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Identification of downstream genetic pathways of *Tbx1* in the second heart field

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Abstract

Tbx1, a T-box transcription factor, and an important gene for velo-cardio-facial syndrome/DiGeorge syndrome (VCFS/DGS) in humans, causes outflow tract (OFT) heart defects when inactivated in the mouse. *Tbx1* is expressed in the second heart field (SHF) and is required in this tissue for OFT development. To identify *Tbx1* regulated genetic pathways in the SHF, we performed gene expression profiling of the caudal pharyngeal region in $Tbx1^{-/-}$ and wild type embryos. *Isl1*, a key marker for the SHF, as well as *Hod* and *Nkx2-6*, were downregulated in $Tbx1^{-/-}$ mutants, while genes required for cardiac morphogenesis, such as *Raldh2*, *Gata4*, and *Tbx5*, as well as a subset of muscle contractile genes, signifying myocardial differentiation, were ectopically expressed. Pan-mesodermal ablation of *Tbx1* resulted in similar gene expression changes, suggesting cell-autonomous roles of *Tbx1* in regulating these genes. Opposite expression changes concomitant with SHF-derived cardiac defects occurred in *TBX1* gain-of-function mutants, indicating that appropriate levels of *Tbx1* are required for heart development. When taken together, our studies show that *Tbx1* acts upstream in a genetic network that positively regulates SHF cell proliferation and negatively regulates differentiation, cell-autonomously in the caudal pharyngeal region.

Keywords: Tbx1; Second heart field; VCFS/DGS; Gene expression profiling

Introduction

The development of the cardiac outflow tract (OFT) is vulnerable to genetic and environmental insults resulting in morphological defects of the heart. OFT anomalies, including abnormal alignment or septation, account for 30% of all cases of congenital heart disease (Kelly and Buckingham, 2002), some of which co-occur in genetic syndromes. Velo-cardio-facial syndrome/DiGeorge syndrome (VCFS/DGS) (DiGeorge, 1965; Shprintzen et al., 1978) is associated with OFT defects due to de novo hemizygous 1.5–3 million base pair (Mb) 22q11.2 deletions (Morrow et al., 1995; Shaikh et al., 2000). Studies in the mouse have demonstrated that *Tbx1*, a member of T-box containing transcription factor family present within the 1.5 Mb 22q11.2 region, is required for OFT development (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001). Functional mutations in *TBX1* have been found in rare non-deleted patients with the syndrome as well (Paylor et al., 2006; Stoller and Epstein, 2005; Yagi et al., 2003).

Tbx1 is expressed in the non-neural crest derived mesoderm of the caudal pharyngeal region, which is part of the second heart field (SHF) (Hu et al., 2004; Xu et al., 2004). Unlike the first heart field (FHF) cells, which differentiate at the cardiac crescent stage and give rise to the atria and left ventricle of the heart, SHF cells differentiate later and give rise to the OFT, right ventricle and part of the atria (reviewed by Buckingham et al., 2005). Inactivation of *Isl1*, a LIM homeodomain transcription factor expressed in the SHF, resulted in failed formation of the OFT, right ventricle and most of the atria due to failed proliferation and migration of this population of cells (Cai et al., 2003). Thus, the SHF, via *Isl1*, contributes significantly to heart development.

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Lineage tracing-experiments showed that the progeny of *Tbx1*-expressing cells in the SHF contribute to the OFT and right ventricle. In $Tbx1^{-/-}$ embryos, although *Tbx1*-traced cells are present normally in the SHF, the contribution to the OFT is lower than in wild type embryos (Xu et al., 2004). Cell proliferation is also modestly reduced in the SHF of $Tbx1^{-/-}$ embryos (Xu et al., 2004; Zhang et al., 2006). Tissue-specific inactivation of *Tbx1* in the SHF by Nkx2-5-Cre (Xu et al., 2004) or Mesp1-Cre (Zhang et al., 2006) resulted in persistent truncus arteriosus (PTA) and ventricular septal defect (VSD), same as those in $Tbx1^{-/-}$ embryos.

Two fibroblast growth factor (Fgf) genes expressed in the SHF, *Fgf8* and *Fgf10* are hypothesized to act downstream of *Tbx1* in cardiovascular development (Hu et al., 2004; Vitelli et al., 2002b). Both genes are downregulated in the SHF of $Tbx1^{-/-}$ mutants, but surprisingly, $Fgf10^{-/-}$ embryos do not have OFT defects (Marguerie et al., 2006) nor did *Tbx1/Fgf8/Fgf10* triple heterozygous mice (Aggarwal et al., 2006), indicating that other genes are responsible for these defects. Recently, *Pitx2* was shown to act downstream of *Tbx1* in the early SHF, but *Tbx1/Pitx2* double heterozygosity affected only OFT alignment but not septation, and it occurred with reduced penetrance (Nowotschin et al., 2006).

It has been difficult to identify genes downstream of *Tbx1* in the SHF. Here, we report a systematic genome-wide search for genes regulated directly or indirectly by *Tbx1* in the caudal pharyngeal region containing the SHF. Our data show that global or pan-mesodermal inactivation of *Tbx1* disrupts a network of genes maintaining cell proliferation in the SHF and causes ectopic expression of known genes required for differentiation, whereas overexpression causes opposite expression changes of a subset of these genes. These findings for the first time delineate a potential molecular network downstream of *Tbx1*.

Materials and methods

Mouse mutants

Generation of $Tbx1^{+/-}$, BAC transgenic and $Tbx1^{flox/-}$ mice has been described previously (Arnold et al., 2006; Merscher et al., 2001). *T-Cre* transgenic mice (Perantoni et al., 2005) were obtained from Dr. Mark Lewandoski. A ROSA26 reporter strain (Soriano, 1999) was obtained from Jackson Laboratories. $Tbx1^{+/-}$ and BAC transgenic mice are congenic in the FVB genetic background. ROSA26 mice were maintained in the C57BL6 background. All the other strains were maintained in a mixed C57BL6/129 background.

Mouse embryo dissections

Mouse embryos in the FVB background at E8.75, E9.75 and E10.75 were isolated in cold PBS. Somite pairs were counted to define the stages: 10–12 pairs of somites were defined as E8.75; 23–25 pairs of somites were defined as E9.75 and 35–37 pairs of somites were defined as E10.75. The caudal pharyngeal region without the first pharyngeal arch and the neural tube was dissected and placed in RNAlater RNA stabilization reagent (QIAGEN) at 4 °C.

Total RNA preparation

To obtain enough RNA for the microarray hybridization experiments, dissected pharyngeal tissue from 5–10 embryos were pooled. The tissue was homogenized in Buffer RLT (QIAGEN). Total RNA was isolated with either RNeasy Mini Kit (QIAGEN) (for E9.75 and E10.75 tissue) or the RNeasy Micro Kit (QIAGEN) (for E8.75 tissue) according to the manufacturer's protocol. Quality and quantity of total RNA was determined using an Agilent 2100 Bioanalyzer (Agilent) and a ND-1000 Spectrophotometer (NanoDrop) respectively.

Target preparation

For total RNA isolated from E9.75 or E10.75 tissue, target preparation was carried out according to Affymetrix's One-Cycle Eukaryotic Target Labeling Assay. Specifically, using One-Cycle cDNA Synthesis Kit (Affymetrix), 1–5 μg total RNA was first reverse transcribed to obtain first-strand cDNA and then second-stranded cDNA was synthesized in an RNase H-mediated reaction. The double-stranded cDNA was purified with the Sample Cleanup Module (Affymetrix) and this served as a template in the subsequent in vitro transcription (IVT) reaction using the IVT Labeling Kit (Affymetrix). The resulting biotinylated cRNA targets were then cleaned up and fragmented with Sample Cleanup Module (Affymetrix).

For the E8.75 tissue, the biotinylated single-strand cDNA targets were amplified from 20–50 nanograms (ng) starting total RNA using The Ovation Biotin RNA Amplification and Labeling System (NuGEN).

Microarray processing

A total of 20 μg fragmented, biotin labeled cRNA (for E9.75 or E10.75 tissue) or 2.2 μg cDNA (for E8.75 tissue) from the last step was hybridized to the GeneChip Test3 array (Affymetrix) to test the quality of the labeled target. Targets which passed the test were then hybridized to the GeneChip Mouse Genome 430A 2.0 Arrays (Affymetrix). Hybridization, washing, staining and scanning was performed in the AECOM Affymetrix Facility according to the Affymetrix manual.

Data analysis

GeneChip data were analyzed with ArrayAssist Lite (Stratagene). Briefly, the original Affymetrix GeneChip CEL files generated by the AECOM Affymetrix Facility were imported into ArrayAssist Lite and a master data table based on them was created by GC-RMA. Then the data was converted to logarithmic scale and the significance analysis was performed using two classes, unpaired *t*-test with a cut-off of fold change >2 and *p*-value <0.05.

Quantitative RT-PCR (qRT-PCR)

To obtain enough total RNA and minimize the variability of gene expression in individual embryos, each sample contained microdissected tissue from two to five embryos. Total RNA was isolated using the RNeasy Protect Mini Kit (Qiagen), and used for the first-strand cDNA synthesis with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). The PCR reactions were performed in the LightCycler apparatus (Roche) using LightCycler-FastStart DNA Master SYBR Green I (Roche). All values were normalized to the level of *Gapdh*, which was used as an internal control in each sample. For each experimental group, three to five samples were tested and the average of them was calculated. Statistical significance between different groups was calculated by unpaired Student's *t*-test. Gene-specific primer pairs used for PCR amplification are listed in Table S4.

In situ hybridization (ISH)

Digoxigenin-labeled RNA probes for *Tbx1* (Nowotschin et al., 2006), *Tbx5* (Chapman et al., 1996), *Gata4*, *Isl1* (Cai et al., 2003), and *Nkx2-5* (Lyons et al., 1995) were prepared by standard methods (Roche). All the other probes were amplified by PCR from either mouse genomic DNA or cDNA, using the primers listed in Table S5. All forward primers contained T3 polymerase priming sequence and all reverse primers contained T7 polymerase priming sequence. The PCR product was purified by the PCR Purification Kit (Qiagen), and anti-sense RNA was in vitro transcribed and labeled with T7 RNA polymerase