**Tbx1 Regulates Proliferation and Differentiation of Multipotent Heart Progenitors**

Li Chen, Filomena Gabriella Fulcoli, Susan Tang, Antonio Baldini

**Rationale:** *Tbx1* encodes a T-box transcription factor implicated in DiGeorge syndrome, which affects the development of many organs, including the heart. Loss of *Tbx1* results into hypoplasia of heart regions derived from the second heart field, a population of cardiac progenitors cells (CPCs). Thus, we hypothesized that *Tbx1* is an important player in the biology of CPCs.

**Objective:** We asked whether *Tbx1* is expressed in multipotent CPCs and, if so, what role it may play in them.

**Methods and Results:** We used clonal analysis of *Tbx1*-expressing cells and loss and gain of function models, in vivo and in vitro, to define the role of *Tbx1* in CPCs. We found that *Tbx1* is expressed in multipotent heart progenitors that, in clonal assays, can give rise to 3 heart lineages expressing endothelial, smooth muscle and cardiomyocyte markers. In multipotent cells, *Tbx1* stimulates proliferation, explaining why *Tbx1*−/− embryos have reduced proliferation in the second heart field. In this population, *Tbx1* is expressed while cells are undifferentiated and it disappears with the onset of muscle markers. Loss of *Tbx1* results in premature differentiation, whereas gain results in reduced differentiation in vivo. We found that Tbx1 binds serum response factor, a master regulator of muscle differentiation, and negatively regulates its level.

**Conclusions:** The Tbx1 protein marks CPCs, supports their proliferation, and inhibits their differentiation. We propose that *Tbx1* is a key regulator of CPC homeostasis as it modulates positively their proliferation and negatively their differentiation. (Circ Res. 2009;105:842-851.)

**Key Words:** cardiac progenitor cells □ cardiac differentiation □ T-box transcription factors □ serum response factor

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T-box transcription factors have important roles in development, and their mutation is associated with developmental disorders in humans and mice. In particular, several members of this family are critical for heart development and are implicated in congenital heart disease. However, an association between T-box factors and stem cell biology is yet to be made. *Tbx1* encodes a T-box transcription factor involved in DiGeorge syndrome, which is associated with cardiac malformations, as well as other developmental anomalies of organs and structures derived from the pharyngeal apparatus.

*Tbx1* is expressed in several tissues but its mesodermal domain (but not cardiac tissue) is critical for heart development, suggesting that the major role of T-box transcription factor 1 (*Tbx1*) in heart development is effected in precursors destined to populate the heart, rather than in cells resident in the heart. Consistent with this idea, loss of *Tbx1* downregulates cell proliferation in a region of the splanchnic mesoderm that includes the second heart field (SHF). The SHF is a population of migratory cardiac progenitors destined to populate most of the heart and continues to provide progenitors to the heart at least until embryonic day (E)9.5 in the mouse. The expression of *Tbx1* in this migratory population was confirmed by cell fate mapping using a Cre-loxP strategy. Not only is it unknown how *Tbx1* functions within the SHF, but it is also unclear what mechanisms regulate the SHF function in general. In particular, is unclear how this cell population is maintained “active,” ie, capable of proliferating and providing differentiating cells to the heart, over several days of embryonic development, although it appears that fibroblast growth factor (FGF) and bone morphogenetic protein signals have a role in this process.

Recent data have uncovered that different cell types populating the heart (eg, cardiomyocytes, endothelial cells, smooth muscle cells) may derive from a single progenitor. How the homeostasis of this population is regulated remains unknown. In this work, we sought to establish if *Tbx1* is really expressed in cardiac progenitor cells and through what mechanisms it regulates the function of the SHF. Results indicate that Tbx1 is indeed expressed in multipotent cardiac progenitors, and it enhances their proliferation and inhibits their differentiation, thus ensuring the
maintenance of the progenitor population. The mechanisms of cardiac progenitors homeostasis are of relevance for cardiac regeneration as they may indicate strategies to handle and expand cardiac progenitors existing or reprogrammed cells. In addition, the use of multipotent progenitors in cardiac regeneration would have the theoretical advantage of generating several types of damaged cells.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Gene Targeting
The allele Tbx1^Flox^ was generated by homologous recombination in AB2.2 mouse embryonic stem (ES) cells, as shown in Figure 1A. Briefly, an Ires-Egfp cassette was knocked into exon 5 of the Tbx1 locus, in the same site that was previously used to generate the alleles Tbx1^Luc^18 and Tbx1^Cre^11.

Mouse Mutants and Breeding
All the experiments involving mice were done according to a protocol reviewed and approved by the Institutional Animal Care and Use Committee of Institute of Biosciences and Technology, in compliance with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals. The following mouse lines have been described previously: Tbx1^Luc^ (also indicated as Tbx1^1-1^)16 COET12 and Mef2c-Cre19 Mice were genotyped by polymerase chain reaction (PCR) as described in the original reports.

Tissue Culture, Flow Cytometry, Cell Sorting, and Differentiation
Tbx1^Flox^ ES cells were cultured in undifferentiated state on γ-irradiated SNL76 feeder cells. For differentiation, cells were cultured using the "hanging drop" method.21 After 2 days, the aggregates (that we refer to as embryoid bodies or EBs) were resuspended in bacteriologic Petri dishes and cultured for additional 4 to 7.5 days in suspension.

We performed flow cytometric analysis using a 2-laser instrument, FACScan (Becton Dickinson). We carried out flow sorting of in vitro differentiated Tbx1^Flox^ cells using a triple-laser instrument (MoFlow, Cytomation, Ft Collins, Colo). We seeded single Tbx1^Flox^ cells from day 8.5 EBs into individual gelatin-coated wells and cultured them for 2 to 3 weeks. Clones were expanded, stocked, and some of the cells were grown and subjected to a differentiation protocol. Then we carried out immunocytofluorescence staining as indicated. Undifferentiated clones were tested by RT-PCR using the primer pairs listed in the Table.

Quantitative expression analysis of genes during in vitro ES cell differentiation was carried out at E0, E2, E4, E6, E8.5, and E9.5.

Transfection and Cell Cycle Analysis
For cell cycle analysis, early passages clones were cultured to 80% confluence. Then cells were starved for 8 hours for synchronization and transfected with a Tbx1-expressing plasmid for 24 hours. Then, the growth media was added back for 24 hours, followed by propidium iodide staining for cell cycle analysis using flow cytometry. C2C12 mouse myoblast cells were cultured to 70% to 80% confluence and transfected with a Tbx1-c-myc-expressing vector DNA.4 Twenty-four hours after transfection, cells were lysed. RNA was isolated for real-time PCR analysis, and proteins were extracted for Western blotting.

Coimmunoprecipitation and Western Blotting
C2C12 cells were transfected with Tbx1-c-myc DNA plasmid and lysed in immunoprecipitation buffer. For immunoprecipitation assays, we used the Profound Mammalian coimmunoprecipitation (Co-IP) kit (Pierce, 23605) following the instructions of the manufacturer. C2C12 cells were transfected with the Tbx1-c-myc–expressing plasmid or empty vector for 24 hours, followed by MG132 treatment for 2 hours. Then cells were cultured in fresh media for another 4 hours. Cells were trypsinized, and protein was extracted and processed for Western blotting. Co-IP with mouse embryo material was carried out with the same procedure described above, except that nuclear extracts were derived from E9.5 wild-type (WT) or Tbx1^−/−^ embryos. Extracts were immunoprecipitated with an anti–serum response factor (Srf) antibody or mouse IgG (controls) and revealed by Western blotting using an anti-Tbx1 antibody.

Immunofluorescence and Immunohistochemistry
For immunofluorescence, cryosections were briefly fixed, permeabilized, and then blocked. Sections were incubated with the primary antibodies, followed by fluorophore-conjugated secondary antibodies. Sections were mounted and photographed under a Zeiss LSM510 laser scanning confocal microscope.

For Immunohistochemistry, we fixed embryos and dehydrated and embedded them in paraffin for histological sections. For antigen retrieval, we boiled sections in sodium citrate buffer. After peroxidase blocking, sections were blocked and incubated with primary antibodies overnight at 4°C. Then sections were treated with biotinylated secondary antibodies at room temperature for 1 hour, followed by treatment with Vectastain Elite ABC reagent (avidin–horseradish peroxidase; Vector Laboratories). Horseradish peroxidase activity was revealed using the DAB kit (Vector laboratories). Sections were dehydrated, counterstained, mounted, and examined under a Zeiss light microscope.

Results
Tbx1 Is Expressed in Multipotent Progenitor Cells
Cre-loxpP–based fate mapping of Tbx1-expressing cells showed contribution to multiple tissue types of the heart, ie, myocardium, endothelium, and smooth muscle.11 With this method, however, it is not possible to establish whether individual Tbx1-expressing cells have multilineage potential or whether the expression of Tbx1 occurs in different lineages. To clarify this...
Figure 1. Generation of the \textit{Tbx1\textsuperscript{Egfp}} allele and isolation of \textit{Tbx1\textsuperscript{Egfp/+}} ES cells. A, Targeting strategy to generate the \textit{Tbx1\textsuperscript{Egfp}} knock-in allele. An \textit{Ires-Egfp} cassette was knocked into exon 5 of the \textit{Tbx1} locus. Southern blotting analysis confirmed homologous recombination. WT allele: 4.4kb; mutant allele: 5.0kb. B, For EB differentiation, ES cells were cultured in hanging drops for 2 days, followed by culture in bacteriologic Petri dishes for additional 4 to 7.5 days in suspension. Quantitative real-time PCR assays carried out to evaluate the expression of selected genes at different time points of EB incubation (day 0, 2, 4, 6, 8.5, and 9.5). RT-PCR with \textit{EGFP} and \textit{Tbx1} primers in ES cells mRNA shows that the expressions of the 2 alleles are very similar to each other. + indicates positive control; − , negative control. C, Flow cytometric analysis of \textit{Tbx1\textsuperscript{Egfp/+}} cells at day 6.5, 8.5, and 9.5 of differentiation. Left graphs are WT controls (parental ES cell line), and right graphs are \textit{Tbx1\textsuperscript{Egfp/+}} disaggregated EB cells; numbers on the left indicate the percentage of \textit{Egfp} \textsuperscript{+/−} cells at day 6.5, 8.5, or 9.5 EBs. \textit{Egfp} \textsuperscript{−} cells, sorted from day 8.5 EBs, were seeded individually into gelatin-coated 96-well plates for clonal assays.
issue, we have carried out clonal assays of individual Tbx1-expressing cells. To this end, we have generated a Tbx1\textsuperscript{Egfp} knock-in allele in mouse ES cells (Figure 1A). A Tbx1\textsuperscript{Egfp/H11001} clone (named D5) was subjected to in vitro differentiation, and we established that the WT Tbx1 allele, as well as the enhanced green fluorescent protein (EGFP) reporter allele, is turned on at day 6 of the “hanging drop” differentiation protocol (Figure 1B). This result was confirmed by flow cytometry, which indicated the appearance of GFP\textsuperscript{+} cells at day 6.5, and detected the highest percentage of GFP\textsuperscript{+} cells (16%) at day 8.5 (Figure 1C). Next, we carried out fluorescence-activated cell sorting at the same stage of differentiation, and seeded individual cells into 96-well plates without feeder cells. On a sample of sorted cells, we confirmed GFP expression by immunofluorescence with an anti-GFP antibody (Online Figure I). Of 288 cells seeded (1 cell per well), 86 proliferated and formed clones. These clones were

### Table. PCR Primer Pairs Used for Gene Expression Analysis

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequence of Forward Primer</th>
<th>Sequence of Reverse Primer</th>
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<tbody>
<tr>
<td>Isl1</td>
<td>5'-GCC TCA GTC CCA GAG TGA TC-3'</td>
<td>5'-AGA GCC TGG TTC TTC TTC TTC-3'</td>
</tr>
<tr>
<td>Nkx2-5</td>
<td>5'-CAG TGG AGC TGG ACA AAG CC-3'</td>
<td>5'-TAG CGA CGG TTC TTC AGC CA-3'</td>
</tr>
<tr>
<td>GATA-4</td>
<td>5'-CTG TCA TCT CAT TGG GA-3'</td>
<td>5'-CCA AGT CGG AGG AAT TT-3'</td>
</tr>
<tr>
<td>Foxa2</td>
<td>5'-CCC GGG ACT TAA AGC CTG GC-3'</td>
<td>5'-GCG CCC ACA TAG GAT GAC-3'</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>5'-TCT AGG AGT CCT TCA GGA-3'</td>
<td>5'-AGC GCT TGA TTC CAC TT-3'</td>
</tr>
<tr>
<td>SM-MHC</td>
<td>5'-AAG CTG CCG CTA GAG GTC A-3'</td>
<td>5'-CCC TCC CTG TGA TCG AT-3'</td>
</tr>
<tr>
<td>cTnT</td>
<td>5'-CTG AGA CAG AGG AGG CCA AC-3'</td>
<td>5'-TCC TGG AAG TGC GCG AT-3'</td>
</tr>
<tr>
<td>GFP</td>
<td>5'-GGA CTT GGT TTT CTT TTA AA-3'</td>
<td>5'-GAA CTT CAG GGT CAG GAT GC-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-GGG ACG ACA TGG AGA AGA T-3'</td>
<td>5'-GTT TGG GTG ACC CCG TCT-3'</td>
</tr>
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Top left, Examples of immunofluorescence using endothelial (Pecam1) and cardiomyocyte (cTnT) markers in type I and type II clones. Top right, Example of RT-PCR assay to test expression of Tbx1, Pecam1, cTnT, and SM-MHC genes; the latter is a marker of smooth muscle cells. + indicates positive control; --, negative control. Bottom right table, Summary of marker analysis results. C, RT-PCR-based expression analysis of a subset of clones (before differentiation) for the genes indicated. Nkx2.5, Isl1, and Gata4 are cardiac progenitor markers. Foxa2 is an endoderm marker.

Figure 2. Clonal assay of Tbx1\textsuperscript{Egfp/+} cells. A, Schematic procedure of clonal assay and differentiation analysis. Approximately 30% (86/288) of single Tbx1\textsuperscript{Egfp/+} cells were able to form clones. After differentiation, 55% of the clones (type I) expressed an endothelial marker (Pecam1); 21% (type II) expressed endothelial (Pecam1), cTnT, and smooth muscle (SM-MHC) markers; 24% (type III) were not positive for any of these markers. B, Differentiation analysis of clones derived from single Tbx1\textsuperscript{Egfp/+} cells.
expanded and stocked at early passages (P3) (Figure 2A). Next, we subjected these clones to spontaneous differentiation (Figure 2). After 7 to 14 days of culture, we tested markers of cardiac muscle, endothelial, and smooth muscle differentiation. Results showed that of 86 clones tested, 47 (55%) were positive for the endothelial marker platelet/endothelial cell adhesion molecule (Pecam)1, 18 (21%) were positive for the cardiomyocyte specific marker cardiac troponin (cTnT), and 18 (21%) were positive for the smooth muscle specific marker smooth muscle–myosin heavy chain (SM-MHC) (the latter tested by RT-PCR) (Figure 2B). None of these clones expressed Tbx1 by RT-PCR (Figure 2B). Interestingly, all the clones positive for cTnT were also positive for SM-MHC and vice versa. In addition, all cTnT+/ and SM-MHC+ clones were also Pecam1−. In summary, we obtained 3 types of clones, type I, positive only for Pecam1 (55%); type II positive for Pecam1, cTnT, and SM-MHC (21%); and type III negative for all 3 markers (24%). Subsequently, we carried out a retrospective analysis of a subset of these clones before differentiation. We evaluated mRNA expression of the cardiac progenitor markers NK2 transcription factor related, locus 5 (Nkx2.5), islet LIM homeobox 1 (Isl1), and GATA-binding protein 4 (Gata4) and of the endoderm marker Forkhead box a2 (Foxa2) (because Tbx1 is expressed also in the pharyngeal endoderm) by RT-PCR. We found that all 8 Type II clones tested were positive for Nkx2.5, Isl1 and Gata4; a Type III clone was positive for Foxa2, whereas a Type I clone (capable of differentiating into endothelial cells) was negative for all these markers (Figure 2C). All types of clones, at this level of differentiation, expressed Tbx1, as expected (Figure 2C). Thus, in these tissue culture experiments we were able to obtain clones for all the major cell types where Tbx1 is normally expressed in embryos, ie, mesodermally-derived endothelial, smooth muscle and cardiomyocyte progenitors, as well as endodermal cells. Most relevant for the scope of this work is the finding that 21% of the clones express cardiac progenitor markers, and are at least three-potent as they are capable to express differentiation markers of endothelial, smooth muscle and cardiomyocytes.

**Tbx1 Enhances the Mitotic Activity of Multipotent Cardiac Progenitors**

Tbx1 loss of function in mouse embryos is associated with reduced mitotic activity in the mesoderm region that includes the SHF.4,5 Therefore, we tested whether overexpression of Tbx1 can regulate the proliferation of multipotent clones. To this end, we have transfected starved cells from Type II clones (early passages, without further differentiation) with a Tbx1-expression vector, and assayed the cell cycle using a DNA-specific dye and flow cytometry. These 2 clones expressed Tbx1 and cardiac progenitor markers but did not express differentiation markers such as Pecam1, cTnT, and SM-MHC (Figure 3A). Results showed an increased number of mitotic cells compared to cells transfected with an empty vector (Figure 3B and 3C). Consistent results were obtained in 3 repeated experiments and with 2 independent clones. Thus, Tbx1 is sufficient to promote mitotic activity in these cells. To confirm this observation in vivo, we have used a Cre-activatable Tbx1-expressing transgenic line.
We crossed the COET line with an SHF Cre driver, the Mef2c-Cre transgenic line and evaluated cell proliferation in the SHF, compared with controls, Tbx1/H11001/H11002 and Tbx1/H11002/H11002 E9.5 embryos, using an anti–phospho-H3 antibody, which identifies mitotic cells. Results showed a significant increase of the number of mitotic cells in Mef2c-Cre;COET embryos (Online Figure II).

**Tbx1 Negatively Regulates Differentiation in the SHF**

The SHF can be defined as a reservoir of cardiac progenitors, which gradually migrate into the heart and contribute to the growth of the outflow tract (OFT) and other regions of the heart. Immunostaining of Tbx1 on mouse embryos at different stages (5 to 22 somites) showed overlap with the SHF marker Isl1 (Figure 4D, 4H, and 4L). However, Isl1 immunostaining appeared much more extensive than Tbx1 immunostaining, because it was clearly visible also in the myocardial layer of the OFT (Figure 4C, 4G, and 4K). In contrast, Tbx1 appeared restricted to the SHF, especially at 22 somites (Figure 4F and 4J). To confirm this finding, we costained embryos at 16 and 22 somites with anti-Tbx1 and anti–α-SMA (smooth muscle actin) as differentiation marker) antibodies. Results showed that there is essentially no overlap between the 2 markers at both stages (Figure 5A through 5F), confirming that Tbx1 is specific for the (undifferentiated) SHF. Because Tbx1 is only expressed in the undifferentiated domain, we postulated that this factor might also have an inhibitory effect on differentiation. To address this point, we have carried out immunohistochemistry with differentiation markers α-SMA, cardiac actin, MF20, and cTnT in Tbx1−/− embryos. Results showed that indeed the expression domain of these 2 markers was extended dorsally-posteriorly to encroach into the SHF anatomic region (Figure 5G through 5P), consistent with recently reported data. Next, we tested whether expansion of Tbx1 expression in the SHF could cause the opposite effect, ie, expansion of the undifferentiated domain ventrally, into the OFT proper. Thus, we have tested Mef2c-Cre;COET transgenic embryos and confirmed that the expression of the Tbx1 protein is indeed extended into the OFT and that the differentiation markers expression domains were displaced ventrally and had little or no overlap with the extended Tbx1 expression (Figure 5Q through 5Z), indicating that Tbx1 regulates negatively muscle cell differentiation in the SHF. Mef2c-Cre;COET mutants at E18.5 also showed developmental defects of the segment of the heart derived from the SHF. Indeed these embryos exhibited a small right ventricle and OFT defects such as ventricular septal defects, double outlet right ventricle, or truncus arteriosus (in 4 mutants analyzed, Online Figure III).

**Tbx1 Regulates the Level of the Srf Protein**

Because α-SMA and cardiac actin are targets of the serum response factor (Srf), a myogenic transcription factor, we...
tested whether the expression of Srf might also be extended posteriorly in Tbx1\(^{-/-}\) embryos. Immunohistochemistry results showed that this is indeed the case (Figure 6A and 6B). Conversely, in the Tbx1 gain of function mutant Mef2c-Cre;COET, Srf expression receded ventrally (Figure 6C and 6D), similarly to the expression of differentiation markers. These data suggest that Tbx1 functions upstream of the muscle differentiation transcription program. To gain further insight into the effect of Tbx1 on muscle differentiation, we carried out cell culture experiments using the myoblast cell line C2C12. Indeed, transfection of a Tbx1 expression vector into these cells reduced the Srf protein level in a dosage-dependent fashion (Figure 6E). In contrast, Srf mRNA level was not affected by Tbx1 expression (Figure 6F), indicating that the reduced level of the protein is not attributable to transcriptional regulation of the Srf gene. Similarly, in situ hybridization on Tbx1 gain and loss of function embryos at E9.5 could not reveal any significant change of Srf RNA expression in the SHF or other tissues (Online Figure IV), thus confirming that Tbx1 does not regulate, directly or indirectly, Srf gene expression. Therefore, we tested whether Tbx1 and Srf proteins may interact. Co-IP experiments in

Figure 5. Tbx1 negatively regulates differentiation in the SHF. A through F, Confocal images of sections from E9.0 (ST16) and E9.5 (ST22) WT embryos double-stained with anti-\(\alpha\)-SMA and anti-Tbx1 antibodies. Tbx1 is expressed in the SHF but not in \(\alpha\)-SMA\(^{-}\) cardiomyocytes of the OFT in both stages. G through P, Immunohistochemistry of differentiation markers including cardiac sarcomeric actin, cTnT, MF20, and \(\alpha\)-SMA on E9.0 to E10.0 WT embryos (G through K), showing expression in the OFT proper but not in the SHF. However, in Tbx1\(^{-/-}\) embryos (L through P), the expression of these markers extended ectopically into the SHF. The ectopic expression is more prominent at E10 (K and P). Q through Z, Ectopic expression of Tbx1 in the OFT of Mef2c-Cre;COET embryos caused reduced expression of \(\alpha\)-SMA (R, S, W, and X), cTnT (T and Y), and MF20 (U and Z) in the OFT at E9.5. CA indicates common atrium; CV, common ventricle. Scale bar: 100 \(\mu\)m.
Tbx1-transfected C2C12 cells demonstrated that indeed the 2 proteins coimmunoprecipitate, suggesting that they form a complex (data not shown). To confirm this observation in vivo, we carried out Co-IP of the endogenous proteins from tissues of WT and Tbx1/H11002 embryos at E9.5. Western blot analysis showed decreased expression of Srf with increasing dosage of Tbx1 protein in C2C12 cells. F, Real-time quantitative PCR of Tbx1-transfected C2C12 cells showed that the level of Srf transcripts is not affected by increasing amount of transfected Tbx1. G, Co-IP experiment showing interaction of the Tbx1 and Srf endogenous proteins in embryo tissues. Nuclear extracts from E9.5 mouse embryos were immunoprecipitated with an anti-Srf antibody or with mouse IgG and revealed with an anti-Tbx1 antibody and an anti-Srf antibody. Tbx1 coimmunoprecipitates with Srf in WT embryos. H, The proteasome inhibitor MG132 abolishes the Tbx1-induced reduction of Srf level in Tbx1-transfected C2C12 cells. CA indicates common atrium. Scale bar: 100 μm.

Discussion

The developmental history of cells destined to populate and thus build the mammalian heart should be the basis for understanding the biology of cardiac stem cells and to engineer cardiac regeneration strategies. The developmental history of the SHF reservoir should be particularly instructive because it functions over a relatively long developmental time, it provides cells to most of the heart, and it is easier to study because there is a rich portfolio of relevant mutants at our disposal. To provide a sufficient number of cells to the developing heart (which grows by addition of cells and by proliferation of resident cells), SHF cells must proliferate at a sufficient rate before they enter the OFT of the heart and differentiate, because at that point, their proliferation rate will decrease substantially. A possible way to understand the mechanisms by which this process is maintained is to identify genes and proteins expressed in the SHF but not in the OFT. Fgf8, for example, is expressed early in the mesoderm of the SHF but not (or very little) in the OFT. Reduced dosage of Fgf8 in the mesoderm leads to OFT defects typical of impaired SHF function. The transcription factor Isl1 is also required for SHF development. However, it is not only expressed in the SHF but also in the differentiated OFT, as shown here and by other groups. In contrast, we
could not find the Tbx1 protein in SHF-derived cells of the OFT but only in the SHF. Earlier reports of Tbx1 gene expression in the OFT myocardium were mostly based on the visualization of β-galactosidase activity from a Tbx1LacZ reporter, thus probably biased by the stability of the β-galactosidase protein. This finding, combined with the data showing expression of Tbx1 in multipotent heart progenitors and showing the ability of the transcription factor to increase mitotic activity in these cells, strongly supports a role of Tbx1 in maintaining SHF cells proliferating (Figure 7). Because Tbx1 can regulate Fgf8 expression in the mesodermal region that includes the SHF, some of its mitogenic activity could be mediated by the FGF signaling. However, maintaining mitotic activity may not be sufficient to ensure maintenance of SHF function. Loss of Tbx1 is associated with premature differentiation, whereas ectopic expression of Tbx1 in the OFT results in suppression of differentiation. A negative regulation of differentiation could be explained by the negative regulation of the Srf transcription factor, which, in turn, regulates the muscle transcription program. Unexpectedly, Tbx1 does not regulate Srf transcription, but it appears to regulate, directly or indirectly, proteasome-mediated degradation of the Srf protein. The fact that Tbx1 and Srf proteins can be coimmunoprecipitated in vivo suggests that the formation of the complex might reduce Srf protein stability.

Furthermore, we show that Tbx1 identifies 3-potent heart progenitors, suggesting that such cells are present in the SHF (Figure 7A). This is consistent with the identification of common progenitors of at least some of the different cell types populating the heart. Our data indicate that Tbx1, at least in the SHF population of heart progenitors, regulates the balance between proliferation and differentiation (Figure 7B).

Finally, our data raise the question of whether the function of Tbx1 that we have identified in cardiac progenitors may also apply to other tissues where Tbx1 is expressed. Indeed, Tbx1 loss of function in mice, and, to a lesser extent, Tbx1 haploinsufficiency in DiGeorge syndrome patients, is associated with hypoplasia or aplasia of several organs and tissues. Thus, it is tempting to speculate that dysregulation of the balance between proliferation and differentiation of different types of progenitor cells or stem cells may be a basic pathogenetic mechanism for the loss of function phenotype.

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Disclosures
None.

References


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Materials and Methods

Gene targeting.

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Mouse mutants and breeding.

All the experiments involving mice were done according to a protocol reviewed and approved by the Institutional Animal Care and Use Committee of Institute of Biosciences and Technology, in compliance with the USA Public Health Service Policy on Humane Care and Use of Laboratory Animals. The following mouse mutant lines have been described previously: $Tbx^{LacZ/}$ (also indicated as $Tbx^{1/-}$), COET, and Mef2c-Cre. Mice were genotyped by PCR as described in the original reports.

Tissue culture, flow cytometry, cell sorting and differentiation.

$Tbx1^{Egfp/+}$ ES cells were cultured in undifferentiated state on γ-irradiated SNL76 feeder cells in DMEM (Gibco, 12100-046) supplemented with 15% fetal bovine serum (HyClone), 2-mercaptoethanol (Gibco, 21985-023) and Penicillin-Streptomycin-Glutamine (GPS, Gibco, 10378-016). For differentiation, cells were cultured at the concentration of 5x10^4 cells/ml using the “hanging drop” method in DMEM containing 20% FBS. After 2 days in hanging drops, the aggregates (that we refer to as embryoid bodies or EBs) were resuspended in 5 ml medium in bacteriological Petri dishes and cultured for additional 4-7.5 days in suspension. We performed flow cytometric analysis using a two-laser instrument, FACScan (Beckton Dickinson). We carried out flow sorting of in vitro differentiated $Tbx1^{Egfp/+}$ cells (day 2+4, 2+6.5 and 2+7.5 of differentiation, that is 2 days in hanging drop plus 4, 6.5, or 7.5 days in suspension in bacteriological Petri dish) using a triple-laser instrument (MoFlow, Cytomation, Fort Collins, CO). We seeded single $Tbx1^{Egfp}$ cells from day 8.5 EBs into individual gelatin-coated wells of 96-well plates, and cultured the in DMEM containing 20% FBS for 2-3 weeks. Clones were expanded, stocked, and some of the cells were grown and subjected to a differentiation protocol, which is cultured in gelatin-coated 24 well plates in DMEM supplemented with 20% knockout™ serum replacement (Gibco, 10828-028) and GPS for 7-14 days. Then we carried out immunocytofluorescence staining with antibodies anti-cardiac troponin T (1:200, Lab Vision, MS-295-P) and anti-Platelet/Endothelial Cell Adhesion Molecule 1 (PECAM-1) (PharMingen, 550274, 1:200). RT-PCR was done with a primer pair for smooth muscle-myosin heavy chain (SM-MHC, see Tab. 1 for sequence). Undifferentiated clones were tested by RT-PCR for the expression of genes Isl1, Nkx2.5, GATA-4 and Foxa2 using the primer pairs listed on Tab. 1.

Quantitative expression analysis of genes during in vitro ES cell differentiation was carried out at EB day 0, 2, 4, 6, 8.5, and 9.5. For these analyses we used quantitative real time PCR with the following commercial primer pairs $Tbx1$ (Applied Biosystems, Mm00448948_m1), $Isl1$ (Mm00627860_m1), $Nkx2.5$ (Mm00657783_m1), $Flk1$ (Mm01222431_m1) and $T-Brachyury$ (Mm01318252_m1).
Transfection and cell cycle analysis.

For cell cycle analysis, early passages clones were cultured to 80% confluency in 6-well plates in DMEM containing 15% fetal bovine serum. Then cells were starved with DMEM for 8 hours for synchronization, and transfected with a \textit{Tbx1}-expressing plasmid. We used FuGENE® 6 transfection reagent (Roche) and 0, 1, or 3 μg of DNA of \textit{Tbx1}-expressing plasmid, normalized by 3, 2, 0 μg, respectively, of DNA of empty plasmid for 24 hrs. The transfection efficiency was 54-56% as tested by co-transfection with a GFP expressing plasmid. Then, DMEM containing 15% FBS was added back for 24 hrs, followed by Propidium Iodide staining for cell cycle analysis using flow cytometry.

C2C12 mouse myoblast cells were cultured to 70-80% confluency in 6-well plates, and transfected with 0, 1, 3, or 5 μg of a \textit{Tbx1-c-myc} -expressing vector DNA. Twenty-four hours after transfection, cells were lysed, RNA was isolated using Trizol (Invitrogen, 15596-026) for real-time PCR analysis, and proteins were extracted after cell lysis with the RIPA buffer (1% NP40, 0.5% NaDoc, 0.1% SDS in PBS, protease inhibitor (Roche, 04 693 159 001)) for western blotting.

Co-Immunoprecipitation and western blotting.

C2C12 cells were transfected with \textit{Tbx1-c-myc} cDNA plasmid and after 24 hrs cells were lysed in immunoprecipitation buffer (20 mM Tris·HCl, pH 8.0/0.5 M NaCl/5 mM MgCl₂/0.5% Triton X-100 and protease inhibitor (Roche)). For immunoprecipitation assays we used the ProFound Mammalian Co-Immunoprecipitation kit (Pierce, 23605) following manufacturer instructions. Briefly, after pre-clearing, lysates were incubated with gel-immobilized rabbit anti-Srf antibody (Santa Cruz Biotechnology, sc-335) or gel-immobilized rabbit IgG as control for 16 hrs at 4°C. Immunoprecipitates were washed with the immunoprecipitation buffer before elution. C2C12 cells were transfected with the \textit{Tbx1-c-myc} expressing plasmid or empty vector using the PolyFect® transfection reagent (Qiagen) for 24 hours, followed by treatment with 3 μM of MG132 for 2 hrs. Then cells were washed before addition of fresh media for another 4 hrs. Cells were trypsinized, protein extracted and processed for western blotting. Western blotting was done using XCell II TM Blot Module (Invitrogen, EI9051) following manufacturer instructions. The primary antibodies were rabbit-anti-Srf (Santa Cruz Biotechnology, sc-335, 1:1,000), rabbit-anti-Tbx1 (Zymed, 34-9800, 1:500), mouse-anti-c-myc (Developmental Studies Hybridoma Bank, 9E10, 1:200) and monoclonal anti-α-Tubulin (Sigma, T9026, 1:1,000). The secondary antibodies were as follows: horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Thermo scientific, 31464, 1:10,000), HRP-conjugated anti-mouse antibody (GE Healthcare, NA931V, 1:10,000). The HRP-derived signal was detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, 34078).

Co-IP with mouse embryo material was carried out with the same procedure described above, except that nuclear extracts were derived from E9.5 WT or \textit{Tbx1}⁻/⁻ embryos. Extracts were immunoprecipitated with an anti-Srf antibody or mouse IgG (controls), and revealed by western blotting using an anti-Tbx1 antibody.

Immunofluorescence and immunohistochemistry.

For immunofluorescence, cryosections were briefly fixed in cold acetone, treated with 1XPBS/0.2% triton and then blocked in 2% bovine serum albumin (Vector Laboratories, SP-5050)/PBST (1XPBS/0.05% Tween20). Sections were incubated with the primary antibodies anti-Tbx1 (Zymed, 34-9800, 1:200) and anti-Isl1 (Developmental
Studies Hybridoma Bank, 39.4D5, 1:50) or anti-α-SMA (Sigma, Clone 14A, 1:200) in blocking solution at 4°C overnight and washed, followed by 1:500 Alexa fluor® 594 goat anti-rabbit secondary antibody (Invitrogen, A31632) and Alexa fluor® 488 donkey anti-mouse secondary antibody (Invitrogen, A21202) in blocking solution at RT for 30 mins. Sections were mounted with Vectashield® with DAPI (Vector Laboratory) and photographed under a Zeiss LSM510 laser scanning confocal microscope.

For Immunohistochemistry, we fixed embryos in 4% Paraformaldehyde/PBS, dehydrated and embedded them in paraffin for histological sections. For antigen retrieval, we boiled sections in sodium citrate buffer (10mM, PH6.0) for 10 mins. After peroxidase blocking (3% H2O2 in PBS), sections were blocked in 2% goat serum/PBS and incubated with primary antibodies to α-sarcomeric actin (monoclonal, Sigma, A2172, 1:200) anti-cardiac troponin T (1:200, Lab Vision, MS-295-P), MF20 (hybridoma bank, 1:50) or Srf (Santa Cruz Biotechnology, sc-335, 1:200) in blocking solution overnight at 4°C. Then sections were treated with biotinylated anti-mouse (1:200) or anti-rabbit (1:200) secondary antibodies (Vector Laboratories) at RT for 1 hr, followed by treatment with Vectastain Elite ABC reagent (avidin–horseradish peroxidase; Vector Laboratories). Horseradish Peroxidase (HRP) activity was revealed using the DAB kit (Vector laboratories). For the alkaline phosphatase-conjugated anti-α-smooth muscle actin (α-SMA) antibody (Sigma, Clone 14A), sections were blocked in 1% sheep serum/PBS and incubated with anti-α-SMA antibody (1:100 in 1% sheep serum/PBS) at 4 ºC overnight. NBT/BCIP (Roche) was used to visualize the alkaline phosphatase activity. Sections were dehydrated, counter-stained with NFR as needed, mounted with Permount and examined under a Zeiss light microscope.

Supplementary Figure 1.

Confirmation of GFP expression by immunofluorescence with an anti-GFP antibody before (A) and after (B) flow sorting.
Supplementary Figure 2. Proliferation of SHF cells in Tbx1 loss and gain of function embryos at E9.5.

(A-D) Phospho-Histone H3 immunohistochemistry shows reduced proliferation in the SHF of Tbx1+/− embryos (ST28) and increased proliferation in Mef2c-Cre; COET embryos (ST31). Arrows indicate phospho-Histone H3 positive cells in the SHF. (E) Mitotic Index (MI) shows statistical significance between control (Tbx1+/− or Mef2c-Cre) and mutants (Tbx1−/− or Mef2c-Cre; COET) (p<0.05; n=3). Approximately 500 cells were scored per embryos, and 2 embryos were scored for each experimental point. OFT: outflow tract; SHF: second heart field; CA: common atrium. Scale bar: 100 μm.
Supplementary Figure 3. Developmental defects of SHF-derived structures of the heart of *Mef2c-Cre; COET* mutants at E18.5.

(A-C) Heart sections of control embryos; (D) heart section of a mutant embryo with small right ventricle (RV) and a ventricular septal defect (VSD); (E) heart section of a mutant embryo with small RV, VSD and truncus arteriosus; (F) heart section of a mutant embryo with small RV and double outlet right ventricle (DORV). RV: right ventricle; LV: left ventricle; RA: right atrium; LA: left atrium; PT: pulmonary trunk; Ao: aorta. All sections were coronal. Scale bar: 100 μm.
Supplementary Figure 4. Similar Srf gene expression in Tbx1 loss and gain of function mouse models at E9.5.

*In situ* hybridization with an Srf probe on control (A-B), Tbx1^{+/−} (C), and Mef2c-Cre; COET (D) embryos. There is no detectable difference among genotypes. OFT: outflow tract; SHF: second heart field; RA: right atrium. Scale bar: 100 μm.