Tbx20 dose-dependently regulates transcription factor networks required for mouse heart and motoneuron development

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Development

Summary

To elucidate the function of the T-box transcription factor Tbx20 in mammalian development, we generated a graded loss-of-function series by transgenic RNA interference in entirely embryonic stem cell-derived mouse embryos. Complete *Tbx20* knockdown resulted in defects in heart formation, including hypoplasia of the outflow tract and right ventricle, which derive from the anterior heart field (AHF), and decreased expression of *Nkx2-5* and *Mef2c*, transcription factors required for AHF formation. A mild knockdown led to persistent truncus arteriosus (unseptated outflow tract) and hypoplastic right ventricle, entities similar to human congenital heart defects, and demonstrated a critical requirement for *Tbx20* in valve formation. Finally, an intermediate knockdown revealed a role for *Tbx20* in motoneuron development, specifically in the regulation of the transcription factors *Isl2* and *Hb9*, which are important for terminal differentiation of motoneurons. Tbx20 could activate promoters/enhancers of several genes in cultured cells, including the *Mef2c* AHF enhancer and the *Nkx2-5* cardiac enhancer. The *Mef2c* AHF enhancer relies on Isl1- and Gata-binding sites. We identified a similar Isl1 binding site in the *Nkx2-5* AHF enhancer, which in transgenic mouse embryos was essential for activity in a large part of the heart, including the outflow tract. Tbx20 synergized with Isl1 and Gata4 to activate both the *Mef2c* and *Nkx2-5* enhancers, thus providing a unifying mechanism for gene activation by Tbx20 in the AHF. We conclude that Tbx20 is positioned at a critical node in transcription factor networks required for heart and motoneuron development where it dose-dependently regulates gene expression.

Key words: Heart, Tbx20, T-box, Transcription factors, Mouse, Embryo, Motoneurons, RNAi, Optical projection tomography

Introduction

The T-box transcription factors are a family of developmentally regulated DNA-binding proteins that play important roles in organogenesis (Papaioannou, 2001). Their function is conserved throughout evolution, and they participate in developmental processes such as cell lineage choices, terminal differentiation and proliferation. Notable in both mice and humans is the marked dependency on T-box transcription factor dose, which is evident in the case of dominant human or mouse mutations that lead to several inherited syndromes affecting the heart and other organs (Basson et al., 1997; Bruneau et al., 2001; Li et al., 1997; Lindsay et al., 2001; Merscher et al., 2001; Packham and Brook, 2003; Yagi et al., 2003).

In the heart, T-box genes play important roles in the development of specific cardiac structures and in the transcription of specific cardiac genes. *Tbx5* specifies the formation of the posterior segments of the heart, the atria and left ventricle; decreased Tbx5 dose results in defective septation and conduction system formation (Ahn et al., 2002; Brown et al., 2005; Bruneau et al., 2001; Garrity et al., 2002; Horb and Thomsen, 1999). Misexpression studies in the chick

have suggested that expression of *TBX5* dictates the location of the interventricular septum (Takeuchi et al., 2003). *Tbx2* is required for the molecular and morphological distinction between the cardiac chambers and the atrioventricular canal (Christoffels et al., 2004; Harrelson et al., 2004), while *Tbx1* is important for aortic arch formation and also contributes to the development of the outflow tract (Hu et al., 2004; Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001; Xu et al., 2004). Biochemical studies have suggested that specific activities of T-box proteins are dictated in part by interactions between different T-box proteins or between T-box proteins and other types of transcription factors: Tbx5 interacts with the homeodomain transcription factor Nkx2-5 to activate cardiac genes (Bruneau et al., 2001; Hiroi et al., 2001), while Tbx2/Tbx5 and Tbx3/Tbx5 counterbalances are important for the proper expression of genes restricted to the cardiac chambers (atria and ventricles) and for their exclusion from the atrioventricular canal (Habets et al., 2002; Harrelson et al., 2004; Hoogaars et al., 2004).

Tbx20 is a recently described T-box transcription factor that is expressed most notably in the heart, retina and motoneurons in vertebrates (Ahn et al., 2000; Brown et al., 2003; Carson et al., 2000; Kraus et al., 2001; Meins et al., 2000; Plageman and Yutzey, 2004; Stennard et al., 2003; Takeuchi et al., 2003). *Tbx20* knockdown by morpholino antisense RNA in zebrafish or *Xenopus* results in abnormal cardiac morphogenesis (Brown et al., 2005; Szeto et al., 2002), but the role of Tbx20 in mammalian heart formation, or its mechanism of action, have not been elucidated. Furthermore, conflicting data exist regarding the activity of Tbx20 as a transcription factor: depending on the target gene and on the presence of other cardiac transcription factors, Tbx20 can either activate or repress transcription (Plageman and Yutzey, 2004; Stennard et al., 2003; Takeuchi et al., 2003).

In order to investigate the roles of *Tbx20* in mammalian embryonic development, we have inhibited *Tbx20* function by transgenic RNA interference (RNAi) in embryonic stem (ES) cell-derived mouse embryos (Kunath et al., 2003; Lickert et al., 2004). We find that *Tbx20* is essential for normal cardiac chamber formation, especially that of the outflow tract and right ventricle, the anterior derivatives of the secondary/anterior heart field (AHF) (Cai et al., 2003; Kelly et al., 2001; Meilhac et al., 2004). Incomplete knockdown of *Tbx20* results in hypoplastic right ventricle and persistent truncus arteriosus, as well as severely compromised valve formation. In the central nervous system, *Tbx20* is required for differentiation of motoneurons, in particular expression of *Isl2* and *Hb9*, genes that encode transcription factors essential for motoneuron differentiation (Thaler et al., 2004). Tbx20 can interact with Isl1, which is crucial for AHF formation (Cai et al., 2003), to activate the AHF enhancers of both *Mef2c* and *Nkx2-5*. These results indicate that Tbx20 is a dose-sensitive regulator of terminal differentiation events in cardiogenesis and neurogenesis, and that it does so via interactions with and regulation of transcription factor networks.

Materials and methods

In vivo RNAi and transgenesis

In vivo RNA interference was performed as previously described (Kunath et al., 2003; Lickert et al., 2004), except that 129S6B6 F1-hybrid (G4) ES cells (Vintersten et al., 2004) were used. Two short interfering (siRNA) sequences (siTbx20-a and siTbx20-b) were designed to specific and distinct regions of the *Tbx20* mRNA. Tbx20-a target sequence was 5′-CACCATCAAACCCCTGGAA-3′; Tbx20-b target sequence was 5′-TCATCATGTGCCCAGCCAC-3′. Real-time reverse transcriptase-mediated PCR was employed for quantitation of *Tbx20* mRNA levels in undifferentiated ES cells to select knockdown ES cell lines. The *Tbx20* siRNA cassettes were also introduced into a vector that allows expression of dsRed under control of the EF1a promoter. Aggregations with tetraploid embryos were performed using knockdown ES cells to generate embryos composed entirely of knockdown ES cells (Nagy et al., 2003; Nagy et al., 1993). Tetraploid embryos providing extra-embryonic tissues in the complementation assay expressed enhanced green fluorescent protein (EGFP) (Hadjantonakis et al., 1998), thereby allowing visual confirmation that only embryos totally derived from ES cells were used in the study. Wild-type embryos from natural matings were used as controls. All embryos were stage-matched to controls by somite count. Transient transgenic mouse embryos were generated by pronuclear injection as previously described (Dodou et al., 2004; Nagy et al., 2003).

In situ hybridization and antibody staining

In situ hybridization was preformed according to standard protocols. For fluorescent whole-mount in situ hybridization, embryos were hybridized with a mixture of digoxigenin-labeled *Mybpc3* and *Actc* probes, and detection was performed using rhodamine tyramide amplification reagents (Perkin-Elmer). Whole-mount immunofluorescence was carried out on embryos fixed in Dent's fixative, using MF20 antiserum directly conjugated to Alexa 594 (Molecular Probes) and unconjugated rat anti-PECAM monoclonal antibody (Pharmingen) visualized with an Alexa 488-conjugated secondary antibody (Molecular Probes). The MF20 antibody developed by D. A. Fischman was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa (Department of Biological Sciences, Iowa City, IA 52242). Western blot for Tbx20 was performed using affinity-purified rabbit antiserum raised against Tbx20.

Optical projection tomography

Optical projection tomography (OPT) was performed essentially as described (Sharpe, 2004; Sharpe et al., 2002), on embryos fluorescently labeled by whole-mount in situ hybridization or immunofluorescence. Analysis and visualization of OPT data was performed with Amira V.3.0 (TGS).

Transactivation assays and reporter constructs

Transactivation assays were performed essentially as described (Bruneau et al., 2001; Durocher et al., 1997). The *Nppa-luc*, *Nkx2-5-luc* (FL construct) and *Mef2c-lacZ* reporters have been previously described (Brown et al., 2004; Dodou et al., 2004; Durocher et al., 1997). Expression constructs for Tbx20 were generated by introducing a full-length *Tbx20* cDNA with an N-terminal Myc epitope tag into pcDNA3.1. All other expression constructs were previously described (Lickert et al., 2004). The *Nkx2-5* 2.5 kb *lacZ* reporter (Lien et al., 1999) (a kind gift from Dr E. Olson, UT Southwestern, Dallas, TX) consisted of nucleotide residues –9700 to –6187 relative to the transcriptional start site upstream of the hsp68-*lacZ* reporter gene (Kothary et al., 1989). Mutations in the putative Isl1-binding site were introduced by PCR.

Co-immunoprecipitation assays

HeLa cells were transfected with an Isl1 expression construct together with FLAG-Gata4, Myc-Tbx20, Myc-Tbx1 or FLAG-Tbx5 expression constructs. Co-immunoprecipitation was preformed as previously described (Lickert et al., 2004).

Development

Development

Results

Expression of Tbx20

As a prelude to functional studies, we examined *Tbx20* expression in detail, and have identified several novel aspects of *Tbx20* expression in the mouse. The first is during early heart formation, when *Tbx20* is expressed in the cardiac crescent as previously described (Carson et al., 2000; Kraus et al., 2001; Meins et al., 2000; Plageman and Yutzey, 2004; Stennard et al., 2003), but also in a more medial field of cells that appears to correspond to a region of the AHF (Fig. 1A). Indeed, at E7.75, E8.5 and E9.5 (not shown), *Tbx20* expression overlaps with the AHF domain of *Nkx2-5* (Fig. 1B-B″) and partly overlaps with expression of *Isl1* (Fig. 1C-C″), which defines the AHF (Cai et al., 2003). Second, during heart chamber formation, as in chicken (Takeuchi et al., 2003), *Tbx20* is expressed more strongly in the right ventricle (RV) than in the left ventricle (LV) (Fig. 1D-D″), and its highest expression abuts that of *Tbx5* at the interventricular septum. We also confirmed the later stage expression of *Tbx20* in cushions of the atrioventricular (AV) junction and outflow tract (Fig. 1E-E″), which later is confined to valves of the great vessels and AV junction (Fig. 1F-F″). Third, *Tbx20* is expressed in dorsal aorta, but not cardinal vein (Fig. 1G,H), similar to zebrafish *Tbx20* (Ahn et al., 2000; Lawson et al., 2001). Zebrafish dorsal aorta differentiation relies on sonic hedgehog (*Shh*) (Lawson et al., 2002). To investigate if *Tbx20* in the dorsal aorta relies on *Shh* in the mouse, we analyzed expression of *Tbx20* in *Shh*-null embryos. As with zebrafish, expression of *Tbx20* relies on *Shh* in the dorsal aorta of the mouse (Fig. 1G,H). *Tbx20* also depends on Shh signaling in the spinal cord (Fig. 1G,H), similar to other markers of differentiating motoneurons (Chiang et al., 1996; Litingtung and Chiang, 2000).

We also examined expression of *Tbx20*-a, which includes a dual activation and repression domain (Stennard et al., 2003), using a cDNA fragment specific to this splice variant (Fig. 2A). We found that unlike the more widespread expression detected by a probe directed to all splice variants of *Tbx20*, *Tbx20a* is expressed in a more-restricted pattern, which in the heart is primarily confined to the developing outflow tract (Fig. 2B).

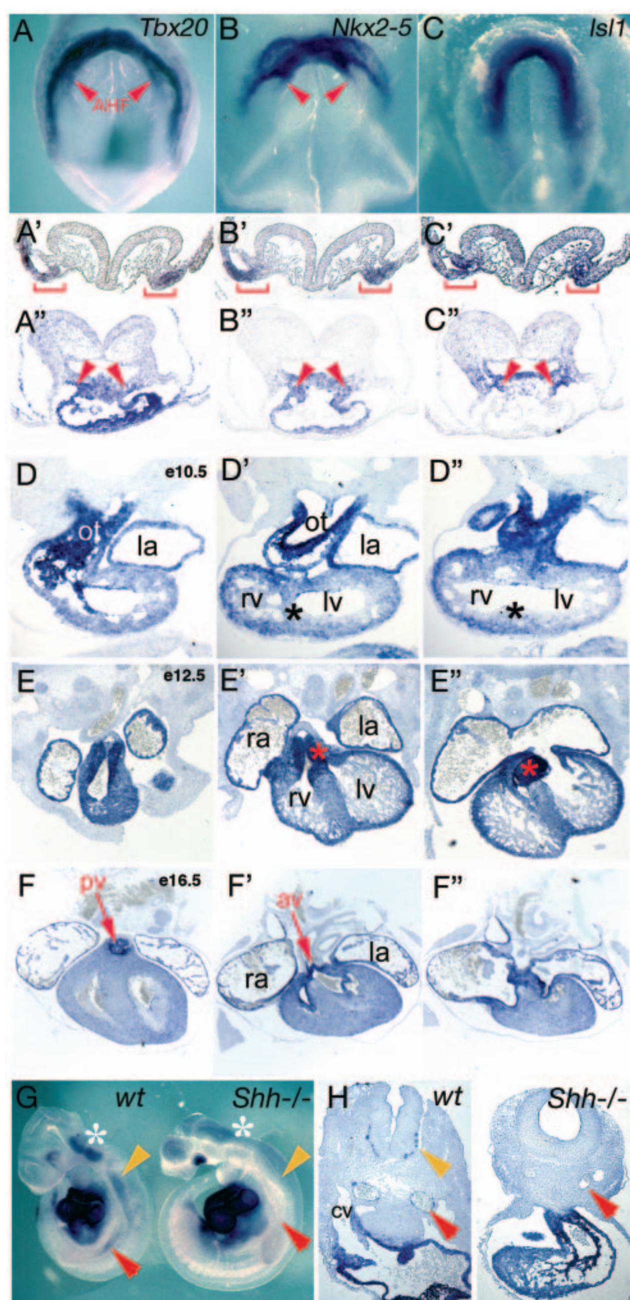

Fig. 1. Expression of *Tbx20* in mouse development. *Tbx20* is expressed in cardiac progenitors at E7.75 (A), including a medial domain of cells corresponding to a similar domain (B) of expression of *Nkx2-5* (red arrowheads). These medial domains are a part of the anterior heart field (AHF), and overlap with expression of *Isl1* (C). Sections of the embryos shown in A-C are shown in A′-C′. Sections of embryos at E8.5 are shown in A″-C″; arrowheads show overlapping *Tbx20*, *Nkx2-5* and *Isl1* expression. (D-F) Dynamic expression of *Tbx20* at E10.5 (D-D″), E12.5 (E-E″) and E16.5 (F-F″). At E10.5 (D-D″), *Tbx20* is expressed in the myocardium and cushion of the outflow tract (ot), in the myocardium and endocardium of the right ventricle (rv), and in the atria (la), with weaker expression in the left ventricular (lv) myocardium. Asterisk indicates the interventricular septum. *Tbx20* is expressed most strongly in the endocardial cushions (red asterisks) at E12.5 (E-E″); at E16.5 (F-F″), its expression is primarily confined to cushion derivatives such as the pulmonary valve (pv) and the aortic valve (av). (G,H) *Tbx20* expression in motoneurons (asterisk and ochre arrowhead) and dorsal aorta (red arrowhead) is lost in $Shh^{-/-}$ embryos at E9.5. Whole mounts are shown in G, while transverse sections are shown in H.

In vivo RNAi of Tbx20

To address the roles played by *Tbx20* in development, we employed an in vivo RNAi strategy (Kunath et al., 2003; Lickert et al., 2004). We designed two different siRNA sequences directed to different regions of the *Tbx20* mRNA (see Fig. S1A in the supplementary material). These were cloned into an H1 short hairpin RNA (shRNA) expression vector that also had a dsRed expression cassette (EF1a-dsRed) to visualize cells transfected with the plasmids. Transient transfection of the *Tbx20* shRNA vectors with a mycTbx20;IRES-enhanced green fluorescent protein (EGFP) expression plasmid into 10T1/2 cells demonstrated efficient reduction of *Tbx20* mRNA, as assessed by Western blot and EGFP fluorescence (see Fig. S1B,F,F′ in the supplementary material). Expression of FLAG-Tbx5, Tbx5;IRES-EGFP or

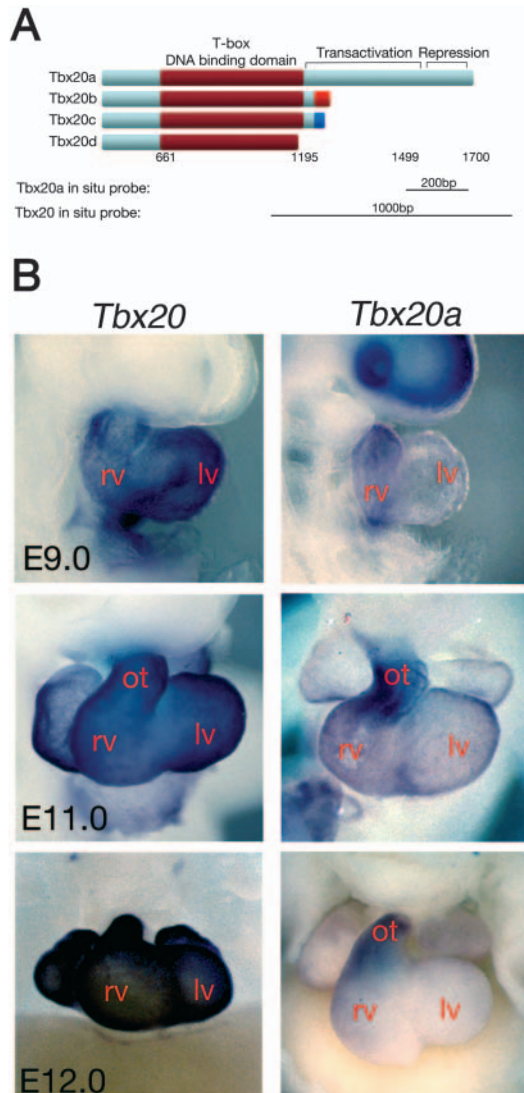

Fig. 2. Restricted expression of *Tbx20* isoform a. (A) cDNAs encoding Tbx20 isoforms a-d [adapted, with permission, from Stennard et al. (Stennard et al., 2003)]. Location and size of the in situ probes are indicated, along with nucleotide positions along *Tbx20* cDNA. (B) In situ hybridization using *Tbx20* (left) or *Tbx20a* (right) in situ probes on E9.5, E11.0 and E12.0 embryos. Apparent *Tbx20a* staining in brain at E9.5 is due to artefactual probe trapping. lv, left ventricle; rv, right ventricle; ot, outflow tract.

mycTbx1;IRES-EGFP was not affected by the Tbx20 shRNAs (see Fig. S1C,D,G-H′ in the supplementary material), demonstrating specificity of the siRNA sequences.

Vectors expressing shRNAs directed against *Tbx20* (without the dsRed cassettes) were electroporated into a hybrid ES cell line (G4), derived from crosses of 129SvJ and C57Bl/6 mice. Quantitative real-time RT-PCR was used to identify ES cell clones with a significant reduction in *Tbx20* mRNA levels (*Tbx20* knockdown clones). For each shRNA sequence, several knockdown clones were identified, with a range of efficiency of reduction of *Tbx20* mRNA (Fig. 3A). Aggregations with tetraploid embryos were performed to generate entirely ES cell-derived embryos from several knockdown lines. Because RNAi can be used to generate an epiallelic series (Hemann et al., 2003; Kunath et al., 2003; Lickert et al., 2004), we examined the effect of reducing *Tbx20* to varying levels on mouse embryo development. The following knockdown ES cell lines were used to generate entirely ES cell-derived embryos: b12, which has a 96% reduction in *Tbx20* mRNA levels; a6 and b2, which have an 80-85% reduction in *Tbx20* mRNA levels; and a4 and b3, which have a 65% reduction in *Tbx20* mRNA levels. Western blot on protein extracted from E9.5 embryos (Fig. 3B) revealed that line b12 had less than 5% wild-type Tbx20 protein, a6 8% and a4 40% wild-type Tbx20 levels. In situ hybridization showed residual *Tbx20* mRNA in the heart of line a6-derived embryos, but no detectable *Tbx20* mRNA in b12 embryos (Fig. 3C).

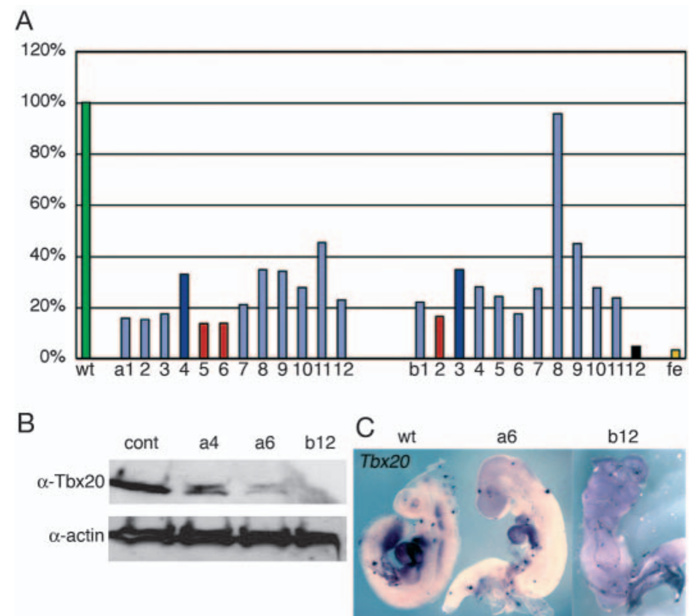

Fig. 3. Efficient *Tbx20* mRNA knockdown in embryonic stem (ES) cells (A) and ES cell-derived embryos (B,C). (A) Quantitative RT-PCR of *Tbx20* mRNA levels in wild-type ES cells (wt) or ES cells stably transfected with constructs expressing *Tbx20* shRNA a or b. Tetraploid aggregations were performed with ES cells with a 95% reduction in *Tbx20* mRNA levels (b12), an 85% reduction in *Tbx20* mRNA levels (a5, a6, b2) or a 65% reduction in *Tbx20* mRNA levels (a4, b3). (B) Western analysis of protein extract from whole E9.5 embryos derived from lines a4, a6 and b12, and from wild-type embryos. (C) In situ hybridization for *Tbx20* on wild-type, a6 and b12 embryos.

Tbx20 and cardiac morphogenesis

Embryos derived from line b12 ('b12 knockdown embryos') died around E9, presumably from severe defects in cardiac morphogenesis (Figs 4, 5). For this line, as well as for all other lines examined, consistent defects were observed in >90% of GFP negative embryos obtained from at least three aggregations (each generating 8-12 embryos). The defects in b12 knockdown embryos consisted of impaired cardiac chamber formation, especially of the RV and outflow tract. *Tbx20* b12 knockdown embryos had no morphologically distinguishable outflow tract. A chamber anterior to the prospective LV, most probably the RV, was connected to the LV by an abnormal constriction. Marker analysis confirmed

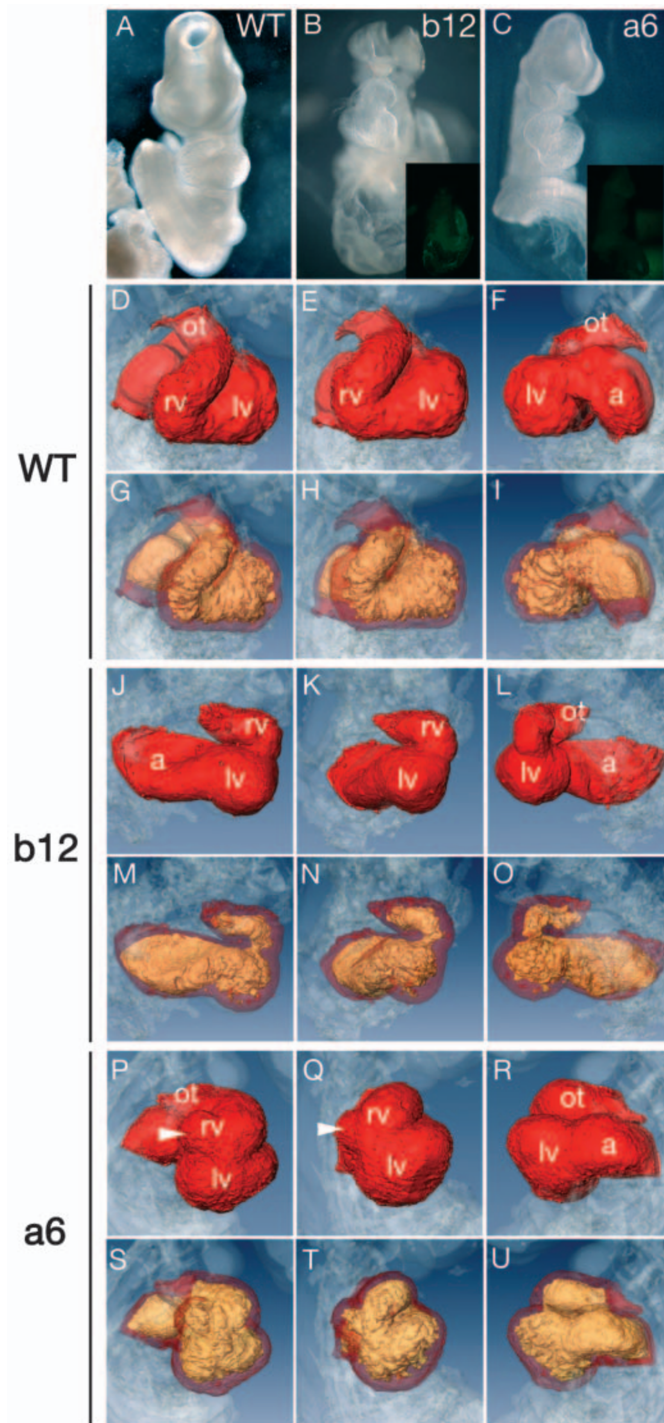

Fig. 4. Altered cardiac morphology in *Tbx20* knockdown embryos. (A-C) Bright-field frontal views of E9.25 embryos. (A) Wild-type, (B) b12 knockdown and (C) a6 knockdown. Insets in B,C show the absence of EGFP signal in the embryo. (D-U) Rendered optical projection tomography of embryos stained for *Actc* and *Mybpc3* to label cardiac myocytes. Embryo is rendered translucent white; heart is red (D-F,J-L,P-Q); chamber fill is yellow (G-I,M-O,S-U). Views are from the right (D,G,J,M,P,S), front (E,H,K,N,Q,T) or left (F,I,L,O,R,U) sides. (D-I) Wild-type embryo; (J-O) b12 knockdown embryo; (P-U) a6 knockdown embryo. a, atrium; lv, left ventricle; ot, outflow tract; rv, right ventricle. White arrowhead indicates unusual bulging of the rv.

that the constriction was the anteriormost boundary of the LV (see below, Fig. 7). This abnormality, combined with an absence of rightward heart looping, resulted in a linear heart tube with an hourglass appearance (Fig. 4B,J-O). Atrial development was less affected, although there was no distinction between left and right atria, resulting in a smaller single atrium (Fig. 4J-O; Fig. 5E,K). Line a6 had more residual *Tbx20* mRNA, and its defects in cardiac morphogenesis were less pronounced, resulting in lethality at E11-E12. In a6 knockdown embryos, the hearts looped, but chamber formation was abrogated, leading to hypoplastic RV (Fig. 4C,P-U), with occasionally an additional bulge protruding from the RV (arrowhead in Fig. 4P,Q). Similar results were obtained for line b2. Histological analysis (Fig. 5) revealed very thin myocardium in line a6, perhaps secondary to hemodynamic insufficiency. At E9.5, increased TUNEL staining in the compact layer and endocardium indicated an increase in apoptosis in these cell layers; overall decreased Ki67 staining was observed, with more pronounced reduction in Ki67+ cells in the endocadium, reflecting decreased proliferation (see Fig. S2 in the supplementary material). In both b12 and a6 *Tbx20*

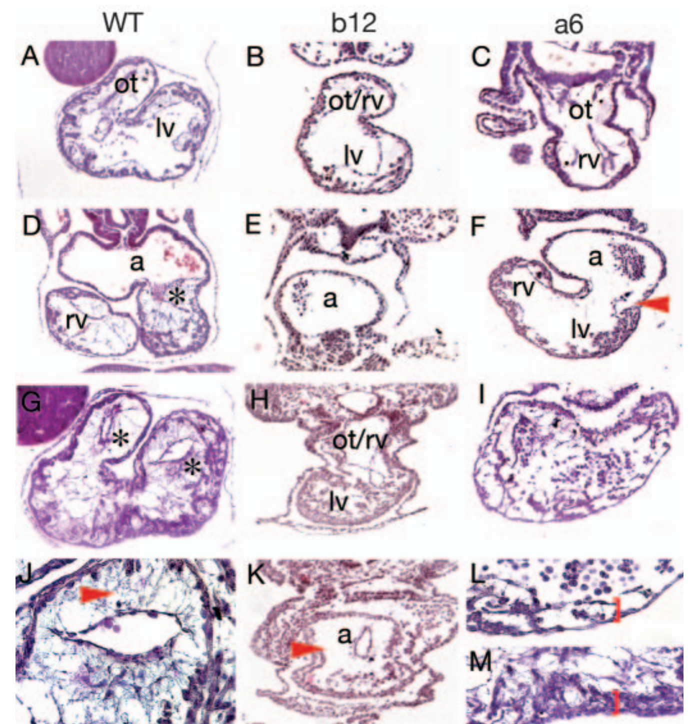

Fig. 5. Histological analysis of altered cardiac morphology in *Tbx20* knockdown embryos at E9.5. (A,D,G,J) Wild-type embryos. (B,E,H,K) *Tbx20* b12 knockdown; B,E and H,K correspond to two distinct embryos. (C,F,I,L) *Tbx20* a6 knockdown. The outflow tract and right ventricle (ot/rv) are combined in b12 embryos. Epithelial to mesenchymal transformation (EMT) of both the outflow and AV cushions is impaired (arrowheads in F,K; see ot in C) compared with wild-type embryos (asterisks in G, arrowhead in J). (J) Higher magnification of the atrioventricular canal in wild type compared with a b12 knockdown (K) embryo, showing the lack of EMT in the b12 embryo (arrowhead). (L,M) Higher magnification of left ventricular wall of a6 (L) and wild-type (M) embryos, showing thinned ventricular wall in a6 (compare red brackets). a, atrium; lv, left ventricle; ot, outflow tract; rv, right ventricle.

knockdown embryos, there was a complete lack of endocardial cushion formation, presumably owing to impaired epithelial to mesenchymal transformation (Fig. 5).

Congenital heart defects due to partial inhibition of Tbx20

Embryos derived from lines a4 and b3, which had a milder (60%) reduction in Tbx20 levels, survived until E13 (Fig. 6A). These milder knockdown embryos had distinct defects in cardiac morphogenesis (Fig. 6B-N). All embryos examined had an unseptated outflow tract, resulting in persistent truncus arteriosus and double outlet RV (Fig. 6C,D,F,K,L). The distal connections to the presumptive aorta and pulmonary artery were appropriately maintained. Most embryos examined also had a hypoplastic RV. These phenotypes reflect a dose-sensitive role for *Tbx20* in RV growth and outflow tract remodeling. An important finding in line a4 *Tbx20* knockdown embryos was a severe defect in valve formation. *Tbx20* is very strongly expressed in the endocardium, and later in valve primordia (Fig. 1) (Lincoln et al., 2004; Plageman and Yutzey, 2004; Stennard et al., 2003). Line a4 knockdown embryos had no identifiable pulmonic valves, and had only very rudimentary aortic and tricuspid valve formation (Fig. 6L-N). Thus, our epiallelic series allowed us to unmask this very important function of *Tbx20* in regulating valve formation.

Cardiac gene expression in Tbx20 knockdown embryos

Marker gene analysis revealed that *Tbx20* is important for fundamental transcriptional pathways in heart development (Fig. 7). Expression of *Nkx2-5*, a homeodomain transcription factor required for activation of several cardiac genes (Lyons et al., 1995; Tanaka et al., 1999), was greatly reduced in *Tbx20* b12 knockdown embryos (Fig. 7A). This decrease was apparent at E8.5, but was not evident at cardiac crescent stages (E7.75, data not shown), suggesting that Tbx20 is required for maintenance, but not initiation, of *Nkx2-5* expression. Expression of *Tbx5*, which delineates the boundary between the LV and RV (Bruneau et al., 1999), was normal, providing a clear marker for the identity of the chambers in the knockdown embryos. *Nppa* is a marker of differentiating working myocardium (Christoffels et al., 2000). *Nppa* mRNA levels were significantly decreased in *Tbx20* b12 ventricular myocardium, although atrial expression appeared normal (Fig. 7C). Expression of *Nppa* was not significantly affected in line a6, thus demonstrating that Tbx20 is important in a dose-dependent manner for chamber myocardium differentiation. Expression of *Hand1* and *Hand2*, which mark the LV and RV, respectively, was decreased in b12 knockdown embryos (Fig. 7E,J). Expression of *Bmp4*, *Mef2c* and *Pitx2*, which are involved in outflow tract development (Delot et al., 2003; Kioussi et al.,

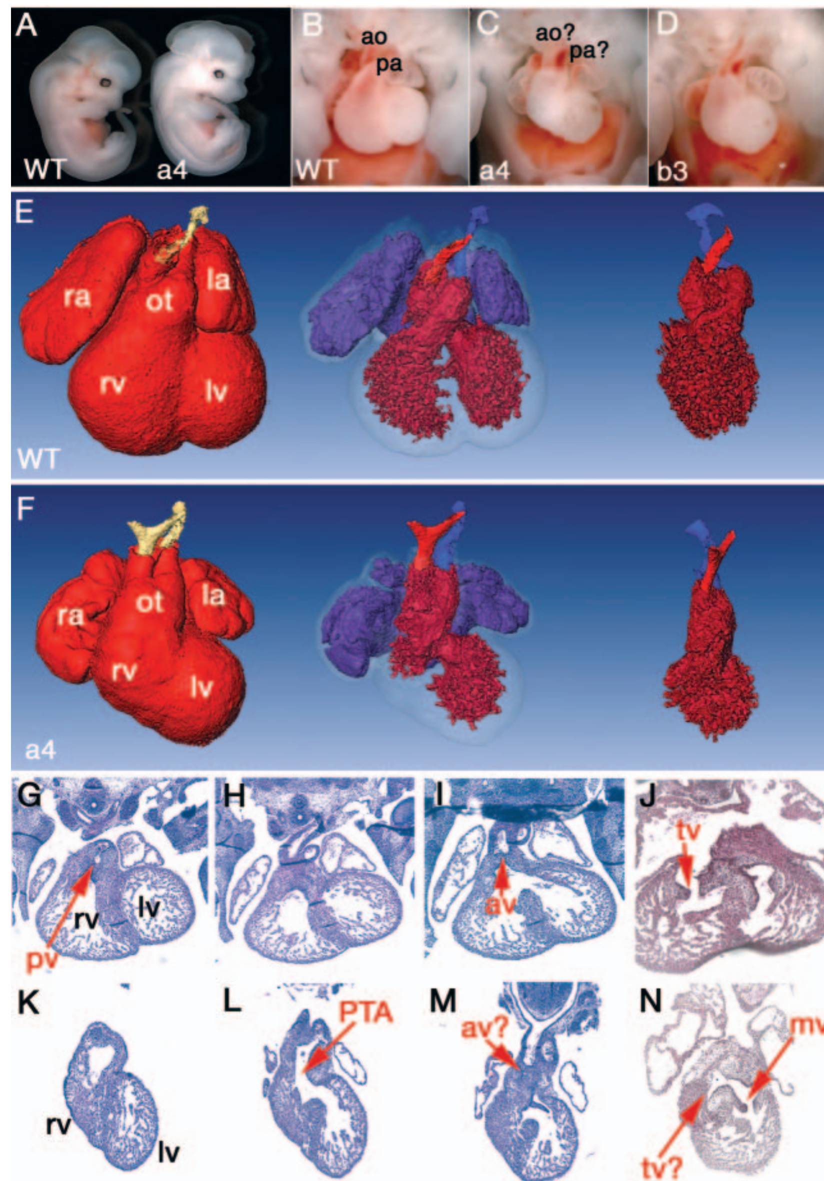

Fig. 6. Cardiac defects resulting from a mild knockdown of *Tbx20* resemble human congenital heart defects. (A) External view of E12.5 wild type and a4 knockdown embryos, showing exencephaly in the a4 knockdown embryo. (B-D) Bright-field view of E12.5 hearts from wild-type (B), and a4 (C) and b3 (D) knockdown embryos. The right ventricle exhibits hypoplasia and there is abnormal septation of the outflow tract in a4 and b3 knockdown embryos. (E,F) Rendered optical projection tomography (OPT) of wild-type (E) and a4 knockdown (F) embryos. (E,F) Surface rendered views of OPT scan (left), followed by rendered chamber fills of OPT scans (middle; atrial chambers are purple, ventricular chambers are dark red, pulmonary artery is blue and aorta is red); and lateral view of the chamber fills (right), with the atria removed. The outflow tracts are spiral and straight in the wild-type and knockdown embryo, respectively. (G-N) Histology of wild-type (G-J) and a4 knockdown (K-N) embryos. There is a lack of outflow valves in L, and very primitive aortic and tricuspid valves in M,N. ao, aorta; av, aortic valve; la, left atrium; lv, left ventricle; mv, mitral valve; pa, pulmonary artery; PTA, persistent truncus arteriosus; pv, pulmonic valve; ra, right atrium; rv, right ventricle; tv, tricuspid valve.

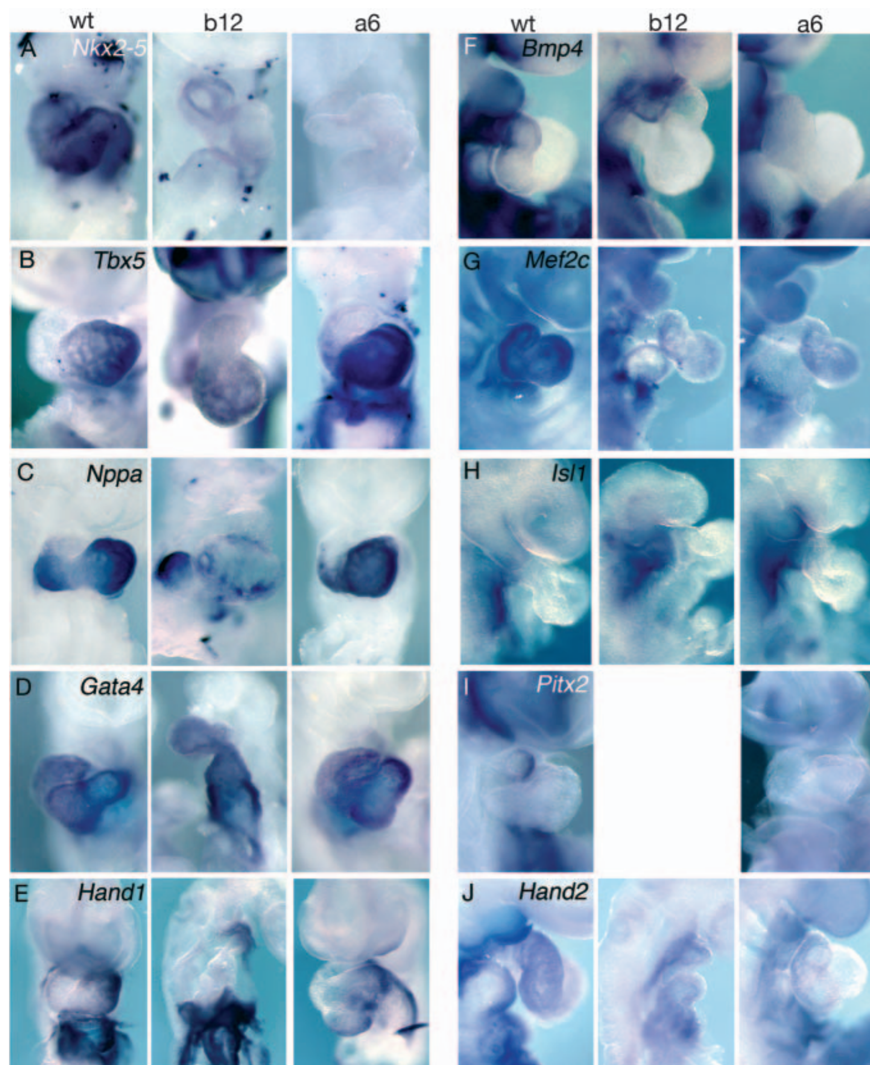

Fig. 7. Altered cardiac gene expression in *Tbx20* knockdown embryos at E9.0. Expression of *Nkx2-5* (A), *Tbx5* (B), *Nppa* (C), *Gata4* (D), *Hand1* (E), *Bmp4* (F), *Mef2c* (G), *Isl1* (H), *Pitx2* (I) and *Hand2* (J) by whole-mount in situ hybridization of E9.0 wild-type (WT), b12 knockdown (b12) and a6 knockdown (a6) embryos are shown.

2002; Lin et al., 1997; Liu et al., 2002; Liu et al., 2004), was also reduced in *Tbx20* knockdown embryos (Fig. 7F,G,I), consistent with a failure of this structure to develop. *Bmp4* expression was maintained in the distal outflow tract, but its expression was reduced in the RV and proximal outflow tract (Fig. 7F). Expression of *Isl1* was not affected by loss of Tbx20 (Fig. 7H). The decreased expression of both *Nkx2-5* and *Mef2c* in *Tbx20* knockdown embryos suggests a fundamental defect in the AHF, which contributes to the anterior segment of the heart, i.e. the outflow tract and RV (Cai et al., 2003; Kelly et al., 2001; Meilhac et al., 2004; von Both et al., 2004), and an impairment of chamber differentiation.

Tbx20 and motoneuron development

Tbx20 is expressed in post-mitotic motoneurons of the spinal cord in mouse and chick (Fig. 1G,H; Fig. 8A,C) (Iio et al., 2001; Kraus et al., 2001). The delayed lethality and pronounced decrease in neural *Tbx20* mRNA levels in a6 knockdown embryos allowed the examination of markers of motoneuron development at E9.5 to assess a potential role for *Tbx20* in these cells (Fig. 8). Isl1, Isl2 and Hb9 are LIM-homeodomain transcription factors that are collectively required for differentiation of somatic and visceral motoneurons (Arber et al., 1999; Pfaff et al., 1996; Thaler et al., 1999; Thaler et al., 2004). Expression of *Isl2* and *Hb9* was decreased in *Tbx20* knockdown embryos, while expression of *Isl1* was intact (Fig. 8A). Dorsoventral patterning of the spinal cord was not affected in *Tbx20* knockdown embryos, as *Pax6* and *Irx3* expression was normally patterned (Fig. 8B). These results suggest that Tbx20 is a crucial determinant of motoneuron differentiation, via activation of motoneuron-specific transcription factors. The activation may be indirect, as we did not detect complete overlap of *Tbx20* expression with that of *Isl2* and *Hb9* at E9.5 (Fig. 8A). However, earlier (E9.0) the expression patterns of *Tbx20*, *Isl1*, *Isl2* and *Hb9* overlapped almost completely (Fig. 8C), suggesting that the effect of Tbx20 on *Isl2* and *Hb9* might be direct at this stage.

Tbx20 activates AHF enhancers in cooperation with Isl1 and Gata factors

Tbx20 may be involved in directly regulating the expression of cardiac genes (Plageman and Yutzey, 2004; Stennard et al., 2003; Takeuchi et al., 2003). We examined the potential for Tbx20 to activate the promoter/enhancers of *Nppa*, *Pitx2*, *Fgf10*, *Nkx2-5*, *Gja5* (encoding Cx40) and *Myh7* (β-myosin heavy chain) in 10T1/2 cells. Tbx20 expression resulted in potent activation of *Nkx2-5*, *Pitx2*, *Fgf10* and *Myh7* regulatory elements, but failed to significantly activate the *Nppa* and *Gja5* promoters (Fig. 9A). This was in contrast to Tbx5, which could activate all promoters/enhancers tested. Tbx1, which is also important for activation of specific genes in the outflow tract and AHF (Hu et al., 2004; Xu et al., 2004), could modestly activate the *Nkx2-5*, *Pitx2* and *Fgf10* reporters, but could not significantly activate the *Myh7*, *Nppa* and *Gja5* promoter/enhancers. Thus, Tbx20 can activate specific cardiac enhancers.

Expression of *Mef2c* and *Nkx2-5*, transcription factors required for AHF formation, was reduced in *Tbx20* knockdown embryos. AHF expression of *Mef2c* relies on a defined enhancer that requires Isl1-, Nkx2-5- and Gata-binding sites (Dodou et al., 2004; von Both et al., 2004). Co-transfection in 10T1/2 cells of a Tbx20 expression construct and the *Mef2c-lacZ* reporter, which comprises the AHF enhancer and the endogenous *Mef2c* promoter (Dodou et al., 2004), resulted in strong activation of *Mef2c-lacZ* (Fig. 9B). Isl1 expression could also activate *Mef2c-lacZ*, and co-transfection of both Isl1

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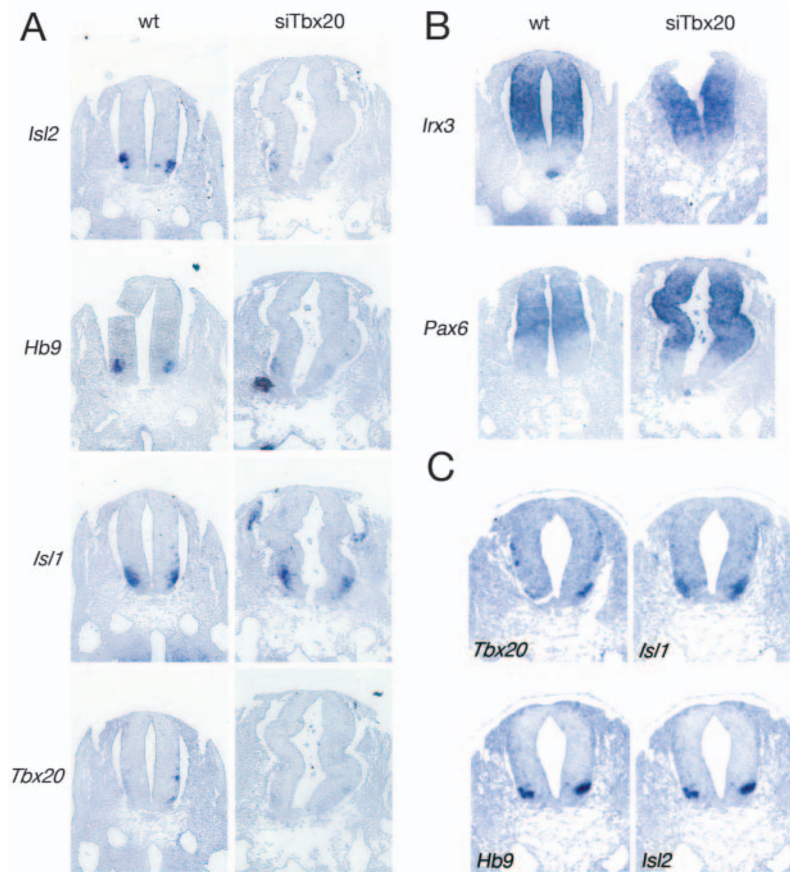

Fig. 8. Abnormal motoneuron differentiation in *Tbx20* knockdown embryos. (A) Expression of *Tbx20* and motoneuron markers *Isl2*, *Hb9* and *Isl1* in sections of E9.5 embryos at the thoracic level. *Isl2* and *Hb9* expression is decreased in *Tbx20* knockdown embryos (siTbx20) compared with wild-type embryos (wt). (B) Normal dorsoventral patterning of the spinal cord in *Tbx20* knockdown embryos, as shown by expression of *Irx3* and *Pax6*. (C) Expression of *Tbx20*, *Isl1*, *Isl2* and *Hb9* in overlapping domains in the spinal cord at E9.

and Tbx20 expression constructs resulted in synergistic activation of *Mef2c-lacZ*. Similarly, Gata4 or Nkx2-5 expression (but not Foxh1) could activate *Mef2c-lacZ* and could synergize with Tbx20.

A proximal enhancer required for *Nkx2-5* expression in the AHF and its derivatives has been shown to require Gata-binding sites (Lien et al., 2002; Lien et al., 1999; Searcy et al., 1998). Gata-binding sites at a more distal enhancer are also required for expression throughout the heart tube (Brown et al., 2004). We examined the upstream regulatory regions of the proximal *Nkx2-5* enhancer and identified a conserved Isl1-binding site, located adjacent to the Gata site required for AHF expression (Fig. 9C). This arrangement of Isl1- and Gata-binding sites is identical to that of the *Mef2c* AHF enhancer (Dodou et al., 2004). We mutated the Isl1-binding site and assessed the activity of the enhancer linked to a *lacZ* reporter gene. Unlike the intact *Nkx2-5-lacZ* construct that expressed β-gal primarily in the RV and outflow tract (Fig. 9D,F), mutation of the Isl1 enhancer disrupted expression of *Nkx2-5-lacZ* (Fig. 9E,G).

Co-transfection in 10T1/2 cells of a Tbx20 expression construct with *Nkx2-5-luciferaseFL*, which includes several cardiac enhancers and the endogenous *Nkx2-5* promoter (Brown et al., 2004), resulted in strong activation of the *Nkx2-5* reporter gene (Fig. 9A). Co-transfection of a Gata4 expression construct along with the Tbx20 expression construct resulted in synergistic activation of *Nkx2-5-luciferase* (Fig. 9H). No synergistic activation was observed with activated bone-morphogenic protein receptor (aAlk3), but the combination of Tbx20, Gata4, and aAlk3 resulted in strong synergy, indicating that Tbx20 can activate the *Nkx2-5* gene via interactions with Gata4 (Fig. 9H). We also co-transfected in 10T1/2 cells the *Nkx2-5-luciferaseFL* construct with Tbx20 in combination with an Isl1 expression construct (Fig. 9H). As with the *Mef2c* enhancer, Tbx20 could readily activate the *Nkx2-5* enhancer synergistically with Isl1. Co-immunoprecipitation experiments in HeLa cells revealed physical interactions between Isl1 and Tbx20 (Fig. 9I). We conclude that Tbx20 is a potent activator of transcription factors that regulate the AHF, and that Tbx20 can activate AHF genes via interactions with Isl1, Nkx2-5 and Gata4, which are crucial for AHF expansion and gene expression (Cai et al., 2003; Dodou et al., 2004; Tanaka et al., 1999).

Discussion

We have demonstrated that the T-box transcription factor Tbx20 is a key upstream regulator of transcription factor networks essential for cardiac and motoneuron development. Our results also indicate that Tbx20 dose is crucial for specific morphogenetic events, and thus *TBX20* is an excellent candidate gene for human congenital heart defects. Furthermore, the cooperative interaction of Tbx20 with the LIM-homeodomain transcription factor Isl1 on enhancers active in the AHF provide a unifying mechanism of action for Tbx20 as a crucial transcription factor in the morphogenesis of this segment of the developing heart.

Tbx20 and cardiac morphogenesis

Loss of Tbx20 resulted in severe defects in cardiac morphogenesis, most notably of the anterior segment of the heart, the RV and outflow tract. In particular, a pronounced lack of outflow tract was observed, both morphologically and by genetic marker analysis. The dysmorphogenesis of the RV appeared less pronounced in the most severe knockdown lines compared with embryos with slightly more remaining Tbx20, perhaps because the embryos died at a stage at which RV development is rudimentary. Alternatively, the more restricted expression of the *Tbx20a* splice variant in the outflow tract suggests that this isoform may have specific functions that are more important than the other more widely expressed isoforms. In embryos derived from line a6, which survived slightly longer than those from the severe b12 line, clear defects in RV formation were observed, indicating that Tbx20 is also important for RV formation. Furthermore, marker analysis revealed defects in cardiac chamber differentiation. Therefore, loss of Tbx20 affects morphogenesis and differentiation of individual segments of the developing heart. The phenotypes

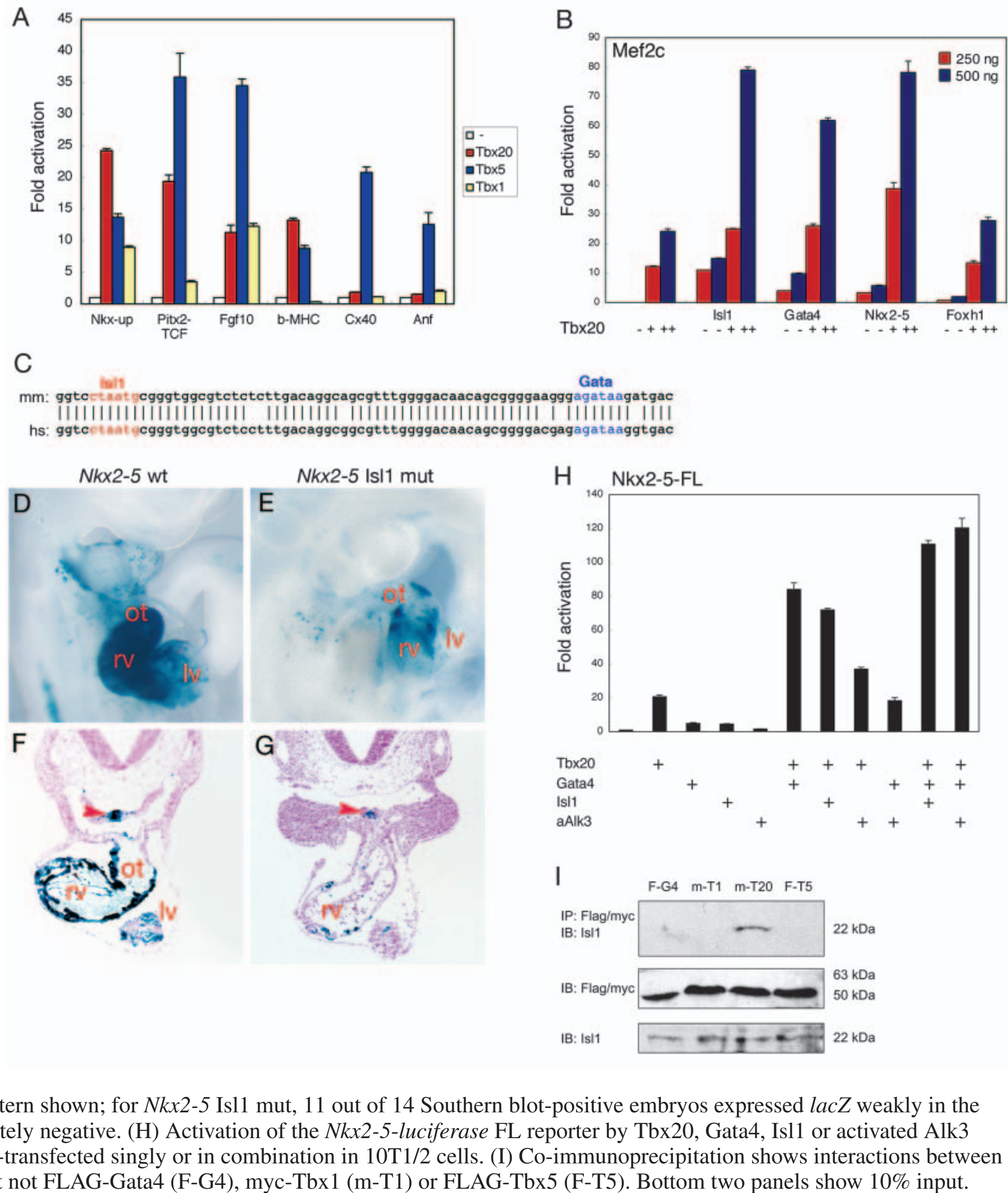

Fig. 9. Tbx20 cooperatively activates transcription. (A) Activation of various reporter constructs by Tbx20, Tbx5 or Tbx1 expression constructs co-transfected into 10T1/2 cells. Reporter constructs used are *Nkx2-5-luciferase*FL (Nkx-up), *Pitx2-luciferase* (Pitx2), *Fgf10-luciferase* (Fgf10), *Myl7-luciferase* (b-MHC), *Gja5-luciferase* (Cx40) and *Nppa-luciferase* (ANF). (B) Activation of the *Mef2c-lacZ* #3 reporter by Isl1, Gata4, Nkx2-5 or Foxh1 expression constructs, with (+, ++) or without (–) a Tbx20 expression construct. Red bars indicate 250 ng of the indicated expression construct, blue bars indicate 500 ng of the expression construct. +, 250 ng Tbx20 expression construct; ++, 500 ng Tbx20 expression construct. (C) Alignment of mouse (mm) and human (hs) sequence of part of the *Nkx2-5* enhancer that comprises the Isl1-binding site (Isl1, red) and the Gata-binding site (Gata, blue). (D-H) Transgenic mouse embryos (E9.75) carrying the *Nkx2-5-lacZ* transgene (*Nkx2-5* wt, D,F), or the *Nkx2-5-lacZmutIsl1* transgene (*Nkx2-5* Isl1 mut, E,G) shown as whole-mounts (D,E) or sections of the whole-mount stained embryos (F,G). Arrowhead shows expression in pharynx. For *Nkx2-5* wild type, nine out of 12 Southern blot-positive embryos expressed *lacZ* strongly in the pattern shown; for *Nkx2-5* Isl1 mut, 11 out of 14 Southern blot-positive embryos expressed *lacZ* weakly in the pattern shown, three were completely negative. (H) Activation of the *Nkx2-5-luciferase* FL reporter by Tbx20, Gata4, Isl1 or activated Alk3 (aAlk3) expression constructs, co-transfected singly or in combination in 10T1/2 cells. (I) Co-immunoprecipitation shows interactions between Isl1 and Myc-Tbx20 (m-T20), but not FLAG-Gata4 (F-G4), myc-Tbx1 (m-T1) or FLAG-Tbx5 (F-T5). Bottom two panels show 10% input.

of *Tbx20* RNAi embryos are reminiscent of those observed in embryos lacking *Smarcd3*, which encodes Baf60c, a muscle-restricted subunit of the BAF chromatin remodeling complex (Lickert et al., 2004). Indeed, significant downregulation of *Tbx20* was observed in *Smarcd3* knockdown embryos.

Atrioventricular and outflow tract cushion formation was abrogated in *Tbx20* knockdown embryos. *Tbx20* is expressed both in the myocardium and in the endocardium, both of which are crucial cell types in cardiac cushion formation (Barnett and Desgrosellier, 2003; Chang et al., 2004). At the present time we cannot distinguish whether the defects in cushion formation are due to a primary defect in endocardial function, or are secondary to the loss of Tbx20 in the adjacent myocardium. However, the severe defects in development of valves in embryos with a mild knockdown of *Tbx20* (line a4) in which myocardial differentiation is not greatly affected, indicates that this may be a primary defect due to loss of Tbx20 in the endocardium. In support of this hypothesis, Tbx20 has been shown to interact with Gata5 (Stennard et al., 2003), which is required for endocardial cell differentiation (Nemer and Nemer, 2002).

Transcription factor networks in heart development

Tbx20 is important for the expression of several key regulators of cardiac morphogenesis, and thus we propose that the loss of Tbx20 affects heart development via a breakdown of transcription factor networks. The complexity of these interactions is further enhanced by the observation that Tbx20 can interact with other transcription factors to regulate high-level tissue-specific expression of essential cardiac transcription factors. Indeed, Tbx20 regulates expression of both *Nkx2-5* and *Mef2c*, and Tbx20 can interact with Isl1 and

Gata4 to activate the AHF enhancers of both genes. As the AHF enhancers of both *Nkx2-5* and *Mef2c* rely on Isl1- and Gata-binding sites and on Tbx20, this provides a common mechanism for the integration of transcription factor inputs for the AHF. The persistent expression of Isl1 in cardiac progenitors (Laugwitz et al., 2005), or the establishment of active chromatin at Isl1-dependent enhancers, may explain the widespread effect of loss of the Isl1-binding sites in the *Nkx2-5* enhancer. Tbx20 has been shown to interact with other important cardiac transcription factors, including Gata4, Nkx2-5 and Tbx5 (Plageman and Yutzey, 2004; Stennard et al., 2003; Takeuchi et al., 2003), and thus the roles of Tbx20 in chamber differentiation may similarly rely on these interactions. However, some degrees of specificity of interactions must exist, as not all enhancers tested for activation by Tbx20 could respond, while they were responsive to Tbx5. This specificity extends to T-box transcription factors expressed in overlapping domains: Tbx1 is also important for AHF formation, but appears to do so via direct activation of fibroblast growth factor and forkhead transcription factor genes, instead of cardiac transcription factors such as those affected by loss of Tbx20 (Hu et al., 2004; Xu et al., 2004). A combinatorial interaction in myocardial development is also observed for Nkx2-5 and Tbx5, which regulate and interact with several other cardiac transcription factors, including Gata4 (Bruneau, 2002; Bruneau et al., 2001; Garg et al., 2003; Tanaka et al., 1999). Thus, self-reinforcing transcription factor networks are central to cardiac gene expression and morphogenesis. Tbx20 appears to be a crucial co-activator in this process, as its nodes of interaction are widespread and positioned at key transition points in heart development, including positive interactions with Nkx2-5, Gata4 and Isl1 in AHF and chamber differentiation.

Tbx20 dose and congenital heart defects

An intermediate (60%) reduction in Tbx20 levels resulted in grossly normal heart morphology, but with impaired outflow tract septation, RV hypoplasia and defective valve formation. These defects resemble several human congenital heart defects, such as persistent truncus arteriosus, hypoplastic RV and Ebstein's anomaly of the tricuspid valve. This suggests that as for several other cardiac transcription factors (Bruneau, 2003; Lickert et al., 2004), partial loss of function of *Tbx20* may be an important etiology of human congenital heart defects. Dominant mutations in *NKX2-5*, *GATA4*, *TBX5* and *TBX1* have been shown to cause congenital heart defects in humans (Basson et al., 1997; Garg et al., 2003; Li et al., 1997; Schott et al., 1998; Yagi et al., 2003), and Tbx20 can interact with several of these transcription factors. Thus, the combined interactions between the cardiac transcription factors implicated in human disease create an interacting network that depends on precise stoichiometry of protein-protein interactions. Interestingly, several individuals with *NKX2-5* mutations have congenital heart defects that affect the tricuspid valve and the outflow tract (Benson et al., 1999; Goldmuntz et al., 2001; McElhinney et al., 2003), structures that are the most affected in the mild *Tbx20* knockdown embryos. It is possible that impaired interaction of the mutant NKX2-5 protein with Tbx20 would be a contributory factor to the tricuspid and conotruncal defects in these individuals. Thus, it is very likely that Tbx20 can play important roles in dose-sensitive transcriptional regulatory complexes, and that dose-dependent tissue-specific transcription is disrupted in human congenital heart malformations because of *TBX20* mutations or mutations in genes required for interactions with Tbx20. *TBX20* is therefore a strong candidate gene for human congenital heart defects.

Tbx20 and motoneuron development

Motoneuron development relies on patterning cues and cell-type specific transcriptional programs, to yield the complexity of regulatory neurons that innervate somatic and visceral muscles. In particular, dorsoventral patterning by Shh signaling established domains of transcription factors that are crucial for the initiation of motoneuron differentiation (Briscoe et al., 2000; Litingtung and Chiang, 2000). This is subsequently accomplished by the localized expression of specific transcription factors that together initiate the terminal differentiation of specific motoneuron subtypes (Price and Briscoe, 2004; Shirasaki and Pfaff, 2002; Thaler et al., 2004). We have demonstrated that Tbx20 plays an important role in motoneuron development. This appears to be primarily in regulating the expression of *Isl2* and *Hb9*, which are essential regulators of motoneuron differentiation downstream of Isl1 (Arber et al., 1999; Thaler et al., 1999; Thaler et al., 2004). As Isl1 expression was not affected in Tbx20 knockdown embryos, it is likely that Tbx20 is an important parallel or interacting modulator of the activity conferred upon the *Isl2* and *Hb9* enhancers by Isl1. As with most markers of differentiating motoneurons, *Tbx20* is regulated by Shh patterning. Therefore, Tbx20 lies at the interface between patterning and differentiation, and probably has a role as a reinforcing factor in the transcriptional regulation of motoneuron differentiation.

Conclusions

In conclusion, we have demonstrated that Tbx20 dose is a crucial determinant of heart morphogenesis, and particularly of the AHF derivatives, the RV and outflow tract. Tbx20 interacts with and regulates important cardiac transcription factors, leading to the conclusion that it plays a central role in coordinating a crucial transcription factor network in heart formation. Future studies will identify the extent of these interactions and how altered Tbx20 dose modifies these networks.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/10/2463/DC1

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