in RIPA buffer. Bradford dye assays were performed to determine total protein concentration, and equal amounts of protein were loaded onto 7.5% SDS-PAGE gels, transferred to nitrocellulose, and blotted with 1:500 anti-9E10 (anti-myc, Sigma) or 1:500 anti-N-terminal Tbx6 antibody (White and Chapman, 2005) in blocking buffer (TBTT containing 5% NFDM), and subsequently incubated in goat anti-rabbit:HRP-conjugated secondary antibody (Jackson ImmunoResearch; 1:2500 dilution in blocking buffer). Blots were stripped and re-probed with 1:500 anti-tubulin (Sigma) or 1:500 anti-actin (Cytoskeleton) in blocking buffer as a loading control.

Skeletal preparations

Skeletons from e13.5 embryos were stained with alcian blue as described by Nagy et al. (2003), except that the staining was performed at 37 °C. Stained embryos were cleared in benzyl benzoate: benzyl alcohol.

Plasmid constructs

Full-length *Tbx6*, *Tbx18*, and *Tbx15* cDNAs were cloned in-frame with the N-terminal myc tag of the mammalian expression vector pCS3mT. PCR amplified Tbx15-DBD and Tbx18-DBD were cloned in frame with an N-terminal myc tag and a nuclear localization signal (NLS) of pCS2mTNLS. The luciferase reporter plasmid contained a 200 basepair region of the *Dll1-msd* enhancer element cloned upstream of the β-globin minimal promoter in pGL4 vector (Promega), where one