Tbx20 is essential for cardiac chamber differentiation and repression of *Tbx2*

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Summary

***Tbx20*, a member of the T-box family of transcriptional regulators, shows evolutionary conserved expression in the developing heart. In the mouse, *Tbx20* is expressed in the cardiac crescent, then in the endocardium and myocardium of the linear and looped heart tube before it is restricted to the atrioventricular canal and outflow tract in the multi-chambered heart. Here, we show that *Tbx20* is required for progression from the linear heart tube to a multi-chambered heart. Mice carrying a targeted mutation of *Tbx20* show early embryonic lethality due to hemodynamic failure. A linear heart tube with normal anteroposterior patterning is established in the mutant. The tube does not elongate, indicating a defect in recruitment of mesenchyme from the secondary heart field, even though markers of the secondary heart field are not affected. Furthermore, dorsoventral patterning of the tube, formation of working myocardium, looping, and further differentiation and morphogenesis fail. Instead, *Tbx2*, *Bmp2* and vinexin α (*Sh3d4*), genes normally restricted to regions of primary myocardium and lining endocardium, are ectopically expressed in the linear heart tube of *Tbx20* mutant embryos. Because *Tbx2* is both necessary and sufficient to repress chamber differentiation (Christoffels et al., 2004a; Harrelson et al., 2004), *Tbx20* may ensure progression to a multi-chambered heart by repressing *Tbx2* in the myocardial precursor cells of the linear heart tube destined to form the chambers.**

Key words: T-box, Heart, Myocardium, Anterior heart field, Bmp

Introduction

Cardiac development starts shortly after gastrulation, when two bilaterally symmetrical regions in the anterior lateral plate mesoderm are specified and form the cardiac crescent (for reviews, see Brand, 2003; Moorman and Christoffels, 2003). The cardiac crescent folds towards the ventral midline to form a linear heart tube that initiates rhythmic contractions shortly thereafter. The myocardium of the elongating and looping heart tube has a primary phenotype and proliferates slowly (Christoffels et al., 2004b). The early heart tube contains the future left ventricle and atrioventricular canal (Davis et al., 2001; Cai et al., 2003). The outflow tract, right ventricle, atria and inflow tract form during looping of the heart tube by addition of mesenchymal precursor cells from the mesoderm of the secondary heart field, which includes the anterior heart field that gives rise to the OFT and right ventricle (Kelly and Buckingham, 2002; Cai et al., 2003; Abu-Issa et al., 2004; Zaffran et al., 2004).

Chamber formation is a localized process (Christoffels et al., 2000; Meilhac et al., 2004). A ventral region of myocardium of the linear heart tube that comes to lie at the outer curvature of the looping heart initiates a chamber-specific program of gene expression that directs a 'ballooning' growth to form the ventricular chambers. Likewise, the atrial chamber myocardium differentiates and expands from the dorsolateral portion of the heart tube. Increased rates of proliferation and subsequent trabeculation, a high conduction velocity and fast contractions characterize chamber (early working) myocardium. Patterned expression of several genes encoding transcription factors and signalling molecules provide evidence for the presence of anteroposterior (AP) and dorsoventral (DV) patterning in the early heart tube that may control these localized differentiation processes (Christoffels et al., 2000). Myocardium outside of these distinct regions, in the inflow tract (IFT), the atrioventricular canal (AVC), the outflow tract (OFT) and the connecting inner curvatures, does not initiate the chamber-specific program of gene expression and retains its primary character. Endocardium lining these regions undergoes an epithelial-mesenchymal transition to form the endocardial cushions. These cushions are pivotal to the formation of the septa of atria and ventricles, and to the formation of the valves (Eisenberg and Markwald, 1995).

Several T-box (Tbx) genes have been implicated in the regulation of vertebrate heart development. Tbx genes encode a family of proteins sharing a highly conserved DNA-binding region, the T-box. T-box proteins act as transcription factors that exert distinct transcriptional activation and repression

functions depending on the molecular context of the conserved DNA-binding site. Members of the gene family are conserved throughout metazoan evolution. In mammals, 18 T-box genes have been identified. Gene targeting experiments in mice have revealed their crucial functions during gastrulation and the development of various organ systems (for reviews, see Papaioannou, 2001; Tada and Smith, 2001). In addition, mutations in several T-box genes cause congenital human diseases demonstrating the importance of the gene family both in development and disease (for a review, see Packham and Brook, 2003). Functional analyses suggest that four of the six T-box genes identified in vertebrate heart development, namely *Tbx1*, *Tbx2*, *Tbx5* and *Tbx20* are important regulators of formation and maturation of the heart. Functional relevance of cardiac expression of *Tbx3* and *Tbx18* has not yet been reported (reviewed by Plageman and Yutzey, 2004a).

Tbx20 is a member of the Tbx1-subgroup of T-box transcription factors. *Tbx20* expression was reported in the allantois, dorsal part of the retina, motoneurons, lateral plate mesoderm, cardiac crescent, primitive heart tube and four-chambered heart of mouse and chick embryos (Carson et al., 2000; Iio et al., 2001; Kraus et al., 2001a). More detailed analyses have revealed differential expression in the developing tetrapod heart. After widespread activation in the linear and looping heart, expression becomes gradually more enriched in AVC, OFT and tricuspid and mitral valves (Brown et al., 2003; Stennard et al., 2003; Takeuchi et al., 2003; Lincoln et al., 2004; Plageman and Yutzey, 2004b; Yamagishi et al., 2004). Cardiac expression is found both in the myocardium and endocardium, and in endocardial cushion tissues (Carson et al., 2000; Kraus et al., 2001a; Stennard et al., 2003). Bmp2 is a crucial inducer of cardiogenic cell fate. *Tbx20*, *Tbx2* and *Tbx3*, but not *Tbx5*, are induced by Bmp2 in avian cardiogenic mesoderm, suggesting that *Tbx20* acts at least partially downstream of Bmp2 signaling (Yamada et al., 2000; Plageman and Yutzey, 2004b).

Tbx20 acts as a transcriptional repressor on conserved T-box DNA-binding sites in cardiac promotors (Plageman and Yutzey, 2004b). Presence of both transactivation and transrepression domains in the C terminus of the Tbx20 protein was reported, providing evidence for a context-dependent control of gene transcription. Collaboration with other cardiac transcription factors might also contribute to functional specificity. Indeed, physical interaction with the cardiac transcription factors Gata4, Gata5 and Nkx2.5 was reported (Stennard et al., 2003).

Tbx20 expression is also found in developing hearts of lower vertebrates and invertebrates, suggesting conservation of a central cardiogenic program. The *Drosophila* orthologs *midline* and *H15* are expressed in the dorsal heart tube. They are required in a redundant fashion for the normal alignment of cardioblasts and associated pericardial cells in the dorsal vessel (Miskolczi-McCallum et al., 2005; Qian et al., 2005). During zebrafish embryogenesis, the expression of the ortholog *hrt* is found in the anterior lateral plate mesoderm, the heart field and the endothelium of the dorsal aorta (Ahn et al., 2000; Griffin et al., 2000). Functional studies using morpholino antisense oligonucleotides revealed a requirement for *hrt* in cardiovascular development. *hrt* morphant hearts do not undergo looping. Chamber formation and gene expression are perturbed (Szeto et al., 2002). A similar cardiac phenotype was observed in *Tbx20* morphant *Xenopus* embryos (Brown et al., 2005).

In this paper, we address the role of *Tbx20* in cardiac development using a gene targeting approach in the mouse. Mice homozygous for the mutant allele die at E10.5 as a result of hemodynamic failure due to severe cardiovascular malformations. A linear heart tube is established but looping morphogenesis and chamber differentiation fail. We demonstrate that the expression domains of *Tbx2*, and of other markers for primary myocardium and endocardium lining the primary myocardium, are expanded in the mutant heart. We suggest that *Tbx20* promotes progression from the linear to the looping and multi-chambered heart by repressing *Tbx2* in the myocardial precursor cells destined to form the chambers, thus allowing chamber-specific differentiation to occur.

Materials and methods

Generation of *Tbx20* mutant mice

To clone the mouse *Tbx20* locus a 129/Ola genomic cosmid library (obtained from the Resourcenzentrum, Berlin) was screened using the mouse cDNA (Kraus et al., 2001a) as a probe. Four independent cosmid clones were purified. Several genomic fragments comprising 26 kb of the 5′-region of the *Tbx20* locus were subcloned from one of them and characterized by restriction and exon mapping (Fig. 1A). To generate a targeting construct allowing inactivation of the *Tbx20* gene, a *lacZ*-fragment followed by a loxP-flanked *neo*-cassette was inserted into an *Nco*I-site located at the start codon in the first exon, and flanked by a 3.3 kb 5′-homology region and a 5 kb 3′-homology region, respectively, derived from genomic subfragments (Fig. 1A). With this construction, a short fragment (346 bp) harboring the rest of exon 1 with the 5′-translated region was deleted, ensuring the generation of a null allele. The targeting vector was linearized at a unique *Sal*I-site and electroporated in ES cells of 129Sv/ImJ genotype. G418-resistant ES cell clones (n=160) were screened for homologous recombination in the *Tbx20* locus by Southern blot analysis. Three ES cell clones proved to be correctly targeted and were subsequently used for microinjection into FvB mouse blastocysts. Five chimeric males were obtained and mated to NMRI females. One chimeric male gave germ-line transmission. F1 heterozygous males were crossed to NMRI females, heterozygous offspring intercrossed, and embryos and newborns analyzed for phenotypic alterations.

Genotyping of *Tbx20* mutant mice

Genotypic characterization of ES cells, embryos and adult mice was done by Southern blot analysis of restriction-digested genomic DNA. DNA was derived from ES cells, embryonic yolk sacs and adult tails, and hybridized with probes distinguishing wild-type and mutant alleles (Fig. 1A). The 5′-probe is a 374 bp *Kpn*I/*Eco*RV fragment subcloned from the genomic region adjacent but outside the targeting vector. This probe recognizes a 4.8 kb *Kpn*I fragment in the wild type and an 8.2 kb *Kpn*I fragment in the mutant (Fig. 1B). The 3′-probe, a 582 bp *Bam*HI/*Kpn*I fragment, detects a 10 kb *Hin*cII fragment in the wild type and an 8 kb *Hin*cII fragment in the targeted allele (Fig. 1C).

After initial genotyping of E9.5 embryos by RFLP-Southern analysis, *Tbx20* homozygous embryos were identified by phenotype. Genotyping on E7.5-E8.5 embryos was also carried out using a β-galactosidase assay on yolk sac tissue, taking advantage of the *Tbx20* expression in this tissue.

Collection of embryos

For timed pregnancies, plugs were checked in the morning after mating, noon was taken as embryonic day (E) 0.5. Embryos harvested from heterozygous intercrosses were dissected in phosphate-buffered

saline (PBS), fixed in 4% paraformaldehyde (PFA)/PBS overnight, dehydrated in methanol and stored at –20°C.

Histological analysis

Embryos for histological staining were fixed in 4% PFA, paraffin-wax embedded and sectioned to 5 μm. Sections were stained with Hematoxylin and Eosin. Whole-mount histochemistry for β-galactosidase activity was carried out as described (Echelard et al., 1994). For detection of endothelial endocardium. anti-PECAM1 (CD31) monoclonal antibody (Pharmingen) was used at a dilution of 1:100 as primary antibody, and 1:200-diluted HRP-coupled goat-anti-rat IgG was used as a secondary antibody. The detection reaction was performed using diaminobenzidine and hydrogen peroxide as substrates.

Proliferation and apoptosis assays

Cell proliferation in tissues of E8.5-E8.75, and E9.5, embryos was investigated by the detection of incorporated BrdU on 5-μm sections of paraffin wax-embedded specimens, similar to published protocols (Bussen et al., 2004). Four sections each of five embryos of each genotype at E8.5 were used for quantification. The BrdU-labeling rate was defined as the number of BrdU-positive nuclei relative to the total number of nuclei as detected by DAPI counterstain in the heart region. Detection of apoptotic cells in 5-μm paraffin sections of E8.5 and E9.5 embryos was based on the modification of genomic DNA using terminal deoxynucleotidyl transferase (TUNEL assay), and indirect detection of positive cells by Fluorescein-conjugated anti-Digoxigenin antibody. The procedure followed exactly the recommendation of the manufacturer (Serologicals Corporation) of the ApopTag kit used.

In situ hybridization analysis

Whole-mount in situ hybridization was performed, following a standard procedure, with Digoxigenin-labeled antisense riboprobes (Wilkinson, 1992). Stained specimens were transferred into 80% glycerol prior to documentation.

Documentation

Whole-mount specimens were photographed on a Leica M420 microscope with a Fujix digital camera HC-300Z; sections were photographed on a Leica Axioplan microscope with ProgRes C14 digital camera. All images were processed in Adobe Photoshop 7.0.

Results

Heart development is severely abnormal in *Tbx20*$^{-/-}$ embryos

To elucidate the role of *Tbx20* in heart development, we used gene targeting in ES cells to generate mice deficient for the *Tbx20* gene (Fig. 1A). The *lacZ* gene was inserted into the start codon of exon 1 to visualize endogenous *Tbx20* expression from the mutant allele by β-galactosidase activity staining (Fig. 3E-F′). RFLP analysis (Fig. 1B,C), and absence of *Tbx20* mRNA in homozygous mutant embryos (Fig. 1D), confirmed that the targeted modification of the *Tbx20* locus resulted in a functional null allele.

Mice heterozygous for the mutant *Tbx20* allele appear normal and are fertile. Mice homozygous mutant for *Tbx20* show severe growth retardation at E9.5 and die at approximately E10.5. Dysmorphic hearts, an enlarged pericardial cavity, edemas and absence of blood circulation indicate that lethality is due to cardiovascular defects (Fig. 2E and data not shown). We here focus on the role of *Tbx20* in cardiac development. The possible requirement for vasculogenesis will be considered elsewhere in more detail.

Mutant and wild-type hearts are morphologically indistinguishable at the linear heart tube stage (E8.0-E8.25; data not shown). At E8.25-E8.5, heart looping and chamber formation is initiated in the wild type (Fig. 2A,C). In the

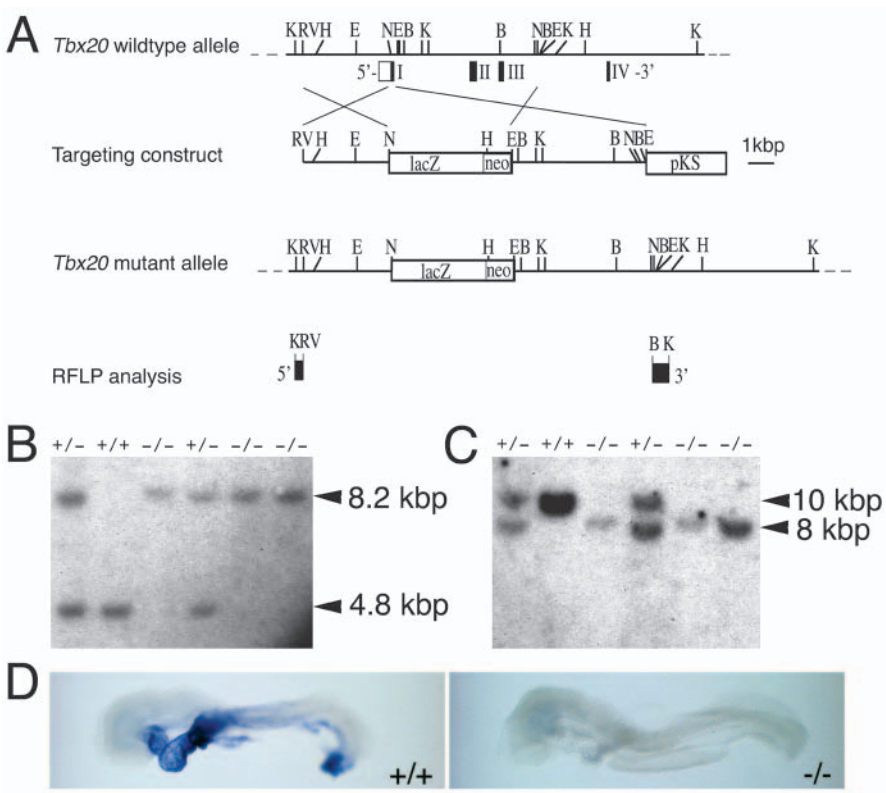

Fig. 1. Targeted disruption of the *Tbx20* locus. (A) Schematic representation of the targeting strategy. Restriction map of the wild-type locus with boxes representing the first four exons of *Tbx20*; coding regions are shown in black, non-coding in white. Fragments used as RFLP probes are shown. The *Kpn*I-*Eco*RV fragment designated as 5′ detects the *Kpn*I-RFLP shown in B. The *Hinc*II-RFLP shown in C is detected by the *Bam*HI-*Kpn*I fragment labelled as 3′. Only *Hinc*II sites relevant for RFLP analysis are shown. B, *Bam*HI; E, *Eco*RI; H, *Hinc*II; K, *Kpn*I; N, *Nco*I; RV, *Eco*RV; neo, loxP-flanked *neomycin* selection cassette. (B) Southern blot analysis of *Kpn*I-digested genomic DNA extracted from E9.5 embryos derived from intercrosses of mice heterozygous for the mutant *Tbx20* allele. Genotypes are indicated above each lane. The 4.8 kb and 8.2 kb bands represent the wild-type and the mutant allele, respectively. (C) Southern blot analysis of *Hinc*II-digested genomic DNA extracted from the same E9.5 embryos. Genotypes are indicated above each lane. The 10 kb and 8 kb bands represent the wild-type and the mutant allele, respectively. (D) In situ hybridization analysis of *Tbx20* expression in wild-type (+/+) and *Tbx20*$^{-/-}$ (–/–) embryos at E8.5 using an antisense riboprobe against the T-box shows complete absence of *Tbx20* mRNA in the mutant embryo.

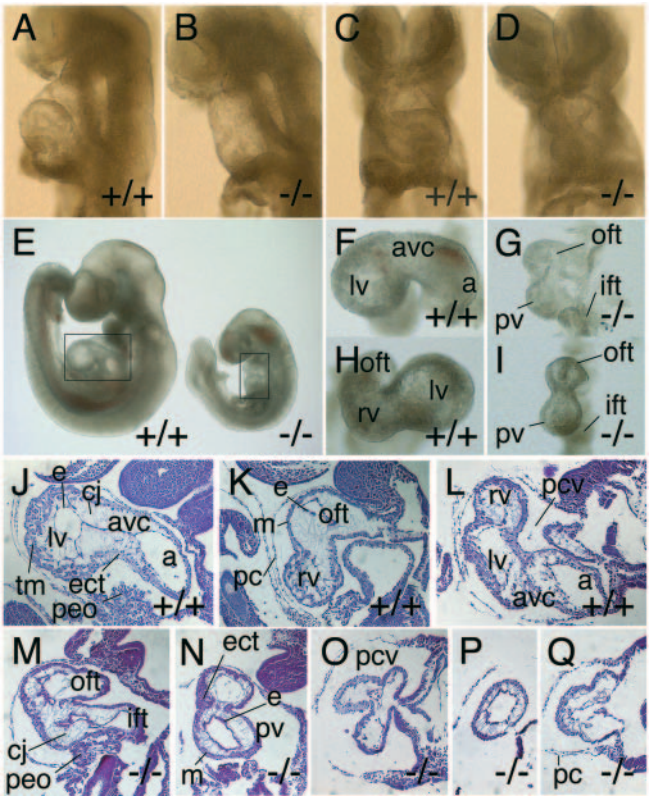

Fig. 2. Cardiac morphology and histology of embryos mutant for *Tbx20*. (A-I) External morphology of embryos and isolated hearts. (A-D) E8.5 heart regions. The wild-type heart (A,C) shows looping, the mutant heart (B,D) remains linear. (E) At E9.5, *Tbx20* mutants are substantially smaller than their wild-type littermates, and exhibit bleeding and edema. Somites are smaller and irregularly organized, as is the neural tube. The first branchial arch is formed. Boxes indicate the heart regions. (F-I) Isolated hearts of E9.5 embryos. (F,H) Wild-type hearts show formation of chambers with right (rv) and left ventricle (lv) and an atrium (a), and of the atrioventricular canal (avc) and outflow tract (oft). (G,I) Mutant hearts feature an outflow tract, a primitive ventricle (pv) and an inflow tract from anterior to posterior. Anterior is up. Views are from the left-lateral side (A,B,E-G), or from the ventral side (C,D,H,I). (J-Q) Histological analysis of E9.5 embryonic hearts by Hematoxylin and Eosin staining of paraffin sections. Differentiation of cardiac tissue in endocardium (e), myocardium (m), cardiac jelly (cj) and endocardial cushion tissue (ect) can be seen in wild-type (J-L) and mutant hearts (M-Q). Trabeculated myocardium (tm) is only formed in the wild-type heart. Sections are sagittal, with anterior up and ventral to the left (J,K,M,N), or transverse, with right up and ventral to the left (L,O,P,Q). Transverse sections of the mutant heart are at the level of the inflow tract (O), primitive ventricle (P) and outflow tract (Q). pc, pericardium; pcv, pericardial cavity; peo, proepicardial organ. Genotypes are indicated in the figure.

mutant, the heart tube fails to loop. Instead, two constrictions appear, separating a putative embryonic ventricle from a posterior inflow tract and an anterior outflow tract region (Fig. 2B,D). By E9.5, the wild-type heart has further elongated and looped, and atrial and ventricular chambers are being formed. By contrast, the mutant heart tube does not elongate further and the architecture of the heart remains unchanged from E8.5 onwards (Fig. 2G,I). Histological analysis confirmed the morphological findings and revealed the presence of myocardium, endocardium, endocardial cushion tissue, and cardiac jelly in the mutant heart (Fig. 2J-Q). Endocardial cushion is accumulated at the anterior constriction compromising the continuity of the endocardial lining of the tube (Fig. 2N). The mutant heart tube shows slow but rhythmic contractions that initiate at the posterior inflow tract region and propagate anteriorly (data not shown).

Anteroposterior patterning of the linear heart tube in *Tbx20* mutants

In an initial attempt to determine cardiac and cardiomyocyte differentiation in *Tbx20* mutant embryos, we analyzed expression of the pan-cardiac marker genes *Nkx2.5* and atrial myosin light chain 2 (*Mlc2a*; *My17* – Mouse Genome Informatics) (Lints et al., 1993; Kubalak et al., 1994). Both genes are expressed throughout the linear heart tube of the mutant at E9.5, suggesting that cardiomyocyte differentiation has occurred normally along the entire extension of the mutant heart (Fig. 3A′-D′). *Tbx20* expression as judged by β-galactosidase expression from the *lacZ* reporter gene was indistinguishable between homozygous and heterozygous mutant hearts (Fig. 3E-F′). This suggests maintenance of cardiomyocyte fate and excludes an autoregulatory requirement for *Tbx20* expression.

We next wished to analyze whether anteroposterior (AP) patterning was established in the mutant heart at E8.5. We used a set of marker genes whose restricted expression along the linear heart tube defines such patterning. α-Myosin heavy chain (*αMHC*) (*Myhca*; *Mhy6* – Mouse Genome Informatics) is expressed in a gradient from the inflow to the outflow tract. β-Myosin heavy chain (*βMHC*) (*Myhcb*; *Mhy7* – Mouse Genome Informatics) is expressed in a reverse gradient from the outflow tract to the inflow tract. Ventricular myosin light chain (*Mlc2v*; *My12* – Mouse Genome Informatics) expression is found in a bilaterally restricted segment that includes the future left ventricle (Christoffels et al., 2000). *Tbx5* expression is high posteriorly in the inflow tract region and declines to low levels in the outflow tract region (Chapman et al., 1996; Bruneau et al., 1999). Finally, *Gata4* is expressed in the posterior heart region and the endoderm (Molkentin et al., 1997). Polarized expression of these markers is normal in the mutant heart at E8.5 and E9.5 (Fig. 3G′-K′ and data not shown), suggesting that AP patterning of the linear heart tube is established and maintained in the mutant. *Pitx2* expression is restricted to the left limb of the inflow tract at E8.5 (Campione et al., 2001). Expression is unchanged in the mutant (arrow in Fig. 3L′) indicating the presence/establishment of left-right signaling in the mutant heart.

The heart tube of *Tbx20*$^{-/-}$ embryos does not elongate, but anterior and secondary heart field markers are not affected

The myocardium of the linear heart tube hardly proliferates, and the 4- to 5-fold elongation of the linear heart tube between E8 and E10.5 primarily results from the recruitment of splanchnic mesoderm of the secondary (including anterior) heart field, which proliferates rapidly (Kelly and Buckingham, 2002; Cai et al., 2003). *Tbx20* is co-expressed with *Mlc2a*, a marker for the primary heart field, but seems to slightly extend anteriorly and posteriorly into the secondary heart field, which suggests a direct control of heart tube elongation (Fig. 3C-F,

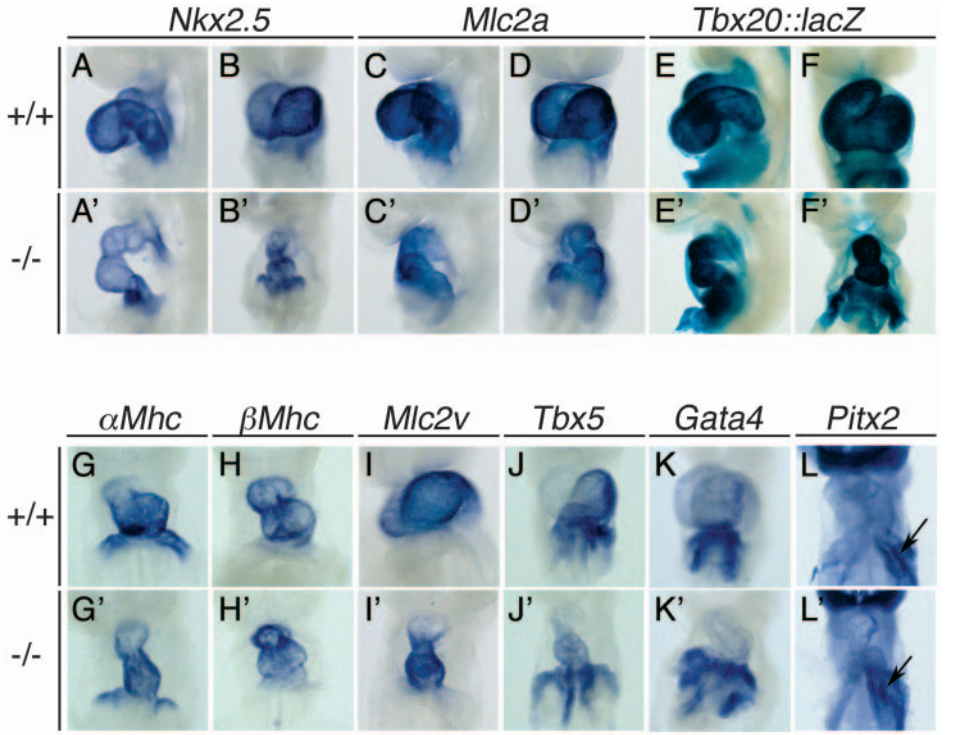

Fig. 3. Cardiac differentiation and patterning in *Tbx20* mutant embryos. Analysis of molecular markers shows that cardiac differentiation and AP patterning of the heart tube occur normally in the *Tbx20* mutant. Expression of pan-cardiac markers by in situ hybridization analysis (A-D) and β-galactosidase activity staining of a *lacZ* reporter gene in the *Tbx20* locus (E,F) in E9.5 hearts of wild-type (A-F) and *Tbx20*$^{-/-}$ embryos (A′-F′). In situ hybridization analysis of markers for AP patterning (G-K′) and left-right asymmetry (L,L′) at E8.5, in wild-type (G-L) and in *Tbx20*$^{-/-}$ (G′-L′) hearts. Views are from the left lateral side (A,A′,C,C′,E,E′) or from the ventral side (B,B′,D,D′,F,F′,G-L′), with anterior up in all cases. Expression patterns are explained in the main text. Markers and genotypes are indicated in the figure.

Fig. 4A-D, and data not shown). The heart tube does not elongate in the *Tbx20* mutant embryo. *Wnt11* expression, a marker for the OFT region of wild-type hearts at E9.5 (arrow in Fig. 4E) (Kispert et al., 1996), is not found in the anterior portion of the mutant heart, the putative OFT (Fig. 4E′). *Hand2* (*Dhand*) is expressed in the entire heart tube at E8.5 but becomes upregulated in the RV and OFT from E9.5 onwards (arrow in Fig. 4F) (Thomas et al., 1998). *Dhand* expression is not detected in the mutant heart tube (Fig. 4F′). This suggests that the ascending limb of the heart tube that gives rise to the RV and OFT of the E9.5 heart has not been added from the anterior heart field. Hence, the OFT of the *Tbx20* mutant heart at E8.5 and E9.5 is a mere functional term for a region of cells fated to contribute to a more upstream (posterior) region in the wild type. We analyzed markers for the anterior (*Fgf8*, *Fgf10*, *Foxh1*, *Mef2C*) (Lin et al., 1997; Kelly et al., 2001; von Both et al., 2004) and secondary heart field (islet 1; *Isl1* – Mouse Genome Informatics) (Cai et al., 2003) to assess whether a specific requirement for *Tbx20* in this process can be unraveled. We studied marker expression in E8.5 embryos, shortly after looping has been initiated in the wild-type heart, to exclude secondary changes. Expression of all of these markers was unaltered in *Tbx20* mutants at E8.5 (Fig. 4G′-M′) suggesting that *Tbx20* does not primarily regulate the formation and differentiation of the secondary heart field.

Dorsoventral patterning and chamber differentiation does not occur in *Tbx20* mutant hearts

In the wild type, ventricular chamber formation starts at E8.25 with the initiation of expression of chamber-specific genes at the ventral side, demarcating the future left ventricular portion. Likewise, atrial chamber formation is first seen as chamber-specific gene expression at the posterior lateral region at E9.25, the late looping stage. This results at E9.5 in the development of chamber compartments at the outer curvatures of the looped heart tube (Christoffels et al., 2000). We analyzed whether chamber specific differentiation programs are initiated in the *Tbx20* mutant heart by using a panel of molecular markers for chamber myocardium, including natriuretic precursor peptide A (*Nppa*, formerly known as *Anf*), *Chisel* (*Smpx* – Mouse Genome Informatics) and *Cx40* (*Gja5* – Mouse Genome Informatics) (Christoffels et al., 2000; Palmer et al., 2001; Delorme et al., 1997) (Fig. 5). Expression of *Nppa* and *Cx40* is completely absent from the mutant heart tube at E9.5 (Fig. 5A′,C′), suggesting that chamber myocardium is not formed in the mutant. Scattered *Cx40* expression is found dorsal to the heart, probably representing remnants of endothelial cells of the dorsal aorta (white arrow in Fig. 5C′). In fact, vessel development is severely affected by loss of *Tbx20*, as seen by absence of *Cx40* expression in all vessels of the E9.5 embryo (data not shown). *Chisel* is weakly expressed in the primitive ventricle of the mutant heart but is not upregulated as it is in the wild type (arrow in Fig. 5B′). Expression of *Hey2* marks ventricular myocardium from E8.5 in the wild-type heart (Leimeister et al., 1999). Absence of *Hey2* expression from the mutant heart (Fig. 5D′) confirms the lack of chamber myocardium.

The failure of chamber differentiation in the embryonic heart tube indicates that the underlying DV patterning may be affected. *Cited1* (also known as *Msg1*) and *Hand1* (*Ehand*) are expressed specifically at the ventral side of the linear heart tube and, subsequently, at the outer curvature of the ventricular portion of the looped heart tube (Dunwoodie et al., 1998; Cserjesi et al., 1995; Biben and Harvey, 1997; Thomas et al., 1998; Christoffels et al., 2000). Expression of neither *Cited1* nor *Hand1* is detected in the heart of stage-matched mutant embryos (Fig. 5E′,F′).

The T-box transcription factor *Tbx3* is hardly detectable at E8.5, but is expressed in the AVC in the E9.5 wild-type heart (Hoogaars et al., 2004). The heart tube of *Tbx20* mutants is devoid of any *Tbx3* signal (Fig. 5G′). Finally, *Tbx18* expression

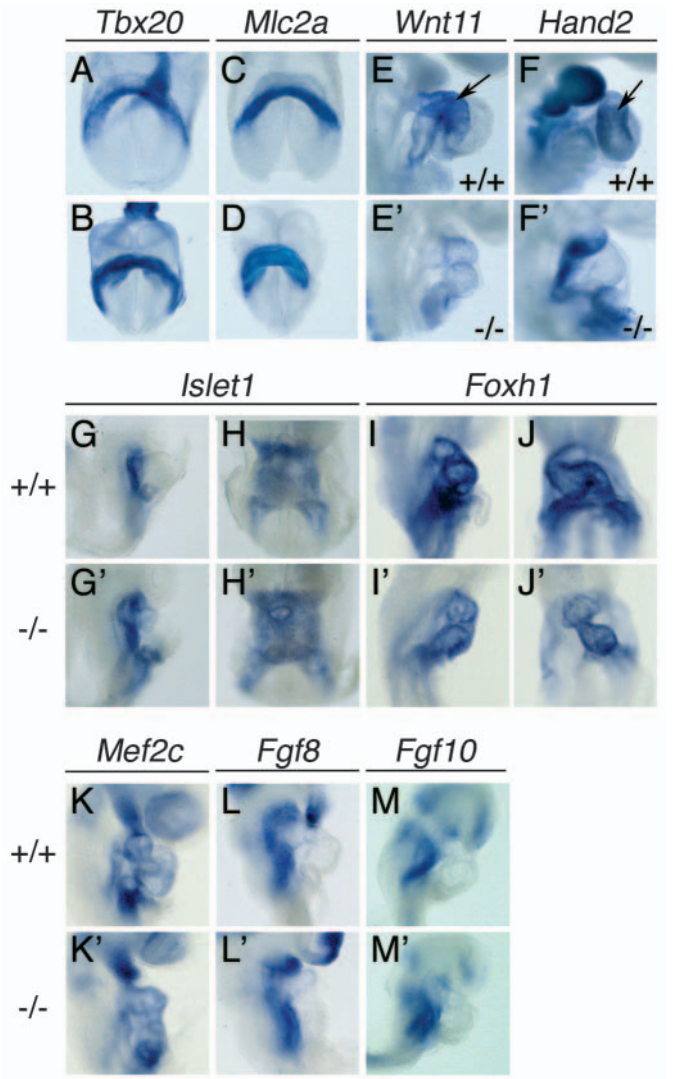

Fig. 4. Secondary heart field development in *Tbx20*$^{-/-}$ embryos. Whole-mount in situ hybridization analysis of *Tbx20* and *Mlc2a* expression in wild-type embryonic hearts at E7.75 (A,C) and E8.0 (B,D); of markers for OFT (*Wnt11*) and OFT/RV (*Hand2*) in E9.5 hearts (E-F′), and of secondary heart field markers in E8.5 wild-type and mutant embryos. Images are magnifications of anterior body regions, including the heart, in views from the right lateral side (E-G′,I,I′,K-M′), or from the ventral side (A-D,H,H′,J,J′). Anterior is always up. Markers and genotypes are as indicated in the figure, for details on expression, see main text.

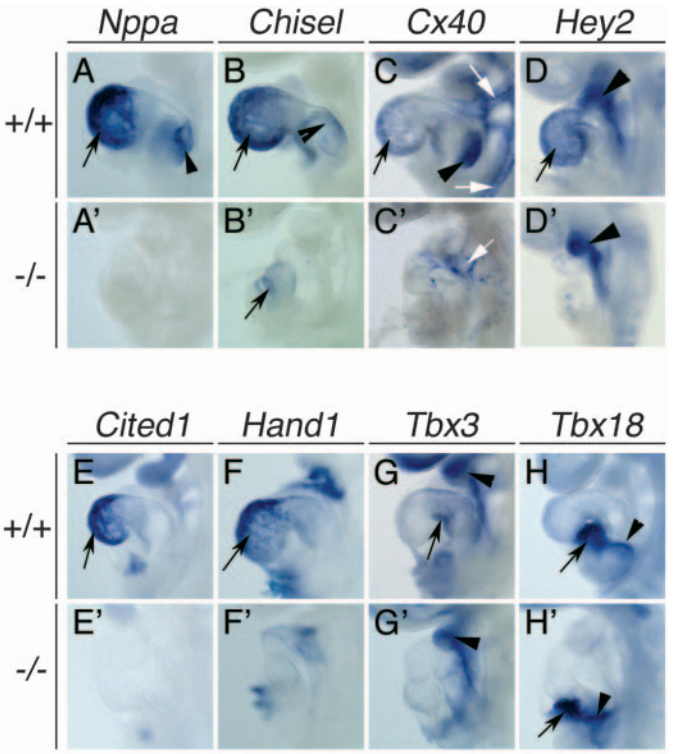

Fig. 5. Chamber formation in *Tbx20* mutant hearts. Whole-mount in situ hybridization analysis of expression of cardiac chamber markers in wild-type and *Tbx20*$^{-/-}$ hearts at E9.5. All views are from the left lateral side, anterior up. Expression of markers for ventricular (arrows in wild-type embryos in A-F) and for atrial chamber myocardium (arrowheads in A-C) are missing in the corresponding stage-matched *Tbx20* mutant hearts. *Chisel* is expressed very weakly in the primitive ventricle of the *Tbx20*$^{-/-}$ heart (arrow in B′). Vessel formation is affected in the mutant, as shown by *Cx40* expression, which marks branchial arch vessels and dorsal aorta in the wild type (white arrows in C), but reveals only scattered cells in the branchial arch region in the mutant (white arrow in C′). *Tbx3* expression is only found in the atrioventricular canal of the wild-type heart (arrow in G). (H,H′) *Tbx18* expression in the proepicardial organ (arrow) and the septum transversum (arrowhead) is unchanged in *Tbx20*$^{-/-}$ embryos. Note that expression of markers for pharyngeal mesoderm, *Hey2* and *Tbx3* (arrowheads in D and G) is preserved in *Tbx20* mutants (arrowheads in D′ and G′). Markers and genotypes are indicated in the figure.

is found in the septum transversum and the proepicardial organ at E9.5 (Kraus et al., 2001b). Expression is unaltered in the mutant, suggesting that proepicardial development is unaffected (Fig. 5H′).

In summary, *Tbx20* mutant hearts do not exhibit any DV patterning or chamber differentiation, but are arrested in the primary linear heart tube stage.

Tbx2 is expressed throughout the linear heart of *Tbx20*$^{-/-}$ embryos

Tbx2 is both sufficient and necessary to prevent differentiation of chamber myocardium (Christoffels et al., 2004a; Harrelson et al., 2004). Therefore, we wondered whether lack of chamber differentiation in the *Tbx20* mutant heart is associated with deregulation of *Tbx2*. In E8.5 wild-type hearts, *Tbx2* expression is found in the myocardium of the IFT (more strongly in its anterior part), in the forming AVC with a sharp border towards the forming ventricle, and in the underlying septum transversum mesenchyme (Habets et al., 2002). At E9.5, *Tbx2* is expressed in the myocardium of the AVC and OFT (Christoffels et al., 2004a; Harrelson et al., 2004). In the *Tbx20* mutant, *Tbx2* expression is strongly upregulated in the cardiac crescent at E7.75-E8.0 (arrow in Fig. 6A′). From E8.25 onwards, *Tbx2* is strongly expressed throughout the linear heart tube, i.e. in the IFT region, the embryonic ventricle and the OFT region (Fig. 6B′-F′). Anti-PECAM immunohistochemistry showed the presence of endothelial

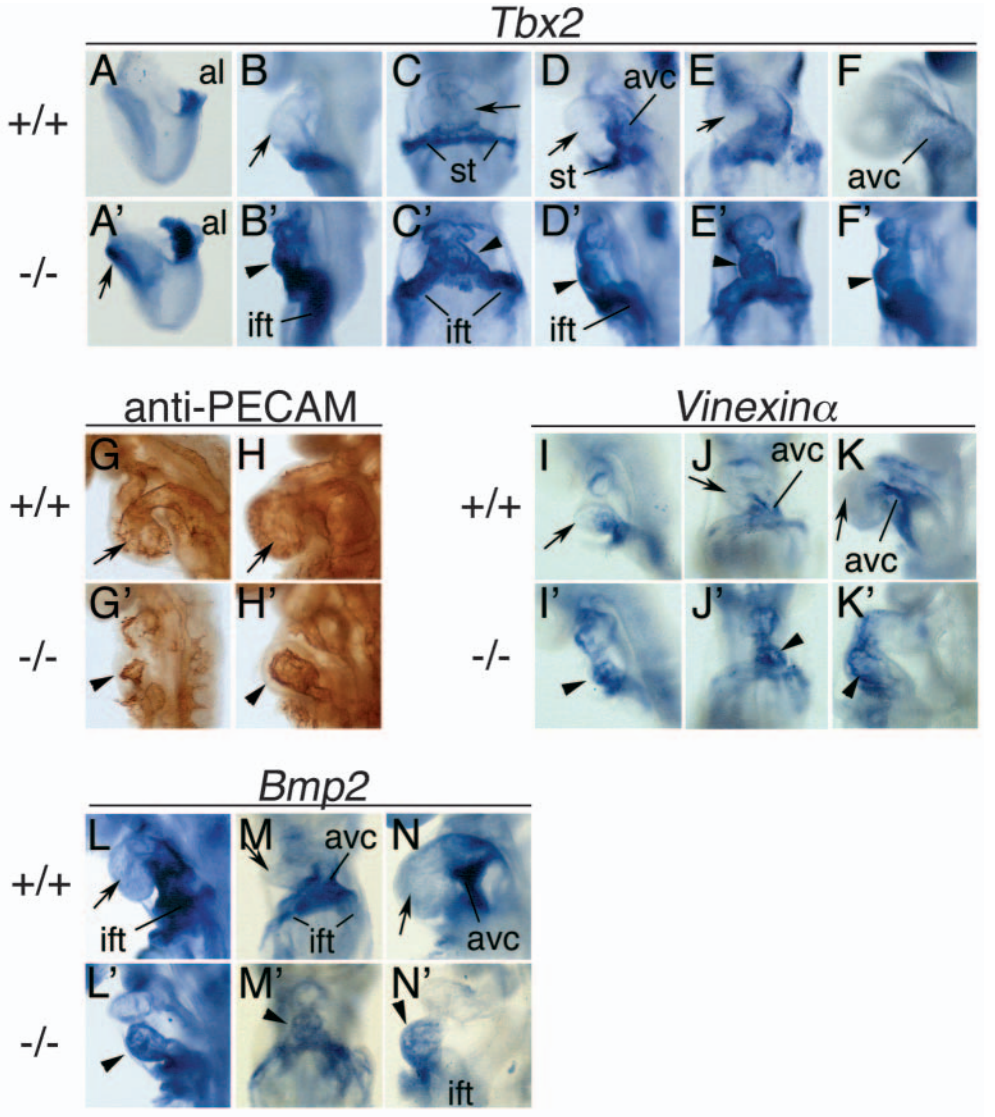

Fig. 6. Analysis of primary myocardium in *Tbx20*$^{-/-}$ hearts. Whole-mount in situ hybridization analysis in E7.5 to E9.5 embryos shows the presence of markers for primary myocardium (*Tbx2*, *Bmp2*) and endocardium (vinexin α) in the heart tube of *Tbx20* mutant embryos. (G-H′) Anti-PECAM immunohistochemistry for endocardial endothelium. Images are of whole embryos (A,A′) and magnifications of anterior body regions, including the heart (B-N′), in views from the right lateral side (A-B′,D,D′,F-I′,K-L′,N,N′), or from the ventral side (C,C′,E,E′,J,J′,M,M′). Anterior is up. Embryos are E7.5 (A,A′), E8.25 (B-C′), E8.5 (D-E′,G,G′,I-J′,L-M′) and E9.5 (F,F′,H,H′,K,K′,N,N′). Arrow in A′ marks *Tbx2* expression in the cardiac crescent in *Tbx20* mutant embryos. Arrows in all other figures point to the left ventricle that is free of hybridization signals for markers of primary cardiac phenotype. By contrast, corresponding mutant hearts show expression of these genes in the primitive ventricle (arrowheads in lateral views of E8.5 and 9.5 mutant hearts). Note the increase of *Tbx2* expression (C′) but decrease of *Bmp2* expression (M′) in the primitive inflow tract of the E8.5 mutant heart. In wild-type embryos, *Tbx2* expression is restricted to the atrioventricular canal (avc) and the septum transversum (st). al, allantois. Markers and genotypes are indicated in the figure.

endocardium in the mutant heart at E8.5 and E9.5. The endothelial lining was found to be discontinuous at the upper constriction. suggesting reduced or absent blood circulation in *Tbx20* mutant embryos (Fig. 6G′,H′). We next investigated whether the mutant endocardium would also be reprogrammed to a type of endocardium lining primary cardiac tissue by analyzing vinexin α (*Sh3d4* – Mouse Genome Informatics) expression. In the wild-type heart, vinexin α expression is restricted to the endocardium of the anterior part of the IFT and AVC at E8.5, and to the OFT and AVC endocardium at E9.5 (Kawauchi et al., 2001). In the *Tbx20* mutant, expression is found throughout the endocardial layer of the linear heart tube at E8.5 and E9.5 (Fig. 6I′-K′). Recently, evidence has accumulated that cardiac *Tbx2* is induced by Bmp2, a secreted protein of the Dpp/Bmp signaling family (Yamada et al., 2000). We reasoned that derepression of *Tbx2* in the *Tbx20* mutant heart may be triggered by spread of *Bmp2* expression from the IFT/AVC region anteriorly into the primitive ventricle and OFT. Analysis of *Bmp2* expression in *Tbx20* mutant hearts at E8.5 and E9.5 revealed a weak but consistent expression of *Bmp2* in the myocardium of the primitive ventricle, but downregulation in the inflow tract, and absence in the outflow tract region (Fig. 6L′-N′). This marker analysis suggests that the *Tbx20* mutant heart, in particular the primitive ventricle, has acquired a primary type of myocardium and endocardium, possibly by Bmp2 induction of *Tbx2*.

Proliferation and apoptosis in *Tbx20* mutant hearts

We next addressed the question whether the impairment of progression from the linear heart tube stage in the *Tbx20* mutant may be caused by reduced cell proliferation and/or an increase in apoptosis. We analyzed programmed cell death by TUNEL assay in wild-type and mutant hearts. Analysis of transverse sections at E8.5 and E9.5 did not reveal any differences in apoptosis between mutant and wild-type hearts at these stages (Fig. 7A-D).

Cellular proliferation was determined by the BrdU incorporation assay on transverse sections of E8.5-E8.75 wild-type and *Tbx20* mutant embryos (Fig. 7E-G). Proliferation in the (primitive) ventricular heart region, as judged by the BrdU-labeling index, was significantly reduced from 0.133±0.0089 in the wild type to 0.03±0.0032 in the mutant (P<0.005; Fig. 7G). By contrast, proliferation in extracardiac regions was obviously unchanged in the mutant embryos (Fig. 7E,F). This

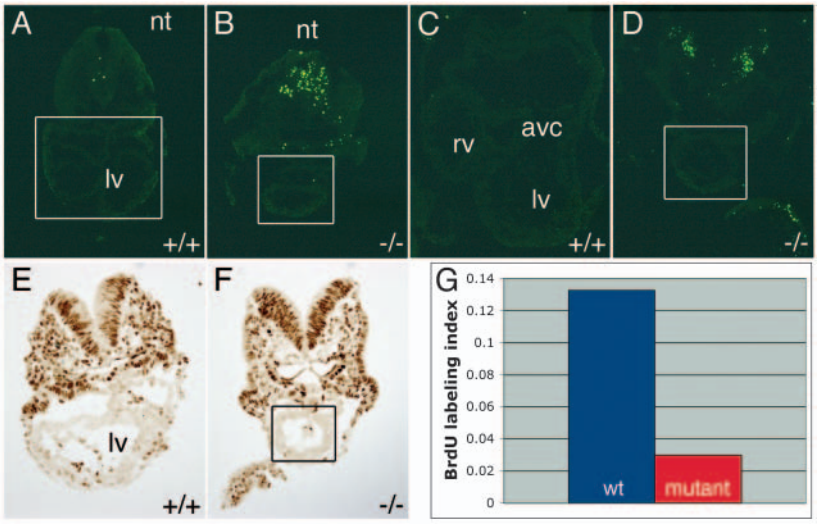

Fig. 7. Apoptosis and proliferation in *Tbx20*$^{-/-}$ hearts. (A-D) TUNEL assay on transverse sections of E8.5 (A,B) and E9.5 (C,D) embryos does not detect a difference in apoptosis between hearts of *Tbx20* mutant embryos (B,D) and wild-type littermates (A,C). However, note the increase in apoptosis in extracardiac tissues such as the neural tube (nt) in the *Tbx20* mutant. White boxes highlight the heart regions. (E,F) Analysis of cell proliferation in transverse sections of the (ventricular) heart region of wild-type (E) and *Tbx20* mutant (F) embryos at E8.75, by BrdU incorporation assay. A black box indicates the mutant heart in F. (G) Quantification of cell proliferation by the ratio of BrdU-positive cells to total cell number, the BrdU-labeling index, in the analyzed heart area. The labeling index is significantly reduced in the mutant heart (0.0298±0.0033) when compared with the wild type (0.1325±0.0089). avc, atrioventricular canal; lv, left ventricle; rv, right ventricle.

suggests that the arrest of heart development in the *Tbx20*$^{-/-}$ embryos is accompanied and probably partly caused by a reduction of cellular proliferation rates. At E9.5, *Tbx20*$^{-/-}$ embryos are characterized by a complete arrest of cellular proliferation in all tissues. We assume that the general arrest in cell proliferation at this stage is due to the severe vascular defects of the *Tbx20*$^{-/-}$ embryos.

Discussion

Vertebrate heart development is a multi-step process comprising patterning, cell differentiation and morphogenesis. Transcription factors and their combinatorial action have been shown to govern many of the underlying molecular pathways. This study shows that the T-box transcription factor gene *Tbx20* is essential for progression from a linear heart tube with AP polarity to a multi-chambered entity with additional polarization and differentiation along the DV axis. The mouse phenotype bears similarities to the phenotypes of zebrafish and *Xenopus* morphants in *Tbx20* orthologs. Our study extends the phenotypic analyses of these morphants, and provides a molecular explanation for the arrest in cardiac development.

Tbx20 regulation of *Tbx2* and formation of cardiac chambers

Chamber formation relies on an integrated patterning program that directs localized differentiation programs along the AP and DV axes of the linear heart tube (reviewed by Moorman and Christoffels, 2003). A linear heart tube with normal AP polarity is established in *Tbx20* mutant embryos. However, DV, i.e. inner-outer curvature, patterning revealed by *Hand1* and *Cited1* expression is absent, and the program for chamber myocardial differentiation is not initiated.

Conceivably, *Tbx20* directly controls DV patterning and subsequent activation of the chamber differentiation program. Loss of *Hand1* expression may contribute to the phenotypic defects in *Tbx20*$^{-/-}$ hearts. *Hand1*, a marker for DV patterning, is required for the formation of the ventrally derived ventricular outer curvature (Biben and Harvey, 1997; Christoffels et al., 2000; Riley et al., 1998). Alternatively, *Tbx20* assures progression from the linear heart tube by preventing the activation or maintenance of the primary myocardial program, specifically in the primitive ventricle. We favor this possibility, and suggest that *Tbx20*-mediated repression of *Tbx2* is pivotal to the normal program of chamber formation.

Tbx2 has a well-established role in maintaining the primary myocardial phenotype. *Tbx2* is expressed in regions of the looped and multi-chambered heart retaining the primary myocardial phenotype (Gibson-Brown et al., 1998; Yamada et al., 2000; Habets et al., 2002; Christoffels et al., 2004a; Harrelson et al., 2004). Loss of *Tbx2* expression leads to expansion of chamber myocardium into the AVC, and subsequent defects in formation of septa and valves (Harrelson et al., 2004). Most importantly, ectopic expression of *Tbx2* in the myocardium of the linear heart tube completely prevents chamber formation (Christoffels et al., 2004a). Thus, loss of *Tbx20* phenocopies misexpression of *Tbx2* in the linear heart tube. This suggests that ectopic expression of *Tbx2* in *Tbx20* mutant hearts accounts for the arrest in cardiac development.

Similar linear heart tube phenotypes have been described for *Nkx2.5* and *Tbx5* mutants (Lints et al., 1993; Tanaka et al., 1999; Bruneau et al., 2001). Cardiac expression of *Nkx2.5* and *Tbx5* is unaltered in *Tbx20*$^{-/-}$ hearts, negating a role for these genes in mediating *Tbx20* function. Expression of *Tbx20* is unaltered in *Tbx5* mutants (Stennard et al., 2003). This and the different signature of molecular markers in all three mutants strongly suggests that *Tbx20*, *Nkx2.5* and *Tbx5* act in distinct cardiogenic programs of chamber formation in the mouse.

Tbx5 and *Nkx2.5* synergistically activate the expression of *Nppa* in the forming chamber myocardium (Bruneau et al., 2001; Hiroi et al., 2001). The expression of *Nppa* is completely abolished in *Tbx20* mutant hearts, although expression of the potential activators Tbx5 and Nkx2.5 is maintained. Habets et al. have recently revealed the ability of Tbx2 to counteract the synergistic activation of *Nppa* by Tbx5/Nkx2.5 (Habets et al., 2002). Thus, ectopic Tbx2 in the *Tbx20*$^{-/-}$ heart may compete with Tbx5 in binding to enhancer elements driving expression of *Nppa* and possibly other chamber myocardial specific genes. In addition, Tbx2 is a direct repressor of connexin 40 and connexin 43 in chamber myocardium (Chen et al., 2004; Christoffels et al., 2004a), which explains the repression of these genes in the *Tbx20* mutant heart.

It is unclear how ectopic activation of *Tbx2* expression in the

Tbx20$^{-/-}$ heart is mediated on the molecular level. *Tbx20* has recently been shown to act as a transcriptional repressor on T-sites in cardiac promotors (Plageman and Yutzey, 2004b), opening the possibility that *Tbx20* directly represses *Tbx2*. However, such a function has not been experimentally confirmed, and is not easy to reconcile with the overlapping expression of *Tbx2* and *Tbx20* in the AVC and OFT from E8.5 onwards. Alternatively, ectopic expression of *Tbx2* could be achieved indirectly. *Tbx2* is induced by Bmp signaling in cardiogenic mesoderm (Yamada et al., 2000). *Bmp2* is co-expressed with *Tbx2* in the AVC. Thus, ectopic expression of *Tbx2* in the primitive ventricle of *Tbx20*$^{-/-}$ embryos could be achieved by activation or derepression of its activator *Bmp2*. Indeed, our analysis has shown that weak but consistent *Bmp2* expression is found in the primitive ventricle in *Tbx20* mutant hearts. Regulation of *Bmp2* by *Tbx20* is likely to be complex. *Bmp2* is co-expressed with *Tbx20* in the AVC from E8.5 onwards (Keyes et al., 2003). However, *Bmp2* expression in the primitive IFT of the *Tbx20* mutant heart is downregulated. In addition, *Tbx2* expression is also found in the outflow tract region of the *Tbx20*$^{-/-}$ heart at E8.5, whereas *Bmp2* is not. Conceivably, combinatorial action of *Tbx20* with other transcription factors will define the regionally restricted expression of potent signaling molecules such as *Bmp2* in the developing heart.

We observed that endocardium of the *Tbx20* mutant heart is also reprogrammed to a type normally lining primary myocardium. At this point it is unclear whether *Tbx20* controls endocardial fate directly. Alternatively, myocardial expression of *Bmp2* and/or *Tbx2* may induce a fate change in the underlying endocardium. Analysis of transgenic embryos ectopically expressing *Tbx2* (Christoffels et al., 2004a) will allow us to discriminate between these possibilities.

Tbx2 is closely related to Tbx3. Both proteins share an identical DNA-binding region and act as transcriptional repressors on conserved DNA-binding sites. *Tbx2* and *Tbx3* are co-expressed in the primary myocardium of the AVC, and can similarly be induced by *Bmp2* signaling (Yamada et al., 2000; Plageman and Yutzey, 2004b). Cardiac defects have not been described in mice homozygous for a null allele of *Tbx3* (Davenport et al., 2003). These experimental findings point to a redundant function of *Tbx3* with *Tbx2* in cardiac development. However, our results suggest that both genes are differentially regulated and might thus exert distinct functions in heart development. *Tbx2* is upregulated in *Tbx20* mutant hearts whereas *Tbx3* expression is lost. Hence, ectopic Bmp2 expression might activate *Tbx2* only. Conceivably, *Tbx3* expression is regulated by other signaling systems or requires higher levels of Bmp2 signaling than *Tbx2*.

At this point, we cannot exclude that other *Tbx2*-independent cardiac functions of *Tbx20* exist. Analysis of the phenotypic consequences of *Tbx20* loss in a *Tbx2* mutant background will be a valuable approach to reveal additional requirements for *Tbx20* in heart development.

Tbx20 and the secondary heart field

Detailed recent analyses suggest that, in the mouse, the right ventricle and the outflow tract, as well as the atria and sinus venosus, originate by continuous recruitment and myocardial differentiation of splanchnic mesodermal cells (Kelly et al., 2001; Cai et al., 2003). Mutations in genes that effect the recruitment, migration, differentiation or proliferation of cells from this secondary heart field show severe hypoplasia of the right ventricle, outflow tract and atria (Lin et al., 1997; Srivastava et al., 1997; Cai et al., 2003; von Both et al., 2004). Similar defects are seen in the *Tbx20*$^{-/-}$ heart, suggesting that *Tbx20* may at least partly regulate secondary heart field development. However, a primary role for *Tbx20* in secondary heart field development seems unlikely for several reasons. First, early *Tbx20* expression overlaps with that of *Mlc2a*, which is considered to mark the primary heart field, but only marginally with that of *Isl1*, a marker for the secondary heart field (Stennard et al., 2003; Cai et al., 2003). Second, markers for the secondary heart field including *Isl1*, *Foxh1*, *Mef2c* and *Fgf10* are unchanged in *Tbx20* mutant hearts, excluding direct regulation of any of these genes by *Tbx20*. Last, the short linear heart tube observed in *Tbx20* mutant embryos and secondary heart field mutants such as *Isl1*, are morphologically similar but molecularly different. Markers for DV patterning and ventricular and atrial differention are not expressed in *Tbx20* mutant hearts. By contrast, DV patterning (*Hand1* expression) and ventricular differentiation (*Hey2* expression) take place normally in *Isl1* and *Foxh1* mutant hearts (Cai et al., 2003; von Both et al., 2004).

However, even if ventricular development is arrested at E8.5 in *Tbx20*$^{-/-}$ embryos, the secondary heart field should still add cells at the poles, resulting in elongation of the heart tube at the arterial and venous ends after E8.5. We think that cells from the secondary heart field are prevented from their normal fate for two reasons. First, *Tbx2* is ectopically expressed throughout the linear heart tube of *Tbx20* mutants. *Tbx2* expression now abuts and possibly also extends into the secondary heart field region. Ectopic *Tbx2* might downregulate proliferation of mesenchymal cells in the secondary heart field region, and/or prevent their myocardial differentiation at the border of secondary heart field and myocardium. This hypothesis gains support from cardiomyocyte-restricted overexpression of *Tbx2* in transgenic mouse embryos. These embryos had short heart tubes as well, supporting the notion that *Tbx2* expression at the border of the secondary heart field interferes with the recruitment of mesenchymal cells. In some cases, we observed transgenic *Tbx2* expression extending into the anterior heart field, as if these cells had turned on the *Mhcb* promoter, but had failed to move in (Christoffels et al., 2004a). Therefore, downregulation of *Tbx2* in cells at the myocardial-secondary heart field border may be required for their subsequent recruitment to the poles of the heart tube. Second, it is likely that impaired vascular development in *Tbx20* mutant embyros dramatically affects cell proliferation, thus preventing expansion of the pool of splanchnic mesodermal cells in the secondary heart field.

A conserved program in vertebrate cardiogenesis?

In all vertebrates analyzed to date, *Tbx20* is expressed in early cardiogenic mesenchyme, in the linear heart tube, during heart looping and chamber formation. Analyses of a *Tbx20* mouse mutant in this study, and of morphants of the zebrafish and *Xenopus* orthologs (Szeto et al., 2002; Brown et al., 2005), suggest that, in vertebrates, *Tbx20* has no unique early function in the induction of cardiac cell fate from the lateral plate mesoderm and the formation of a linear heart tube, but only in the transition to the multi-chambered heart. The late

requirement for a T-box transcription factor is reminiscent of the situation in mesoderm formation. There, brachyury (*T*) is expressed in the mesoderm forming region with the onset of gastrulation, but is only required for mesoderm formation and axial elongation significantly later (Herrmann and Kispert, 1994). In either case, redundancy with another Tbx family member may account for this lack of an early requirement. Alternatively, these T-box transcription factors may need to interact with auxiliary factors that become expressed only later in development. The analyses of zebrafish *hrt* and *Xenopus Tbx20* morphant phenotypes have shown that the heart tube acquires AP patterning, but fails to loop and forms abnormal chambers. Although not addressed in those studies, it is tempting to assume that DV patterning of the heart tube and chamber differentiation fails, similar to the situation in the mouse. Interestingly, it was noted that the *hrt* morphant heart contracted abnormally and slowly. This resembles the change of contraction velocity and rhythm we observed in the *Tbx20*$^{-/-}$ heart tube. In zebrafish heart development, *hrt* may regulate *tbx5* negatively, as *hrt* is both sufficient and required to repress *tbx5* expression in the developing heart (Szeto et al., 2002). In the mouse, we observed unchanged *Tbx5* expression but upregulated *Tbx2* instead. Simplistically, one could suggest that *tbx5* functionally replaces *Tbx2* in the zebrafish. However, this is unlikely. *tbx5* has been shown to be required for looping and maintaining the heart tube in the zebrafish (Garrity et al., 2002), a role that is similar to the requirement for murine *Tbx5* in posterior heart development (Bruneau et al., 2001). As *Tbx2* has not yet been described in the zebrafish, the functional significance of *tbx5* derepression in the *hrt* morphant heart remains unclear. Notably, in *Xenopus Tbx20* morphants cardiac *Tbx5* expression is unchanged, similar to the situation in the mouse. However, a synergistic role for *Tbx5* and *Tbx20* in Xenopus heart development was suggested (Brown et al., 2005). Such a mechanism seems unlikely for mouse cardiogenesis because the cardiac phenotypes of *Tbx5* and *Tbx20* mutants differ significantly (Bruneau et al., 2001) (this study). Together, these findings may provide evidence for the divergence of *Tbx20*-controlled molecular pathways in zebrafish, *Xenopus* and mouse, compatible with the increase in cardiac complexity achieved in tetrapod evolution.

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