Published in final edited form as:
J Mol Cell Cardiol. 2010 November ; 49(5): 836–840. doi:10.1016/j.yjmcc.2010.08.023.

Partial Rescue of the *Tbx1* mutant Heart Phenotype by *Fgf8*: genetic evidence of impaired tissue response to Fgf8

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Abstract

Tbx1 is the candidate gene of DiGeorge syndrome and is required in humans and mice for the development of the cardiac outflow tract (OFT) and aortic arch arteries. Loss of function mutants present with reduced cell proliferation and premature differentiation of cardiac progenitor cells of the second heart field (SHF). Tbx1 regulates *Fgf8* expression hence the hypothesis that the proliferation impairment may contribute to the heart phenotype of mutants. Here we show that forced *Fgf8* expression modifies and partially rescues the OFT septation defects of *Tbx1* mutants but only if there is some residual expression of *Tbx1*. This genetic experiment suggests that Tbx1, directly or indirectly, affects tissue response to Fgf8. Indeed, *Tbx1*$^{-/-}$ mouse embryonic fibroblasts were unable to respond to Fgf8 added to the culture media and showed defective response of Erk1/2 and Rsk1. Our data suggest a coordinated pathway modulating Fgf8 ligand expression and tissue response to it in the SHF.

Keywords

T-box; cardiac outflow tract septation; DiGeorge syndrome; FGF signaling pathway; cardiac progenitors

Introduction

Tbx1 is T-box transcription factor required for the development of several organs, including sections of the heart. Haploinsufficiency of *TBX1* can cause DiGeorge/Velocardiofacial syndrome, often associated with congenital heart disease. Mouse mutants recapitulate most of the human syndrome phenotype. *Tbx1* expression in the mesoderm, and, in particular, in the mesoderm expressing the cardiogenic transcription factor *Nkx2.5*, is required for the elongation and septation of the cardiac outflow tract as well as for the septation of the ventricles (1). Expression analyses and cell fate mapping established that *Tbx1* is expressed in the second heart field (SHF), which is a cardiac progenitor cell (CPC) population that migrates into the cardiac outflow tract (OFT), right ventricle and part of the atria. We have shown that *Tbx1* is

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Disclosures The authors declare that they have no conflict of interest.

expressed in tri-potent heart progenitors and, in these cells, it modulates positively cell proliferation and inhibits differentiation (2). Thus, the molecular pathway(s) regulated by Tbx1 in CPCs could be exploited for the expansion of cardiac stem cells in future applications of regenerative medicine. Therefore, it is important to define the mechanisms by which Tbx1 effects its functions in CPCs and thus define the molecular players. There are at least two mechanisms that appear relevant to a role of Tbx1 in cell proliferation. One is the recently described mechanism of Smad1-Tbx1 interaction (3) that leads to inhibition of Smad1 signaling, which inhibits proliferation in the SHF (4). Another is transcriptional regulation of *Fgf8*, a ligand of the fibroblast growth factor signaling that has, among other functions, mitogenic activity. Although *Tbx1* and *Fgf8* interact genetically (5), forced expression of *Fgf8* in the *Tbx1*-expression domain is insufficient to rescue the main phenotypic abnormalities of *Tbx1*$^{-/-}$ mutants, such as the heart phenotype (6). However, forced expression of *Fgf8* in *Tbx1*-expressing cells is capable of partially rescue the severe thyroid phenotype, which is a cell non-autonomous consequence of *Tbx1* loss (7). This finding and the availability of new mutant alleles have encouraged us to revisit the role of *Tbx1* in FGF signaling. Here we show that forced expression of *Fgf8* in mice that express a small dose of *Tbx1* mRNA rescues partially the heart phenotype, thus we hypothesized that *Tbx1* <u>is necessary to respond to Fgf8</u>. This hypothesis was successfully tested in a tissue culture model.

Materials and Methods

In this work we used the following mouse lines previously described: *Tbx1*^{Cre} (8), *Tbx1*^{neo2} (9), *Tbx1*^{fgf8} (6), *Fgf8*$^{fl/fl}$ and *Fgf8*$^{+/-}$ (10). Phenotypic analysis was carried out by direct examination of dissected embryos and by histological analyses.

Primary mouse embryonic fibroblasts (MEFs) were isolated from individual *Tbx1*$^{-/-}$, *Tbx1*$^{neo2/-}$ and wild-type embryos at E13.5. To this hand, the internal organs, head, tail and limbs were removed. Cells were cultured in Dulbecco's modified Eagle's medium with 20% FBS and 1% NEAA, and used for a maximum of 4 passages. We derived and tested 3 wild type, 5 *Tbx1*$^{-/-}$ and 2 *Tbx1*$^{neo2/-}$ MEF lines, each derived from individual embryos.

Fgf8 treatment was carried out using recombinant Fgf8b (R&D) at 50μg/ml along with 100ng/ml of heparin (Sigma) for 5, 10 or 15 minutes.

Quantitative real time PCR was performed using reverse-transcribed total RNA from MEFs or whole embryos, using SYBR green and an Applied Biosystem 7900H machine, with the following primers: *Etv4* F- cagcaggaagccaccact, R- gggggagtcataggcactg; *Etv5* F-gcagtttgtcccagattttca, R- gcagctcccgtttgatctt; *Rsk1* F- ttcacacggctctcaaagg, R-ccagctcagccaggtaaaac. *Tbx1*: E3/E4-F: ctgaccaataacctgctggatga, E3/E4-R-ggctgatatctgtgcatggagtt. Relative quantification was calculated by the ΔΔCt method.

Western blotting analyses were performed using total protein extracts from MEF cells. The antibodies were purchased from Millipore (pRsk1), SantaCruz (Erk1/2, Crkl, Frs2α, Rsk1), Cell Signaling Technology, Inc (p44/42, pErk1/2), Abcam (Actin), and Sigma (Tubulin).

Results and Discussion

Loss of Fgf8 in the Tbx1 domain causes Tbx1$^{-/-}$-like OFT septation defects

Using a tamoxifen-inducible *Tbx1*-cre (*Tbx1*^{mcm}) driver, we have shown that *Fgf8* dosage reduction in conditional mutants enhances the *Tbx1* haploinsufficiency phenotype at the level of aortic arch defects (6), but we could not find OFT defects. In addition, Brown et al. (11) using a *Tbx1* promoter-enhancer construct as the transgenic Cre driver, observed various types of OFT defects in *Fgf8* conditional mutants, but they did not report truncus arteriosus communis

(TAC) the typical OFT defect observed in $Tbx1^{-/-}$ mutants. Thus, we decided to repeat the experiment using our latest Cre knock-in driver, $Tbx1^{Cre}$, which induces robust recombination and is expressed in the endogenous *Tbx1* domain (8). RNA *in situ* hybridization with an *Fgf8*-deletion specific probe (10) at E9 showed undetectable or very low levels of *Fgf8*-expression in the pharyngeal region of $Tbx1^{Cre/+}$;$Fgf8^{fl/-}$ embryos compared to controls while other domains such as the fronto-nasal process and limb bud showed robust expression (Fig. 1A,B). Analysis of the OFT phenotype in E18.5 $Tbx1^{Cre/+}$;$Fgf8^{fl/-}$ embryos revealed the presence of truncus arteriosus communis (TAC) in 67% (10/15) of them. In 5 of the 10 embryos with TAC, the defect was partial (Fig. 1E–J), while in the remainder, the defect was complete i.e. total lack of septation, similarly to $Tbx1^{-/-}$ mutants. In all conditional mutants with TAC, the truncal valve maintained its continuity to the mitral valve, indicating that the alignment of the OFT with the ventricles is less defective in these mutants than in $Tbx1^{-/-}$ animals. In addition, in all embryos, the TAC was positioned above the inter-ventricular septum straddling a ventricular septal defect (VSD) (Fig. 1H–I). In contrast, in $Tbx1^{-/-}$ mutants, the TAC is positioned on the right ventricle. Thus, conditional ablation of *Fgf8* in the *Tbx1* domain interrupts both conal and truncal septation, but rotation and alignment of the OFT are only mildly affected. Overall, these data demonstrate that the loss of expression of *Fgf8* in *Tbx1*-expressing cells causes OFT developmental abnormalities similar to those caused by loss of *Tbx1*, at least in a $Tbx1^{+/-}$ background.

Fgf8 rescue of OFT septation defects of Tbx1 mutants requires a small amount of functional Tbx1

The $Tbx1^{Fgf8}$ allele is null for *Tbx1* and expresses an *Fgf8* cDNA in the *Tbx1* expression domain (6). Despite the predicted importance of the loss of *Fgf8* expression in the pathogenesis of OFT septation defects of $Tbx1^{-/-}$ mutants, this allele did not modify these defects in *Tbx1*-null embryos (6). Therefore, we considered the hypothesis that the complete absence of *Tbx1* may impair the cellular response to Fgf8. This hypothesis would also explain why the $Tbx1^{Fgf8}$ allele could partially rescue the thyroid phenotype of $Tbx1^{-/-}$ mutants (7). Indeed, Tbx1 is not expressed in the thyroid tissue, targeted by Fgf8, thus it cannot have any role in regulating response to Fgf8 in this tissue.

To address this hypothesis in vivo, we carried out rescue experiments in mice that express a low amount of *Tbx1* (per se insufficient to support normal development of the OFT), but perhaps sufficient to partially restore cellular response to Fgf8.

We used the $Tbx1^{neo2}$ allele that expresses 15–20% of the WT *Tbx1* mRNA level (12). $Tbx1^{neo2/-}$ animals show very similar cardiovascular defects as $Tbx1^{-/-}$ mutants, indicating that this low level of *Tbx1* expression is insufficient to modify significantly the OFT null phenotype (9,12). We maintain the $Tbx1^{Fgf8}$ allele in the $Tbx1^{Fgf8/Dp1}$ background, where *Dp1* is a segmental duplication of the *Tbx1* locus which rescues the high penetrance of aortic arch defects associated with the $Tbx1^{Fgf8}$ allele (6). $Tbx1^{Fgf8/Dp1}$ mice were crossed with $Tbx1^{neo2/+}$ mice, and the offspring examined atE18.5. In total, we have analyzed 97 embryos (18 $Tbx1^{Fgf8/\mathrm{neo2}}$, 29 $Tbx1^{Fgf8/+}$, 19 $Tbx1^{neo2/\mathrm{Dp1}}$, and 31 $Tbx1^{+/\mathrm{Dp1}}$), of these, only $Tbx1^{Fgf8/\mathrm{neo2}}$ and $Tbx1^{Fgf8/+}$ embryos presented with cardiovascular abnormalities. In particular, 19 $Tbx1^{Fgf8/+}$ embryos (66%) exhibited aortic arch artery abnormalities but no OFT defects (consistent with previously reported data (6)). All the 18 $Tbx1^{Fgf8/\mathrm{neo2}}$ embryos presented with OFT defects (Fig 2A–D”). These included TAC (10 embryos, 56%), double outlet right ventricle (DORV) (7 embryos, 39%) and transposition of the great arteries (TGA, 1 embryo or 6%). This phenotype represents a considerable improvement compared to the one observed in $Tbx1^{neo2/-}$ embryos. Indeed, the latter genotype is associated with 95% incidence of TAC (9,12), thus almost all embryos lack OFT septation. In contrast, only 56% of $Tbx1^{Fgf8/\mathrm{neo2}}$ embryos lack OFT septation. We could no find any other phenotypic changes compared to the *Tbx1*$^{neo2/-}$ phenotype. To evaluate whether the partial phenotypic rescue may be caused by up-regulation of the *Tbx1*^{neo2} allele by Fgf8, we performed real time quantitative (qRT) PCR analysis on E9 embryos (Fig. 1K). The average relative expression level was not significantly different (19% for *Tbx1*$^{Fgf8/\mathrm{neo2}}$ vs. 14% for *Tbx1*$^{neo2/-}$; p=0.07 with 2-tailed T-test). These data support the hypothesis that reduced *Fgf8* expression in *Tbx1*$^{-/-}$ embryos contributes to the OFT septation phenotype and it also suggest that Tbx1 is required for tissue response to Fgf8.

Tbx1$^{-/-}$ cells do not respond normally to Fgf8

To support the above in vivo findings that suggest a requirement of *Tbx1* for normal response to Fgf8, we evaluated the response of cultured mouse embryonic fibroblasts (MEFs) from wild type and *Tbx1*$^{-/-}$ embryos, to recombinant Fgf8b protein added to the culture media. We measured the expression of two genes known to respond positively to Fgf8 treatment, *Etv4* (also known as *Pea3*) and *Etv5* (also known as *Erm*) (13). qRT-PCR analysis on two independent wild type MEF lines revealed a robust response to Fgf8, while two independent *Tbx1*$^{-/-}$ lines did not show any significant response (Fig. 2E). Next, we tested the phosphorylation of Rsk1, a downstream component of FGF and other growth factors signaling pathways, responsible for relaying the signal to the nucleus. Results showed a robust response to Fgf8 in wild type MEF cell lines, but very low signal in mutant cells (Fig. 2F). Rsk1 is a phosphorylation target of the MAP kinase Erk1/2. Therefore, we tested the phosphorylation response of Erk1/2 to FGF8. Analogously to the results with Rsk1, we did not find any difference in Erk activation in mutant cells (Fig. 2G). We checked whether loss of *Tbx1* causes any difference in the expression of Rsk1 or Erk1/2. For the latter, we could not demonstrate any difference in expression (Fig. 2G). However, for Rsk1 we saw reduced expression in most cell lines analyzed (each from an individual embryo) at the protein level (Supplementary Fig. 1) and RNA level (Supplementary Fig. 2). However, even in the presence of near normal levels of Rsk1 protein, we could not detect any phosphorylation response to FGF8 in mutant MEFs (Supplementary Fig. 1, lanes 3–4). The observed variability may be due to the heterogeneous nature of the primary MEF cultures. We also tested whether Frs2α (14) and Crkl (15) proteins are expressed differentially in mutant vs. wild type cells, but we could not detect any difference (Supplementary Fig. 3).

Next, we tested whether the small amount of *Tbx1* expression in *Tbx1*$^{\mathrm{neo2}/-}$ MEFs is sufficient to rescue the response to FGF8 in culture. Results showed that indeed *Tbx1*$^{\mathrm{neo2}/-}$ MEFs are capable to respond to FGF8 as revealed by Rsk1 phosphorylation (Fig. 2H).

Taken together, our in vivo and in vitro data strongly suggest an impairment of tissue response to Fgf8 associated with loss of *Tbx1*. Indeed, in vivo, replacement of *Fgf8* expression in *Tbx1* expressing cells is capable of partially rescuing the OFT phenotype only in the presence of a small amount of *Tbx1* expression. Analogously, in vitro, MEF cells require a minimal level of *Tbx1* expression to respond to Fgf8. Further work is necessary to address the mechanism by which loss of Tbx1 impairs response to Fgf8. One can speculate that this may involve direct, cell autonomous interaction with the FGF signal transduction pathway, or an indirect interaction. Mesp1-fated mesodermal cells (which include cardiac progenitors) require Fgfr1 and Fgfr2 to support normal OFT development (16), thus suggesting that cardiac progenitors are targeted by FGF signaling. Because *Tbx1* is expressed in these cells, it is possible that it plays a cell-autonomous role in modulating their response to Fgf8. It is also possible that the loss of *Tbx1*, by affecting differentiation, makes cells unresponsive to Fgf8.

Our data open a new window into the regulation of the response of cardiac progenitor cells to growth factors. Further work will be necessary to fully understand the significance of this mechanism for the proliferation and growth of these cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by NIH grant HL064832, grants from the Italian Telethon Foundation, and grants from the EU AnEUploidy and CardioGeNet programs (to AB).

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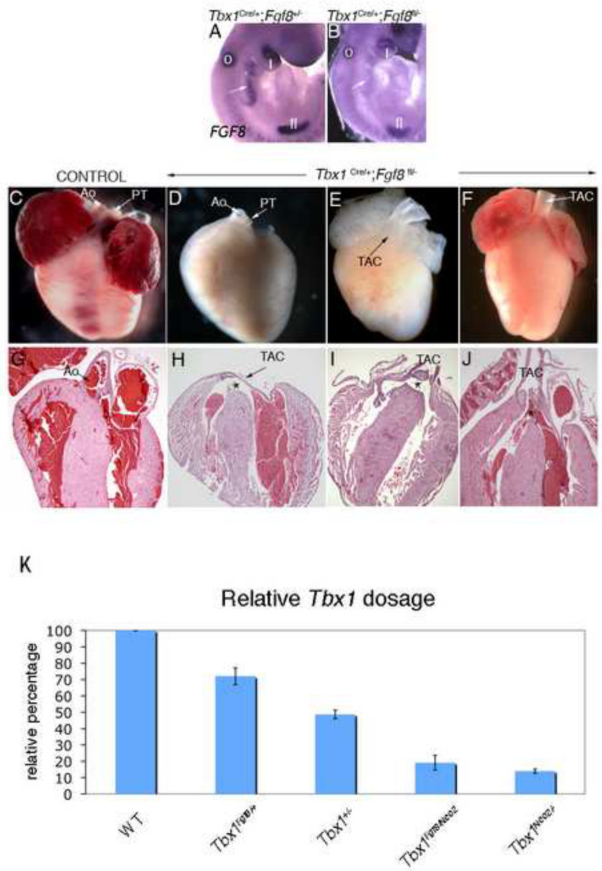

Figure 1. Loss of Fgf8 in Tbx1-expressing cells causes Truncus arteriosus Communis

A–B: *Fgf8 in situ* hybridization in control and conditional mutants (*Tbx1*$^{cre/+}$;*Fgf8*$^{fl/-}$) at E9.5 shows reduced expression in the pharyngeal region (arrow). O, otocyst; I, 1st pharyngeal arch; fl, forelimb bud.

C–J: OFT and intracardiac phenotype in near term control (C,G) and *Tbx1*$^{cre/+}$; *Fgf8*$^{fl/-}$ (D–F; H–J) embryos. Note the interventricular septal defect (*) and truncus arteriosus communis (TAC).

K: Histogram of qRT-PCR data showing the relative expression of *Tbx1* E9.5 embryos (3 embryos per genotype).

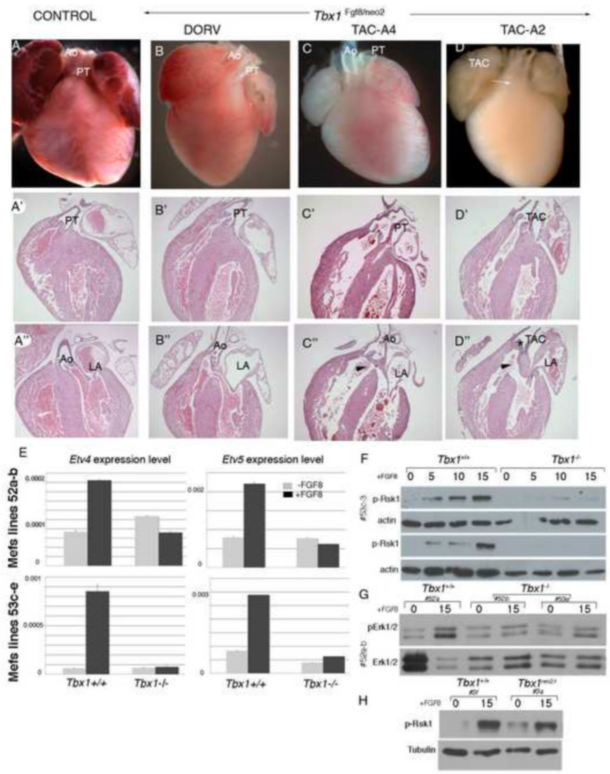

Figure 2. Forced Fgf8 expression in the Tbx1 domain partially rescues cardiovascular defects of Tbx1-hypomorphs

A–D: Isolated hearts from near-term control *Tbx1*$^{dp1/neo2}$ (A) and *Tbx1*$^{fgf8/neo2}$ (B–D) embryos show a range of phenotypes, including alignment and OFT defects. Sections of the corresponding hearts show DORV (B–B”), TAC type A4 (C'–C”, distal septation of aorta and pulmonary trunk with unseptated truncal valve overriding the interventricular septum) or TAC type A2 (D'–D”). Note the continuity between truncal and mitral valves in C” which is lost in D” (asterisk in D”). Ao, aorta; PT, pulmonary trunk; DORV, double outlet right ventricle; TAC, truncus; LA, left atrium; A2, A4 according to van Praagh's classification. Note VSD in C” and D” (arrowheads).

E. qRT-PCR of Fgf8 target genes *Etv4* and *Etv5* expression in mouse embryonic fibroblasts (MEFs) from *Tbx1*$^{+/+}$ and *Tbx1*$^{-/-}$ embryos (two independent lines per genotype) exposed to recombinant FGF8 in culture. Mutant cells do not respond properly to FGF8 stimulation.

F–G. Western blots of proteins extracted from the same cells. Mutant cells express very little or undetectable level of Rsk1 and Erk1/2 phosphorylation.

H: Western blot of proteins extracted from wild type and *Tbx1*$^{neo2/-}$ embryos. Low levels of *Tbx1* are sufficient to rescue Rsk1 phosphorylation response to FGF8.