Tbx5 specifies the left/right ventricles and ventricular septum position during cardiogenesis

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Summary

Extensive misexpression studies were carried out to explore the roles played by *Tbx5*, the expression of which is excluded from the right ventricle (RV) during cardiogenesis. When Tbx5 was misexpressed ubiquitously, ventricular septum was not formed, resulting in a single ventricle. In such heart, left ventricle (LV)-specific *ANF* gene was induced. In search of the putative RV factor(s), we have found that chick *Tbx20* is expressed in the RV, showing a complementary fashion to *Tbx5*. In the Tbx5-misexpressed heart, this gene was repressed. When misexpression was spatially partial, leaving small Tbx5-negative area in the right ventricle, ventricular septum was shifted rightwards, resulting in a small RV with an enlarged LV. Focal expression induced an ectopic boundary of Tbx5-positive and -negative regions in the right ventricle, at which an additional septum was formed. Similar results were obtained from the transient transgenic mice. In such hearts, expression patterns of *dHAND* and *eHAND* were changed with definitive cardiac abnormalities. Furthermore, we report that human *ANF* promoter is synergistically activated by Tbx5, Nkx2.5 and GATA4. This activation was abrogated by Tbx20, implicating the pivotal roles of interactions among these heart-specific factors. Taken together, our data indicate that Tbx5 specifies the identity of LV through tight interactions among several heart-specific factors, and highlight the essential roles of Tbx5 in cardiac development.

Key words: Heart development, Ventricular septum, *Tbx5*, *Tbx20*, *dHand*/*eHand*

Introduction

T-box (Tbx) genes are involved in various aspects of pattern formation in both vertebrate and invertebrate embryos. They encode transcription factors that are characterized by a highly conserved DNA-binding domain (T-box) and an unusual mode of DNA recognition (Kispert and Herrmann, 1993; Muller and Herrmann, 1997; Smith, 1999). The biological functions of Tbx genes have been investigated by misexpression by in ovo electroporation in chick and gene targeting in mice (Takeuchi et al., 1999; Rodriguez-Esteban et al., 1999; Koshiba-Takeuchi et al., 2000; Wilkinson et al., 1990; Chapman and Papaioannou, 1998; Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001; Bruneau et al., 2001; Russ et al., 2000).

One of most well-characterized Tbx genes is *Tbx5*. This gene is expressed in developing forelimb buds and on the dorsal side of the retina. We and another group have reported that *Tbx5* is a crucial determinant of wing (forelimb) (Takeuchi et al., 1999; Rodriguez-Esteban et al., 1999). In addition, *Tbx5* regulates pattern formation of the eye and also the retinotectum projection along its dorsoventral axis (Koshiba-Takeuchi et al., 2000). The roles played by *Tbx5* during heart development, however, remain unclear, although mutations of human *TBX5* have been found in individuals with Holt-Oram syndrome (OMIM 142900) (Basson et al., 1997; Li et al., 1997; Basson et al., 1999). In such individuals, characteristic defects of the upper limb and heart are observed (Holt and Oram, 1960). Precise analysis suggests the haploinsufficiency of *TBX5* in Holt-Oram syndrome. Similar haploinsufficiency was reported for other T-box genes, such as human *TBX3* and *TBX1* (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001; Bamshad et al., 1997; Epstein, 2001), indicating that the levels of T-box proteins are crucial for normal functioning (Bruneau et al., 2001; Hatcher and Basson, 2001).

Recently, mouse *Tbx5* was knocked out to generate heterozygous and homozygous mice (Bruneau et al., 2001). Homozygous $Tbx5^{del/del}$ mice do not survive past embryonic day 10.5 (E10.5) because of the arrest in heart development at E9.5. By contrast, heterozygous $Tbx5^{del/+}$ mice show several morphological alterations in both the heart and the forelimb. In

such deformed hearts, large atrial septum defects (ASDs) and ventricular septum defects (VSDs) are observed as a variety of complex cardiac malformations. In addition, abnormalities of the cardiac conduction system are found. These lines of evidence highlight the multiple roles of *Tbx5* in heart development and the haploinsufficiency of *Tbx5*, providing a valuable model of congenital heart diseases (Bruneau et al., 2001).

Expression patterns of *Tbx5* were reported previously (Bruneau et al., 1999; Yamada et al., 2000). In both chick and mouse, *Tbx5* is expressed in the precardiac mesoderm. This expression then becomes restricted to the posterior part of the looping heart tube. Later, *Tbx5* expression is restricted to the atria and the left ventricle, and a ventricular septum is formed at the boundary of *Tbx5*-expressing and non-expressing domains. Hence, this gene would provide a novel and valuable marker to explore the mechanism of ventricular specification. Recently, interesting heart phenotypes of transgenic mice have been reported. For example, the *Tbx5* gene was overexpressed ubiquitously in the primitive heart tube under the control of a β-myosin heavy chain promoter. Persistent expression resulted in heart looping defects, abnormalities of early chamber development and loss of ventricular-specific gene expression, indicating an essential role for *Tbx5* in early heart development (Horb and Thomsen, 1999; Hatcher et al., 2001; Liberatore et al., 2000). Nonetheless, the premature death of such transgenic mice prohibited precise analysis of cardiac development.

Vertebrates exhibit different heart morphologies: fish have one ventricle/one atrium, whereas birds and mammals have two ventricles/two atria. Considering the left ventricle-specific expression of *Tbx5*, this gene could be useful for exploring the evolution of vertebrate hearts and could provide valuable insights on the ventricle specification, onset of congenital heart diseases and evolution of vertebrate heart morphology. For this purpose, we modified our in ovo electroporation techniques to optimize efficient expression of transgenes in the developing heart and analyzed the functional roles of the *Tbx5* gene during both chick and mouse cardiac development in detail. In addition, we report that chick *Tbx20* is expressed in the right ventricle, showing a mutually exclusive pattern to *Tbx5*. Our data provide important insights on a combinatorial expression patterns of Tbx genes in the developing heart and their putative interaction.

Materials and methods

In ovo electroporation

In ovo electroporation was carried out as described previously (Takeuchi et al., 1999; Momose et al., 1999). We modified our in ovo electroporation techniques to obtain efficient expression of transgenes in the heart. Briefly, a CUY-21 electroporator (Gene System, Osaka, Japan) was used. Fertilized eggs were purchased from Takeuchi and Yamagishi poultry farms (Nara, Japan). A small window was opened for access. PBS(–) was poured over the embryo to obtain appropriate resistance (1.0 kΩ). Two platinum electrodes (Gene System, Osaka, Japan) were fixed in parallel or in a cross to obtain ubiquitous or restricted expression of the transgene, respectively. An anode was inserted beneath the embryonic endoderm. A cathode was placed on the surface of ectoderm, and the DNA solution was injected by a sharp glass pipette into the space between the embryonic ectoderm and the precardiac mesoderm. The positions of electroporation were selected according to the cardiac fate map (Montgomery et al., 1994; Redkar et al., 2001; Fishman and Chien, 1997). Electric pulses were applied (5 V, 40 mseconds, three times) during injection of the DNA solution into the space between the endoderm and the mesoderm. After the eggshells were sealed, the embryos were allowed to develop in humidified incubators. Expression plasmids were constructed in the RCAS retroviral vector as described previously (Motgan and Fekete, 1996).

Whole-mount in situ hybridization and isolation of probes

In situ hybridization was performed as described previously (Wilkinson, 1993). Probes for *ANF*, *BMP2*, *Tll1* and *VEGF* were amplified by RT-PCR using primers derived from the published sequences (GenBank Accession Number X57702, X75914, U75331 and S79680, respectively). Zebrafish probes for Tbx5 and Tbx20 were also amplified by RT-PCR based on the published sequences (GenBank Accession Number AF152607 and AF253325, respectively).

Transient transgenic assay

Transgenic mice were generated by pronuclear injection of plasmids into fertilized eggs as described previously (Saijoh et al., 1999). The injected embryos were transferred into pseudopregnant recipients and allowed to develop in utero. Embryos were examined for the presence of the transgene by PCR. Four primers were used for PCR analysis: 5′-GAGTTCCCCAAGTGAATGAAA-3′ and 5′-GCAGACATTCAG-TGGACT-3′ for the β-*MHC* construct (Liberatore et al., 2000), and 5′-TGGTGAACCGCATCGAGCTGAAG-3′ and 5′-CGTCCTCGAT-GTTGTGGCGGATC-3′ for the *MLC-2v* construct (Ross et al., 1996).

Transfection assay

Zebrafish *Tbx20* full-length sequence was amplified by RT-PCR, then subcloned in pCAGGS expression vector (Niwa et al., 1991). Human *ANF* promoter was amplified by PCR using primers from the published sequence (1539 bp upstream fragment from the initiation codon of *ANF*). This region was been reported to contain three Tbx5-binding sites (Bruneau et al., 2002). Transfection was performed based on the Polyethylenimine (PEI)-mediated gene delivery method (Boussif et al., 1995). Luciferase and β-gal assays were carried out as described previously (Ogura and Evans, 1995).

Results

In the chick, precardiac precursor cells are formed in two crescent areas at the rostral part of the embryo. These cells form the precardiac mesoderm, which enlarges and fuses to form a heart tube. After a looping event, both atrial and ventricular chambers develop, with two septa separating blood flow (Montgomery et al., 1994; Redkar et al., 2001). As reported previously (Bruneau et al., 1999; Yamada et al., 2000), *Tbx5* expression is evident from the very early stages. First, *Tbx5* is expressed in a gradient fashion, with the strongest signal at the caudal part of the heart tube. This expression then becomes restricted to the left ventricle and atria at stage 30 (Fig. 1A). *Tbx5* is expressed in the left side of the ventricular septum, leaving a *Tbx5*-negative half in the right (Bruneau et al., 1999). This expression pattern strongly suggests that *Tbx5* specifies the identity of the left and right ventricles and that the ventricular septum is formed at the boundary of *Tbx5*-positive and -negative regions (black arrowhead in Fig. 1A) to separate the aortic and pulmonary circulation.

In search of putative *Tbx* gene(s) expressed in the *Tbx5*-negative right ventricle, we have found that chick *Tbx20* shows a complementary pattern of expression in the heart ventricle (Fig. 1B). Hence its expression is confined to the

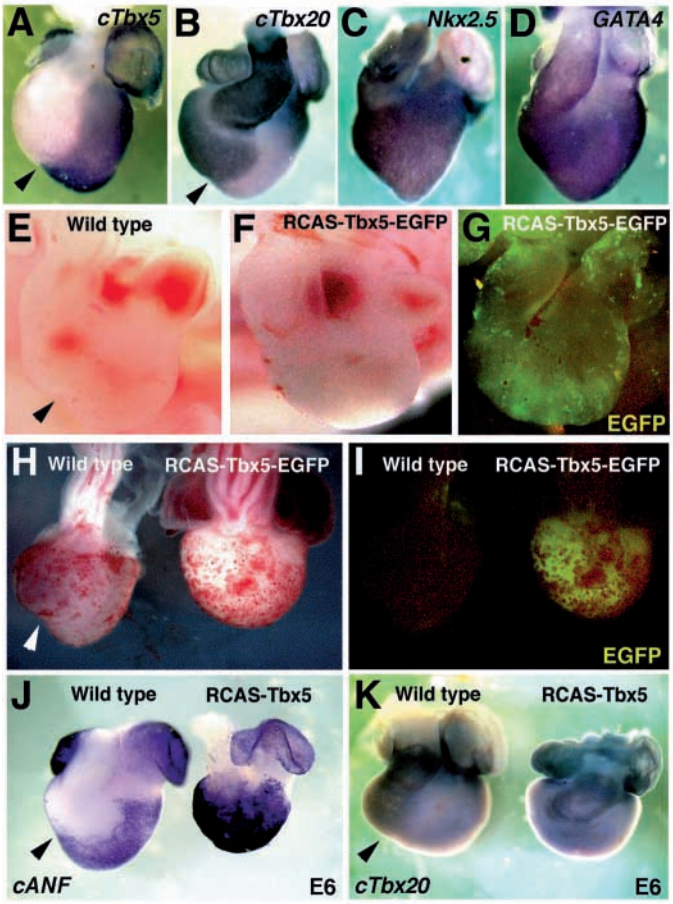

Fig. 1. (A,B) At stage 30, chick *Tbx5* (A) is expressed in the developing left ventricle and *Tbx20* (B) is expressed in the right ventricle. The ventricular septum is formed at the boundary of the two areas (arrowheads, A,B) that give rise to the left and right ventricles. (C,D) Expression of chick *Nkx2.5* (C) and GATA4 (D) is observed throughout the developing heart ventricles. (E) A small indentation can be observed on the surface of the normal heart (arrowhead) at E5. This corresponds to the position of ventricular septum formation. (F) When *Tbx5* was misexpressed, this indentation was not formed. (G) In electroporated hearts, *Tbx5* was misexpressed ubiquitously, as confirmed by the GFP signals derived from the electroporated *Tbx5-EGFP* fusion gene. (H) At E8, the electroporated heart exhibited a round, smooth shape, whereas an indentation (arrowhead) was formed on the surface of the normal heart. (I) In electroporated hearts, GFP fluorescence signals were observed in the entire ventricle. No GFP signal was detected in the normal heart. (J) Normally, the chick atrial natriuretic factor (*ANF*) gene is expressed in the left ventricle. An indentation can be observed at the right border of *ANF* gene expression (arrowhead). When *Tbx5* was misexpressed, the *ANF* gene was induced in the entire ventricle at E6 and (K) expression of *Tbx20* gene disappeared from the right side where normally this gene is expressed.

right ventricle and abuts that of *Tbx5* at the position of the ventricular septum indicated by a black arrowhead in Fig. 1B. These observations strongly suggest that the right and left ventricles are specified by the distinct Tbx genes expressed in an asymmetric fashion.

Recently, physical interactions between several heart-specific transcription factors have been reported to play pivotal roles during cardiac development (Bruneau, 2002; Nemer and Nemer, 2001). Tbx5 associates directly with Nkx2.5 to promote cardiomyocyte differentiation (Bruneau et al., 2001; Hiroi et al., 2001). Nkx2.5 interacts with GATA4 to regulate several cardiac genes (Durocher et al., 1997; Lee et al., 1998). In addition, physical interactions among Tbx2, Tbx5 and Nkx2.5 are crucial for the cardiac development (Habets et al., 2002). These lines of evidence indicate that the tight crosstalks among heart-specific factors control the orchestrated cardiac development. As expected, two key factors, *Nkx2.5* and *GATA4* genes, are expressed ubiquitously in the developing heart ventricles (Fig. 1C,D).

To explore the roles played by Tbx5, we exploited our in ovo electroporation technique to misexpress this gene in the developing chick heart. With our system, the configurations of the electrodes and DNA injection enabled us to obtain rapid and targeted expression of transgenes in the heart (Takeuchi et al., 1999; Koshiba-Takeuchi et al., 2000; Ogura, 2002). Using this approach, we misexpressed a *Tbx5-GFP* fusion gene inserted in the RCAS retroviral vector. As reported previously, we did not detect any functional difference between this fusion gene and the wild-type *Tbx5* (Takeuchi et al., 1999; Koshiba-Takeuchi et al., 2000). In addition, we repeated electroporation of *EGFP* gene alone in chick heart, but we did not detect any morphological alteration. When the *Tbx5-GFP* was electroporated into the precardiac mesoderm of embryos at stage 5, we observed robust GFP signals in the entire heart at E5 (Fig. 1G). At this stage, the position of ventricular septum is already evident as a small indentation on the surface of the normal heart (arrowhead in Fig. 1E). By contrast, when *Tbx5* was misexpressed ubiquitously, as confirmed by the GFP signals (Fig. 1G), such an indentation was not formed, making the contour of the heart round and smooth (Fig. 1F,H). At E8, we obtained robust GFP signals uniformly in the entire ventricle, whereas the normal heart did not show any GFP fluorescence (Fig. 1I), indicating that the *Tbx5* gene was misexpressed in the entire heart. As the indentation that is formed on the surface of a normal heart (arrowhead in Fig. 1H) corresponds to the position of the ventricular septum, this morphological change indicates that septum formation was disrupted in the transgene-electroporated heart (Fig. 1H).

To analyze further, we checked the expression of the chick atrial *ANF* gene in both the normal and electroporated hearts (Fig. 1J). In the normal heart, the *ANF* gene is expressed in the left ventricle without any signal in the right as observed in mouse (Zeller et al., 1987). The position of the arrowhead in Fig. 1J indicates the boundary between the left and right ventricles. Contrary to the normal heart, *ANF* was induced strongly in the entire ventricle when *Tbx5* was misexpressed in the developing precardiac field. This strongly suggests that the formation of the right ventricle was disturbed by the extensive misexpression of *Tbx5* (n=8/11). As reported previously (Bruneau et al., 2001), *ANF* gene is one of direct targets of Tbx5. As *ANF* is expressed in the entire ventricle, this also suggests that Tbx5 was successfully misexpressed in the developing heart ventricle. In mammals, *ANF* gene is induced by cardiac stress as first reported by Burnett et al. (Burnett et al., 1986), ubiquitous induction of *ANF* gene in Tbx5-misexpressed heart might be due to cardiac stress. Nonetheless, we did not observe cardiac overload in these chick hearts. Rather, hemodynamic observation suggests that circulation is

severely disturbed and slow, thereby cardiac overload is not a primary cause of *ANF* induction, although we do not exclude that possibility that subsequent hypoxic stress might partially contribute to this induction.

To check the effects of Tbx5 misexpression on the right ventricle-specific marker gene, we examined the expression of *Tbx20* in the Tbx5-misexpressed heart. As shown in Fig. 1B, in the normal heart, *Tbx20* is expressed in the right ventricle with its left limit located at the small indentation (arrowhead in Fig. 1K). By contrast, when Tbx5 was misexpressed, *Tbx20* expression disappeared from the whole ventricle, which shows again the round and smooth contour (Fig. 1K) (n=4/11). These results strongly suggest that misexpression of Tbx5 in the entire ventricle induces the *ANF* expression, and represses *Tbx20* in the right ventricle, thereby converting the *ANF*-

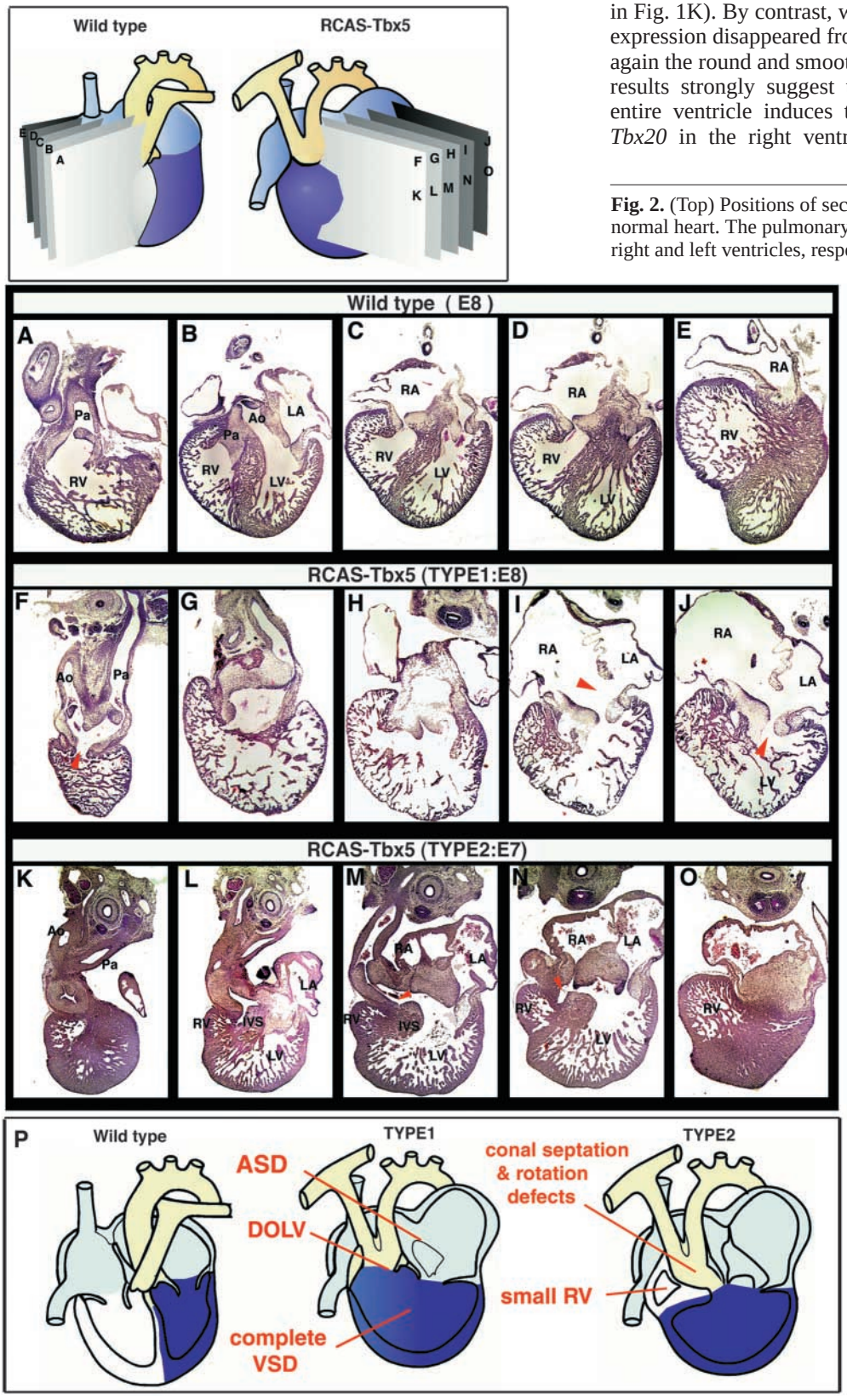

Fig. 2. (Top) Positions of sections A-O. (A-E) Serial sections of normal heart. The pulmonary artery and aorta are connected to the right and left ventricles, respectively. Both right and left ventricles develop normally with the extensive trabecular formation and a ventricular septum. Right and left atrio-ventricular canals are formed at the correct position. (F-J) An abnormal heart in which *Tbx5* was misexpressed ubiquitously (Type 1). Ventricular septum formation was severely disturbed, resulting in a single ventricle. The ventricular wall was thin, and the trabeculae were coarse and rough. Both right and left atria were dilated with an atrial septum defect (ASD; arrowhead in I). The aorta and pulmonary artery were fused at their base and connected to the single ventricle, resulting in a double outlet left ventricle (DOLV; arrowhead in F). Arrowhead in J indicates the atrio-ventricular canal. (K-O) Another type of malformation was observed (Type 2) in which the left ventricle expanded and the right ventricle shrank. The relative sizes of these two ventricles indicate a shift of the ventricular septum formation to the right, although the trabecular formation and the thickness of ventricles were not affected. Conal septation/rotation defects were also observed (arrowhead in M and N). The atrial septum formed, but it was thin and membranous.
(P) Illustrations of the induced malformations. Type 1: atrial and ventricular septum defects (ASDs and VSDs). Alterations in both the aorta and pulmonary artery (DOLV and conal septation/rotation defects). Thin ventricular wall, suggesting abnormal differentiation of cardiac muscle cells. Dilatation of atrium. Type 2: VSD with a shift of the position of septum, resulting in a small right ventricle and expanded left ventricle, the conal septation/rotation defects with coarctation and thin atrial septum.

off/*Tbx20*-on profile of the right ventricle to the *ANF*-on/*Tbx20*-off profile.

To analyze further the morphological changes induced by misexpression of *Tbx5*, we made a series of continuous sections of the normal and electroporated hearts at E8.0. In the normal heart, the ventricular septum formed at the boundary of the left and right ventricles (Fig. 2A-E) (n=6). At this stage, extensive trabecular formation was also evident in both the right and left ventricles that are connected to the pulmonary artery and aorta, respectively (Fig. 2A,B). Both left and right atrio-ventricular canals formed normally (Fig. 2B-D).

In contrast, several anomalous alterations were found in the electroporated hearts (Type 1 in Fig. 2F-J) (n=8/37). First, the formation of the ventricular septum was completely inhibited, resulting in a single ventricle (Fig. 2G-J). Second, a large dilatation of the atria was also observed (Fig. 2I,J). Third, the ventricular wall was thinner than that of the normal heart, and the trabecular formation was coarse and rough, suggesting that differentiation of cardiac muscle cells was also disturbed (Fig. 2G-J). Fourth, the aorta and pulmonary artery were fused at the base and connected to the single anomalous ventricle, resulting in a double outlet left ventricle (DOLV). Although the truncal septum was formed, the conal septation and/or the conal rotation seemed to be abnormal (Fig. 2F). These morphological changes suggest that misexpression of *Tbx5* induces aberrant differentiation of cardiac muscle cells (Hatcher et al., 2001; Liberatore et al., 2000), suppression of ventricular septum formation and malformation of the conotruncal septum, hence resulting in a complex of cardiac defects, as illustrated in Fig. 2P (Type 1).

We observed another type of morphological alteration in electroporated hearts, in which the Tbx5-positive domain was expanded rightward by electroporation (Type 2 in Fig. 2K-O) (n=17/23). In this case, the left ventricle expanded, and the right ventricle shrank (Fig. 2K-O), with a distinct VSD (arrowhead in Fig. 2N). The relative sizes of the two ventricles suggested that the ventricular septum was shifted to the right. Conal septation and rotation defects were also observed (Fig. 2K-M). The atrial septum was formed, but it was membranous

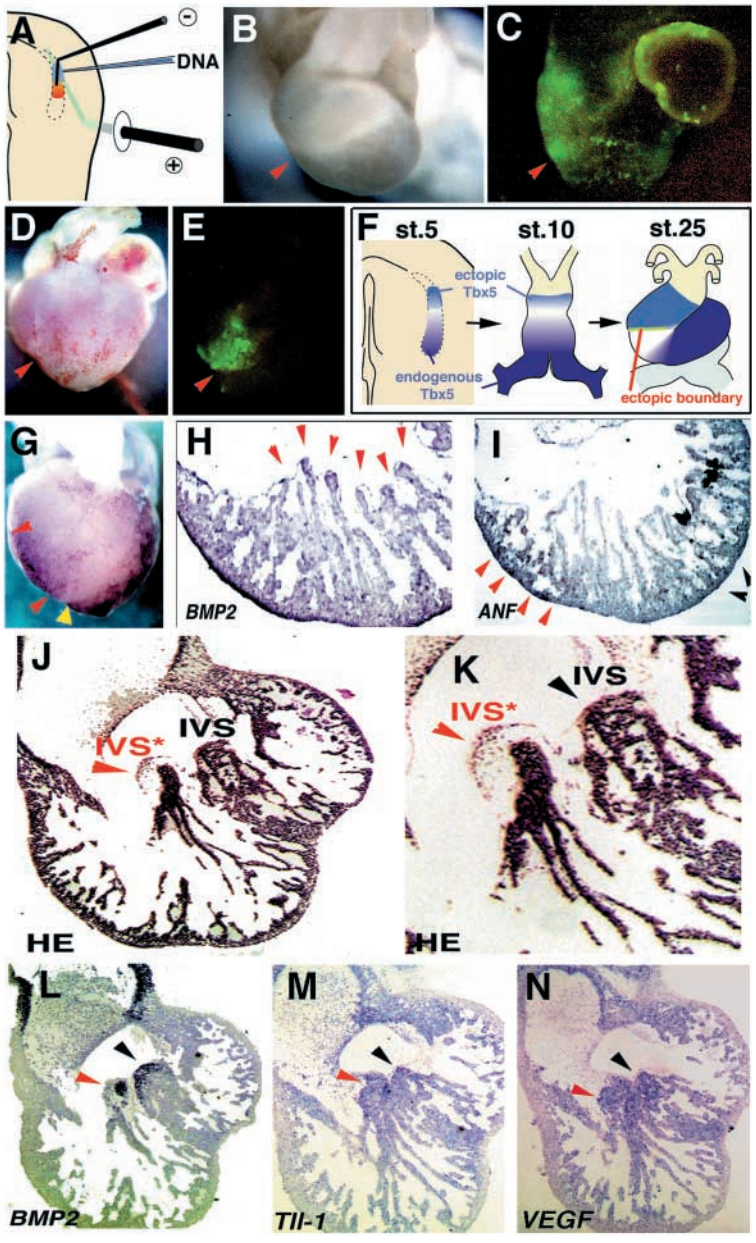

Fig. 3. (A) To obtain restricted expression of the transgene, two electrodes were placed in a cross configuration. With this arrangement, electric pulses hit only a limited region of the precardiac mesoderm. (B) Morphology of a heart, in which the *Tbx5-EGFP* expression construct was electroporated at stage 5. Abnormal indentation is shown by a red arrowhead. GFP fluorescence signals (C) are observed predominantly in the restricted part of the right ventricle (red arrowhead). (D) Morphology of the electroporated heart at E7 showing an abnormal broad indentation (red arrowhead) that shows GFP fluorescence (E, arrowhead). (F) Schematic representation of the experimental design. At stage 5, *Tbx5-EGFP* gene was electroporated at a restricted region of the rostral precardiac field. At stage 10, ectopic misexpression was evident in a rostral part of the prospective right ventricle, making an ectopic boundary of *Tbx 5* expression. (G) At E7, when in situ hybridization using *cANF* probe was performed in electroporated heart, ectopic induction of this gene was evident in a restricted of the prospective right ventricle between two red arrowheads. Normal boundary of *cANF* expression is indicated by a yellow arrowhead. (H,I) At E5, serial sections showed that misexpression of Tbx5 resulted in (H) abnormal growth of trabeculae (red arrowheads). At the tip of each trabecula, ectopic *BMP2* expression was induced, albeit weakly (red arrowheads). (I) *cANF* gene was ectopically induced in the right side of the abnormal trabecula growth (red arrowheads). Normal *cANF* expression is indicated by black arrowheads. Note that abnormal trabecula growth is evident between the ectopic *cANF* expression and the normal *cANF* expression. (J-N) At E6, serial sections were made and examined both histologically (Hematoxylin and Eosin staining) and by marker expression. In the Tbx5-misexpressed heart, an ectopic septum-like structure (IVS*) was observed (J) in addition to the normal septum (IVS). Higher magnification of this section revealed that trabeculae amalgamated to form both the ectopic and the normal septums (K). When *in situ* hybridization was carried out, *BMP2* (L), *Tll1* (M) and *VEGF* (N) genes were found to be expressed in the ectopic septum (red arrowheads) as in the normal septum (black arrowheads).

and thin (Fig. 2N). Trabecular formation and the thickness of the ventricular wall were not affected. Observed cardiac defects are illustrated in Fig. 2P (Type 2).

We also misexpressed the *Tbx5* gene in a restricted domain of developing hearts. In this case, we placed two electrodes in a cross configuration (Fig. 3A) to target electric pulses to a restricted part of the precardiac field. In addition, we used RCAS virus-incompetent chick embryos to prevent expansion of transgene expression. Even in such hosts, stable and long-lasting expression of Tbx5-GFP was obtained. When an RCAS-*Tbx5-GFP* construct was injected at stage 5, discrete GFP fluorescence signals were observed in a limited part of the prospective right ventricle at E4.0 (Fig. 3B,C). At E7.0, an abnormal indentation (red arrowhead in Fig. 3D) was observed on the right ventricle, corresponding to the domain of the restricted GFP signals (red arrowhead in Fig. 3E). Experimental design is illustrated in Fig. 3F. When *Tbx5-GFP* was misexpressed in a restricted region, an ectopic boundary of *Tbx5*-positive and -negative regions was formed in the developing right ventricle, as illustrated in Fig. 3F. In this case, endogenous *Tbx5* expression was stronger in the caudal part of the precardiac mesoderm at stage 5, whereas the electroporated *Tbx5* was misexpressed in the restricted rostral area that gives rise to the right ventricle. At stage 10, endogenous caudal expression is maintained in the developing heart tube, while the expression of the introduced *Tbx5* transgene became evident at its rostral end. At stage 25, three domains were formed from the left side of heart to the right: (1) an endogenous *Tbx5*-positive domain (the prospective left ventricle), (2) a *Tbx5*-negative area (the prospective right ventricle) and (3) an ectopic *Tbx5*-positive area (Fig. 3F). Hence, this restricted expression results in the extra-boundary formation of *Tbx5*-positive and -negative regions in the prospective right ventricle.

As expected, the *ANF* gene was induced in the right ventricle (between two red arrowheads in Fig. 3G) (n=9/14). The boundary of the endogenous c*ANF* expression is indicated by a yellow arrowhead in Fig. 3G. In serial sections of these misexpressed hearts, extension of trabeculae was observed with weak expression of *BMP2* at their tips (Fig. 3H). This accelerated growth of trabeculae suggests that an ectopic septum was induced at the new boundary of *Tbx5* expression, as *ANF* gene was expressed in a complementary manner to the extension of trabeculae (the ectopic and endogenous *cANF* expression is indicated by red and black arrowheads in Fig. 3I, respectively).

To analyze the morphological changes in detail, we made serial sections of electroporated hearts at E6 and carried out in situ hybridization using several ventricular septum markers. Contrary to the normal heart, an ectopic septum-like structure (IVS* in Fig. 3J,K) was formed in the right ventricle (IVS in Fig. 3J,K) (n=8/43). To confirm that this ectopic structure is indeed the ventricular septum, we performed in situ hybridization using several septum markers: *BMP2*, *Tll1* (*tolloid-like 1*) and *VEGF* (vascular endothelial growth factor) (Lyons et al., 1990b; Lyons et al., 1995; Clark et al., 1999; Tomanek et al., 1999; Miquerol et al., 2000). As expected, all of these markers were expressed (Fig. 3L-N, respectively). These lines of evidence strongly suggest that restricted expression of *Tbx5* in the prospective right ventricle induces an ectopic ventricular septum at the new border of *Tbx5* expression in the developing right ventricle.

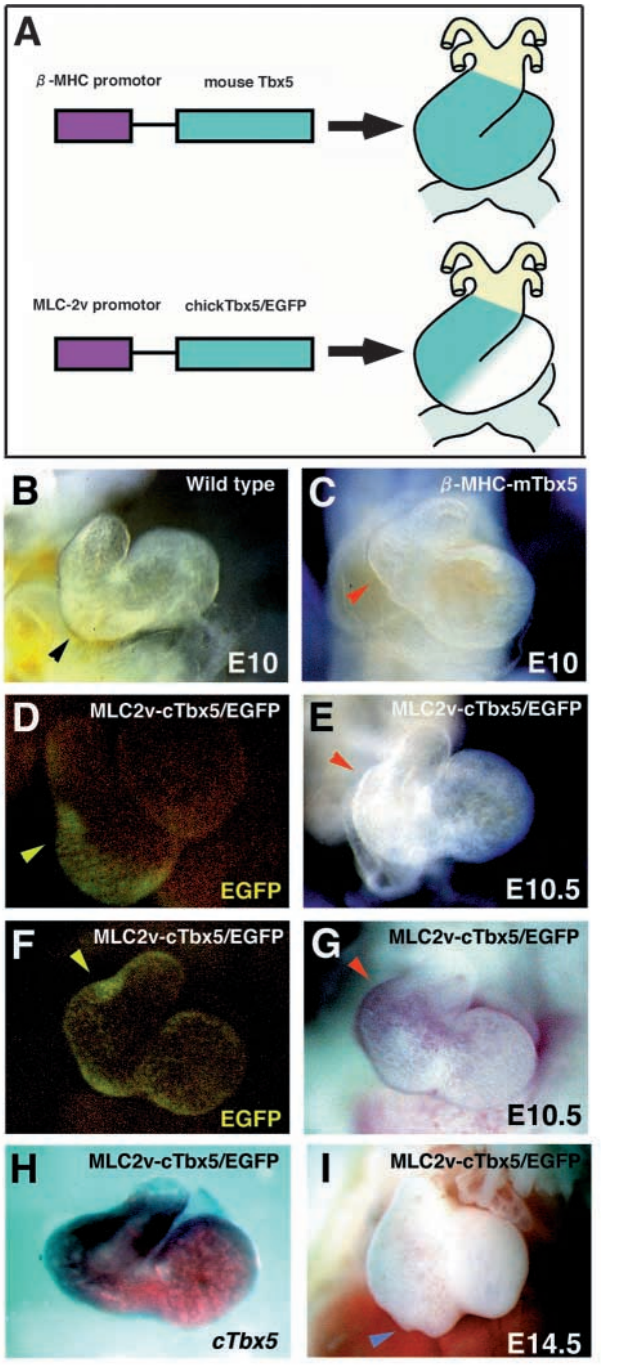

Fig. 4. (A) The *β-MHC* promoter drives expression of mouse *Tbx5* in the entire ventricle. By contrast, the *MLC-2v* promoter targets expression of the chick *Tbx5-EGFP* fusion gene to the right side of heart. (B) In the normal heart, the prospective right ventricle develops as a bulge (black arrowhead). (C) When *Tbx5* was misexpressed in the entire heart, this bulge was disrupted. The prospective left ventricle was connected to the conus cordis with a small junction indicated by a red arrowhead. (D-G) When the *Tbx5-EGFP* fusion gene was expressed by the *MLC-2v* promoter, GFP signals were observed on the right side of the heart (D,F) and a swelling was formed (E,G) at E1.5 on the prospective right ventricle (red arrowheads). (H) When chick *Tbx5* was used to detect its expression in the heart, this transgene was found to be expressed along a gradient in the prospective right ventricle. (I) In some cases, a small protrusion was formed on the surface of the right ventricle (blue arrowhead).

Misexpression experiments in chick hearts indicate that *Tbx5* specifies the left ventricle, and that the ventricular septum is formed at the boundary of *Tbx5*-positve and -negative domains. To confirm this hypothesis, we checked the expression of several markers known to be expressed asymmetrically in the heart. As shown in Fig. 1J, chick *ANF* was induced in the developing right ventricle when *Tbx5* was misexpressed. By contrast, *Tbx20*, which is expressed in the right ventricle, was repressed (Fig. 1K). Nonetheless, in the chick heart, other markers such as the *dHAND* and *eHAND* genes are expressed uniformly in both ventricles (data not shown). This prompted us to carry out misexpression studies in mouse hearts.

For this purpose, we used two expression constructs (Fig. 4A) to target transgene expression in developing mouse hearts. One is a mouse *Tbx5* expression construct in which the *β-MHC* (myosin heavy chain) promoter was used to misexpress this gene uniformly in the ventricle (Liberatore et al., 2000; Lyons et al., 1990a). In another construct, we used the *MLC-2v* (myosin light chain) promoter, which was reported to drive transgene expression in the right ventricle (Ross et al., 1996). In this construct, the chick *Tbx5-EGFP* fusion gene was used to monitor expression. With these two constructs, we made several transgenic mice in which the *Tbx5* genes were misexpressed transiently. We did not establish a stable transgenic line, as we speculated that misexpression of the *Tbx5* gene in the ventricle itself would induce severe abnormalities of heart morphology, and hence cause premature death of the embryos.

When *Tbx5* was misexpressed uniformly with the *β-MHC* promoter, several morphological changes were observed (Fig. 4C). In the normal heart, the developing right ventricle is already formed and evident as a bulge in the right side of heart tube at E10 (black arrowhead in Fig. 4B). By contrast, this bulge was not formed in the hearts in which *Tbx5* was misexpressed by the *β-MHC* promoter (red arrowhead in Fig. 4C). In such hearts, the prospective left ventricle seemed to be connected to the conus cordis with a small junction. In addition, this abnormal junction was shifted rostrally compared with the normal heart (red arrowhead in Fig. 4C). This indicates that uniform expression of *Tbx5* perturbs the normal development of both the right ventricle and the atrio-ventricular connection. We confirmed the uniform expression of the transgene by in situ hybridization (data not shown).

Next, we analyzed the morphological changes induced by the *MLC-2v*/chick *Tbx5-EGFP* construct. When we checked transgene expression by GFP fluorescence signals, GFP expression was evident on the right side of the heart tube as expected (yellow arrowheads in Fig. 4D,F), indicating that the chick *Tbx5* gene was misexpressed in the prospective right ventricle. Contrary to the electroporation experiments in the chick, the *Tbx5-EGFP* gene was expressed in a gradient fashion in this transgenic mouse, as confirmed by both GFP signals (Fig. 4D,F) and whole-mount in situ hybridization using chick *Tbx5* as a probe (Fig. 4H). Although *Tbx5-EGFP* was strongly expressed at the right-most end of the prospective right ventricle, this expression became

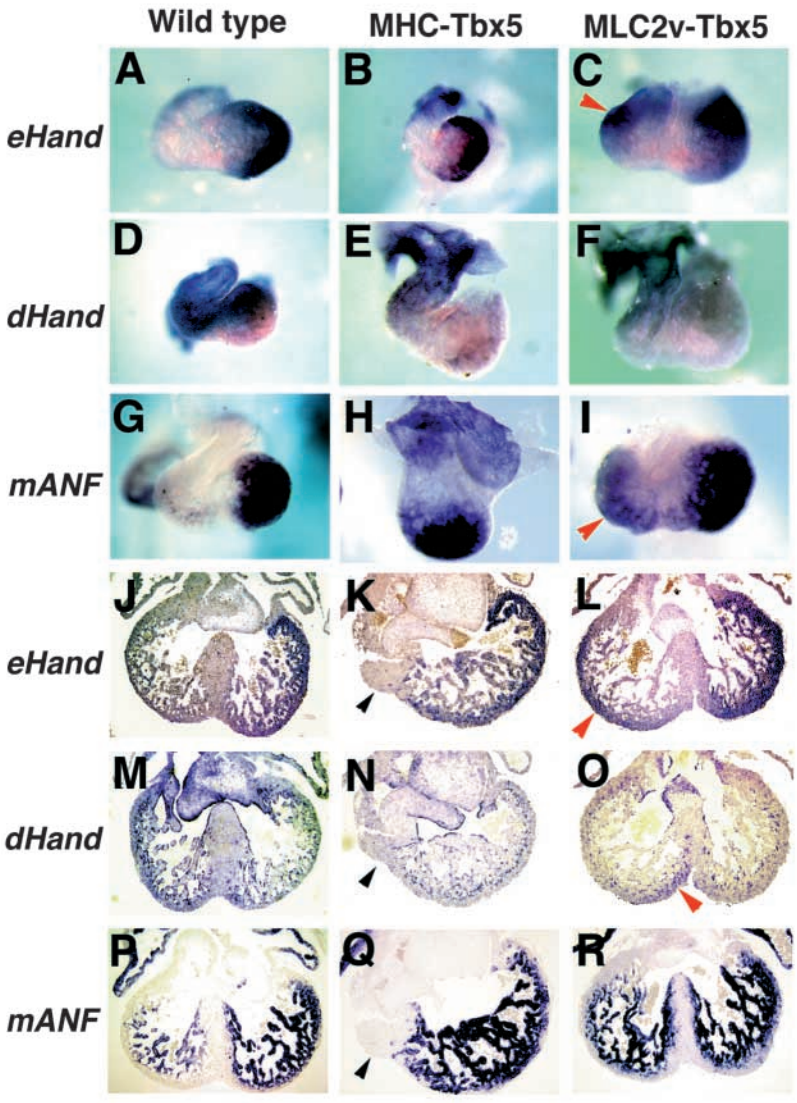

Fig. 5. (A,D,G) Expression of the *eHAND* (A), *dHAND* (D) and mouse *Anf* (G) genes in the normal heart. Both *eHAND* and mouse *Anf* are expressed in the left ventricle, whereas *dHAND* is expressed in the right. (J,M,R) These patterns of expression were also confirmed in tissue sections. (B,E,H) In the *MHC-Tbx5* transgenic mice, both *eHAND* and mouse *Anf* were induced in the entire ventricle, whereas *dHAND* was repressed. (K,N,Q) This was also confirmed in tissue sections. Histological examination of these sections revealed that ventricular septum formation was disrupted with a tiny bulge at the right-most end (black arrowhead). (C,F,I) In the *MLC-2v-Tbx5* transgenic mice, the right ventricle expanded (red arrowhead). In this region, both the *eHAND* and mouse *Anf* genes were induced, and the *dHAND* gene was repressed. (L,O,R) Histological analysis revealed the swelling of the right ventricle (red arrowhead in L). In this swelling, *eHAND* was induced in a gradient manner (red arrowhead in L) and was absent from a region near the septum. (O) In this small region, *dHAND* is expressed (red arrowhead), whereas it is repressed in the rest of right ventricle. (R) Mouse *Anf* was also induced in the right ventricle of this transgenic mouse.

faint at the middle of the ventricle. We did not detect GFP fluorescence at the position of the ventricular septum (Fig. 4D,H). Interestingly, such transgenic hearts exhibit a consistent morphological alteration, namely, swelling of the prospective right ventricle (red arrowheads in Fig. 4E,G). Later, a small protrusion was formed on the surface of the induced swelling in the prospective right ventricle at E14.5 (blue arrowhead in Fig. 4I).

To confirm the nature of these morphological alterations, we checked the expression of several markers by in situ hybridization (Fig. 5). As reported previously, mouse *eHAND* is expressed in the prospective left (Fig. 5A,J). By contrast, *dHAND* genes is predominantly expressed in the prospective right, albeit expanding to the bulbus cordis, the part of the prospective left ventricles and the ventricular septum (Fig. 5D,M) (Srivastava et al., 1997; Firulli et al., 1998; Srivastava et al., 1995). In addition, the mouse *ANF* gene is expressed in the left ventricle (Fig. 5G,R) (Zeller et al., 1987), as is found in the chick. Hence these three genes provide good markers to confirm the identity of the left and right ventricles. When we analyzed transgenic hearts in which *Tbx5* was misexpressed uniformly with the *β-MHC* promoter, we found that the domains of *eHAND* and mouse *Anf* expression were expanded (*n*=3/3, *n*=4/4, respectively), although the gross size of the heart decreased (Fig. 5B,H). By contrast, expression of *dHAND* was repressed, leaving the entire ventricle *dHAND*-negative (Fig. 5E) (*n*=5/5).

We carried out the same analysis on hearts in which the *Tbx5* gene was misexpressed with the *MLC2v* promoter in a gradient manner, with robust expression at the right-most end. In such hearts, the *eHAND* and mouse *Anf* genes were strongly induced in the swelling formed by the expression of this transgene (red arrowheads in Fig. 5C,I) (*n*=4/4, *n*=4/4, respectively), whereas the expression of the *dHAND* gene was suppressed (Fig. 5F) (*n*=3/3).

The same results were obtained when analyzed in tissue sections. This analysis also enabled us to examine the induced morphological changes in detail. As observed with whole-mount in situ hybridization, the *eHAND*, mouse *Anf* and *dHAND* genes are expressed in similar fashions (Fig. 5J,M,R). In addition, we found that the ventricular septum is developing at this stage (E10.5). In the deformed hearts dissected from the *β-MHC-Tbx5* transgenic mice, both *eHAND* and mouse *Anf* genes were induced almost to the right end of the developing ventricle (black arrowhead in Fig. 5K,Q), whereas expression of the *dHAND* gene was found to be repressed completely (Fig. 5N). Furthermore, septum formation was completely suppressed in such hearts (Fig. 5K,N,Q). Instead, a tiny bulge of ventricular wall was formed in a small *eHAND*/mouse Anf-negative region (black arrowheads in Fig. 5K,N,Q).

Conversely, the ventricular septum formed normally in hearts of the *MLC2v-Tbx5* transgenic mice (Fig. 5L,O,R), although the right ventricle appeared to be enlarged. In this enlarged right ventricle, the mouse *Anf* gene was induced strongly (Fig. 5R). Likewise, expression of the *eHAND* gene was induced (red arrowhead in Fig. 5L), albeit expression disappeared near the septum (black arrowhead in Fig. 5L). In this *eHAND*-negative domain near the septum, expression of the *dHAND* gene was detected (red arrowhead in Fig. 5O), although this gene was completely suppressed in the remaining part of the right ventricle (Fig. 5O).

Taken together, these lines of evidence strongly suggest that the forced expression of the *Tbx5* gene in the prospective right ventricle converts expression patterns of several right and left ventricular markers with extensive morphological alterations.

Our embryological data indicate that *Tbx5* specifies the left ventricle and the ventricular septum is formed at the boundary between *Tbx5*-positive and *Tbx5*-negative regions. Interestingly, another T-box gene, chick *Tbx20* is expressed in a complementary fashion, hence expressed in the *Tbx5*-negative right ventricle. As misexpression of Tbx5 in the right ventricle represses *Tbx20* expression, these two Tbx genes may be mutually exclusive. To understand molecular interaction between Tbx5 and Tbx20, we carried out a set of transfection assays using human *ANF* promoter-luciferase reporter construct. As reported previously, Tbx5 and Nkx2.5 synergistically activate this promoter (Bruneau, 2002; Nemer and Nemer, 2001; Bruneau et al., 2001; Hiroi et al., 2001). In addition, another heart-specific transcription factor, GATA4 again synergistically activates cardiac α-actin promoter with Nkx2.5 (Durocher et al., 1997; Lee et al., 1998). In addition, recently, it has been reported that Tbx2 abrogates the synergistic activation of the *ANF* promoter by Tbx5 and Nkx2.5 (Habets et al., 2002). These lines of evidence suggest that the tight interactions and the crosstalks among heart-specific transcription factors play essential roles for the chamber formation of heart (Bruneau, 2002; Nemer and Nemer, 2001).

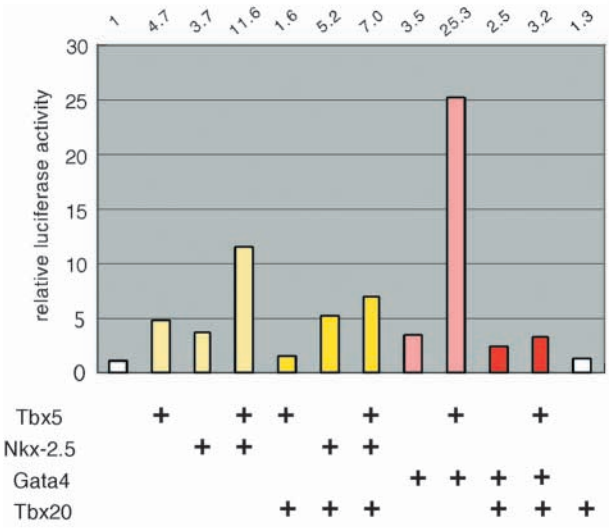

Fig. 6. Human *ANF* promoter-luciferase construct was transfected to COS7 cells along with various expression plasmids. Tbx5 and Nkx2.5 activated this promoter weakly (4.7- and 3.7-fold, respectively). As reported, co-expression of these factors synergistically activated this promoter (11.6-fold). Addition of zebrafish *tbx20* expression vector repressed Tbx5-mediated activation (1.6-fold), although this did not affect Nkx2.5-mediated activation (5.2-fold). Interestingly, Tbx20 abrogated efficiently the synergistic activation by Tbx5 and Nkx2.5 (7.0-fold). When another heart-specific factor GATA4 was expressed, the *ANF* promoter was activated weakly (3.5-fold). When GATA4 and Tbx5 were co-expressed, robust activation was observed (25.3-fold). Tbx20 abrogated this strong synergistic activation efficiently (3.2-fold), whereas Tbx20 did not affect the *ANF* activation by GATA4 alone (2.5 fold). Tbx20 alone did not affect the *ANF* promoter activity (1.3 fold).

When a human *ANF* promoter-luciferase construct was transfected to COS7 cells along with several expression plasmids, Tbx5, Nkx2.5 and GATA4 activated this promoter about 4.7-, 3.7- and 3.5-fold, respectively (Fig. 6). As reported previously, when both Tbx5 and Nkx2.5 were co-expressed, synergistic activation was observed (about 11.6 fold). Interestingly, when both Tbx5 and GATA4 were co-expressed, robust synergistic activation was obtained (25.3-fold), indicating that the synergism between Tbx5 and GATA4 is more potent. By contrast, when full-length zebrafish Tbx20 was misexpressed, these synergistic actions between Tbx5/Nkx2.5 and Tbx5/GATA4 were abrogated (7.0- and 3.2-fold, respectively). As *ANF* gene is expressed in the left ventricle, our data indicate that the left side expression of this gene is regulated in two ways: (1) activation by Tbx5 in the left ventricle and (2) repression by Tbx20 in the right ventricle. These lines of evidence suggest that the specification of chick left/right ventricles and the position of ventricular septum are controlled by the distinct actions of two Tbx genes expressed in the mutually exclusive fashion. When we used chick Tbx20, exactly same data were obtained (data not shown).

Discussion

Results obtained from our experiments are summarized in Fig. 7. Normally, the *Tbx5*-positive/*Tbx20*-negative part develops to become the left ventricle, and the *Tbx5*-negative/*Tbx20*-positive area gives rise to the right ventricle. The ventricular septum is formed at the boundary of these two domains, separating the aortic and pulmonary blood flows completely. These observations indicate that the domains of *Tbx5*/*Tbx20* expression demarcate the left and right ventricles.

As shown in the chick and mouse experiments, when *Tbx5* was misexpressed ubiquitously in the heart tube, ventricular septum formation was disturbed, resulting in a single ventricle and several malformations in the atria and the tissues derived from the bulbus cordis/truncus arteriosus (Type 1 in Fig. 2P and Fig. 7). These morphological changes were accompanied by the complete repression of *Tbx20*. When a small *Tbx5*-negative region was left in the chick heart, the ventricular septum was shifted to the right, with a small right ventricle and an enlarged left ventricle. In such hearts, VSDs and conal septation/rotation defects were frequently observed (Type 2 in Fig. 2P and Fig. 7). When misexpression occurred in a more restricted area in the chick heart, an ectopic septum was formed at the new boundary in the right ventricle (Fig. 4J,K; Type 3 in Fig. 7). These lines of evidence strongly suggest that *Tbx5* specifies the identity of left ventricle through the tight interactions with *Tbx20* and other heart-specific factors during chick cardiac development. In both the chick and mouse experiments, several malformations were observed outside of the ventricle. First, septation defects were frequently found in the conus cordis (Type 1 in Fig. 7, Fig. 2F-J). Second, ASDs were evident with abnormal dilation of atria (Fig. 2I,J). These observations indicate that *Tbx5* has essential roles during development of multiple heart tissues.

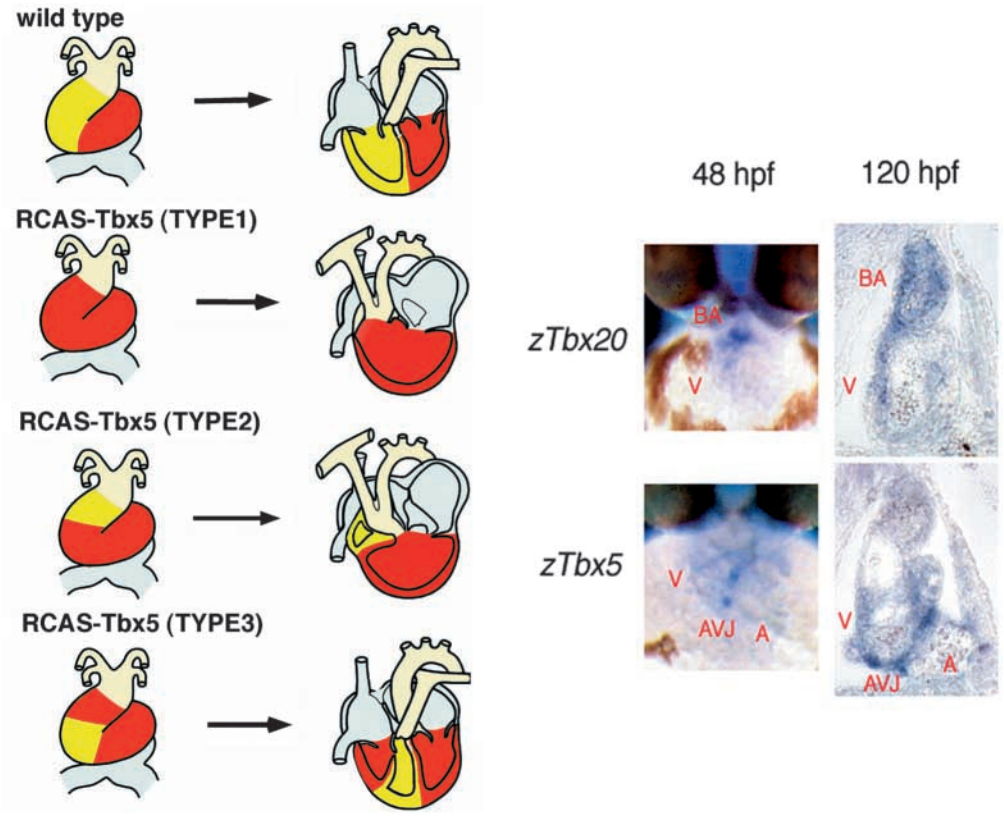

Fig. 7. In the wild-type heart, *Tbx5* is expressed in the prospective left ventricle (red), not in the prospective right ventricle (yellow). At the boundary of these *Tbx5*-positive and -negative regions, an interventricular septum was induced. (Type 1) When *Tbx5* is misexpressed in the entire ventricle, this boundary is not formed and septum formation does not occur, resulting in a single ventricle. (Type 2) When *Tbx5* expression expands to the right, leaving a small *Tbx5*-negative region, the ventricular septum is shifted to the right. In such hearts, the left ventricle expands, and the right ventricle shrinks. (Type 3) When misexpression of *Tbx5* is more restricted to the right ventricle, an ectopic boundary of *Tbx5*-positive and -negative regions is formed, inducing the ectopic formation of the ventricular septum in the right ventricle. (Right panels) In zebrafish heart, *tbx20* is expressed in the bulbus arteriosus (BA) and the ventricular wall near the BA. This gene shows a gradient of expression in the ventricle with strongest signals at the BA, and ending near the atrioventricular junction (AVJ). By contrast, expression of *tbx5* is seen in the AVJ and the ventricle near the AVJ, but expression becomes faint near the BA. This indicates that *tbx20* and *tbx5* are expressed in a complementary fashion, but not discreetly. Expression patterns are basically same at 48 and 120 hours post fertilization (hpf).

In the chick and mouse hearts in which *Tbx5* was misexpressed ubiquitously, the ventricular wall was thinner than normal, and the trabecular formation was coarse and rough. These phenotypic changes indicate that *Tbx5* regulates cardiac muscle differentiation. Recently, it was found that the Tbx5 protein associates and interacts physically with the cardiac homeoprotein Nkx2.5, which is essential in cardiac muscle development (Bruneau et al., 2001; Hiroi et al., 2001). In addition to this, we found that the interaction between Tbx5 and GATA4 synergistically activates the *ANF* promoter. These lines of evidence strongly suggest that Tbx5 possesses multiple interfaces necessary for the multiple protein-protein interactions. Hence, changes of the level of Tbx5 protein in differentiating cardiomyocytes might affect the transcriptional control of cardiac genes by disturbing the balance of multiple interactions. Both the loss and gain of *Tbx5* function disturb the transcriptional control of Tbx5 targets, probably through the abnormal balance between the Tbx5, Tbx20, GATA4 and Nkx2.5 proteins.

Contrary to chick *Tbx20*, it has been reported that mouse *Tbx20* is expressed uniformly in all four heart chambers (Kraus et al., 2001). Similar differences in expression patterns of cardiac genes can be found in *dHAND* and *eHAND*. Mouse *dHAND* (Hand2 – Mouse Genome Informatics) and *eHAND* (Hand1 – Mouse Genome Informatics) genes are expressed differently in the ventricles, whereas chick *HAND* genes are expressed uniformly in the developing heart. In search of putative right ventricle-specific markers, we cloned chick desmin, dystrophin and *SM22α* genes, as mouse counterparts of these genes are expressed in the right ventricle (Kuisk et al., 1996; Kimura et al., 1997; Moessler et al., 1996; Li et al., 1996). However, these genes are expressed uniformly in chick heart (data not shown). These lines of evidence suggest that the mechanism of ventricular specification might be different in species. Because *ANF* gene is the direct target of Tbx5, it might not be adequate to argue that chick *ANF* is the left ventricle-specific marker. As described above, several genes are expressed in different manners in mouse and chick hearts. Hence, it is important to isolate novel left or right ventricle-specific markers. For this purpose, we are performing cDNA subtraction and RDA (representational difference analysis) to isolate region-specific markers in developing vertebrate hearts.

Recently, functions of zebrafish T-box gene *tbx20* (previously known as *hrT*) gene have been reported (Szeto et al., 2002). Interestingly, loss of *tbx20* function resulted in upregulation of *Tbx5*. Conversely, misexpression of *tbx20* induced downregulation of *tbx5*. These data indicate that Tbx20 regulates *tbx5* expression in zebrafish. Our chick data also indicate that Tbx5 represses *Tbx20* when misexpressed (Fig. 1K). These lines of evidence suggest that the tight regulatory interaction between Tbx5 and Tbx20 is crucial in zebrafish and chick, but not in mouse right ventricle. Comparative and comprehensive approaches using various molecular markers should be carried out to uncover the mechanism of development and evolution of different vertebrate hearts.

In addition, Tbx20 represses the synergistic action of Tbx5 and GATA4 on human *ANF* promoter, indicating that Tbx20 represses *ANF* expression in the right ventricle. In mouse heart, *ANF* expression is also restricted to the left ventricle, although mouse *Tbx20* is expressed uniformly. This suggests that different mechanism might operate in the mouse left ventricle to sustain the action of Tbx5 or inhibit the function of Tbx20. As the levels of T-box proteins are crucial for normal development, the levels of Tbx5 and Tbx20 proteins might be important for the development of mouse left ventricle. In addition, we do not exclude the possibility that unknown factor(s) might be involved in this process.

Interestingly, the *ANF* and connexin 40 (*cx40*) genes were found to be direct targets of the Tbx5/Nkx2.5 protein complex (Bruneau et al., 2001; Hiroi et al., 2001). Consistent with this, misexpression of *Tbx5* in the right ventricle induces robust expression of the *ANF* gene in both mice and chicks, as described above (Figs 1 and 5). Although we did not examine the expression of *cx40* in our system, we found that the beating pattern of electroporated chick hearts was abnormal: simultaneous contraction of atria and ventricles instead of normal serial beating (data not shown). This could be related to the abnormal conduction systems found in both the heterozygous $Tbx5^{del/+}$ mice and individuals with Holt-Oram syndrome.

As observed in our gain-of-function approaches, *Tbx5* misexpression disturbs the normal differentiation of cardiac muscle. By contrast, multiple anomalies found in both the heterozygous $Tbx5^{del/+}$ mice and individuals with Holt-Oram syndrome indicate the haploinsufficiency of *Tbx5* in cardiac development. These lines of evidence strongly suggest that the level of *Tbx5* expression in the developing cardiac muscle cell is crucial. As reported, the Tbx5 protein interacts with Nkx2.5 (Bruneau et al., 2001; Hiroi et al., 2001), and another T-box protein Tbr1 makes a complex with CASK, one of the membrane-associated guanylate kinases (MAGUKs), to regulate transcription of target genes (Hsueh et al., 2000). These physical interactions suggest that the balances between Tbx proteins and other interacting partners are important for the orchestrated processes of pattern formation. As genetic analysis of *Drosophila* suggests the interaction between *optomotor blind* (*omb*), one of the *Drosophila* T-box genes, and DPP/WG signaling, the physical interactions between Tbx proteins and other factors including signal transduction factors could be a general characteristic (Srivastava and Olson, 2000; Conlon et al., 2001). Solving these putative interactions would be an important key to understanding the roles played by Tbx genes during development.

As an opposite approach, we electroporated a dominant negative form of *Tbx5* (*EnR-Tbx5*: fusion of *Engrailed* suppressor domain and chick *Tbx5*), expecting a leftward shift of the ventricular septum. Nonetheless, misexpression of EnR-Tbx5 led premature death of embryos from E1.5 to E3 (n=76/76), probably because this type of misexpression itself disturbs cardiac development at very early stages, such as heart tube looping and differentiation.

As shown in Fig. 2F-O, ventricular myocardium was thin when Tbx5 was misexpressed. This would suggest that misexpression of Tbx5 might induce apoptosis or inhibition of cell growth. Nonetheless, when we performed the TUNEL assay to detect apoptotic cell death in differentiating cells, we could not detect any difference between the normal and the Tbx5-misexpressed hearts (data not shown). Even between E4 and E5, at which the ventricular septum starts to form, apoptotic cell death was not evident, indicating that the loss of ventricular septum is not due to Tbx5-induced apoptosis.

Rather, we speculate that retardation of myocardium growth is an indirect effect of abnormal heart development, as septum-less single ventricle hearts exhibited an abnormal beating pattern (data not shown). In addition, both ASD and VSD could cause severe circulation defects, resulting in loss of hemodynamic stimulation to the developing myocardium. We also do not exclude the possibility that overexpressed Tbx5 Inhibits proliferation of myocardiac cells.

Contrary to the phenotypes observed in chick hearts, misexpression of Tbx5 in mouse hearts did not affect growth of myocardium. As shown in Fig. 5L,O,R, misexpression of Tbx5 driven by the *MLC2v* promoter induced swelling of the right ventricle, leaving the size of the left ventricle normal. In addition, thickness of the ventricle was normal, even when Tbx5 was misexpressed in the entire ventricle by the *β-MHC* promoter. These lines of evidence suggest that the abnormal phenotypes observed in both chick and mouse were not due to the proliferation/apoptosis defects. Nonetheless, we do not exclude that possibility that over or high doses of Tbx5 misexpression might affect the growth and/or differentiation of cardiomyocytes, as the balances with other transcription factors, such as Tbx20, GATA4 and Nkx2.5 are pivotal for the normal patterning of vertebrates hearts.

As mentioned previously, vertebrates exhibit different heart morphologies: fish possess one ventricle/one atrium; amphibians have one ventricle/two atria; reptiles have two incomplete ventricles/two atria; and birds/mammals have two complete ventricles/two atria. Although we have not yet expanded our analysis to other vertebrates, the *Tbx5* and *Tbx20* genes could be good markers with which to explore the evolution of heart morphology of various vertebrate animals. To explore further, we checked expression patterns of zebrafish *tbx20* and *tbx5* in developing hearts (right panel in Fig. 7). Interestingly, *zTbx20* is expressed in the Bulbus arteriosus (BA) and the ventricle near the BA, whereas *zTbx5* in the atrioventricular junction (AVJ) and the ventricle near the AVJ. Expression of these Tbx genes in the ventricle is complementary at both 48 and 120 hpf, yet showing gradient expression without making a clear boundary observed in chick heart. These observation are compatible with the mutually repressive actions of these Tbx genes (Szeto et al., 2002) and our observations that the ventricular septum is formed at the distinct expression boundary of *Tbx5*.

Our data provide important insights on cardiac development, onset of human congenital heart diseases, and evolution of vertebrate hearts. Although we are far from a complete understanding, precise molecular analysis of the *Tbx5* gene would provide valuable information for the comprehensive understanding of vertebrate pattern formation.

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