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Tbx1 regulates proliferation and differentiation of multipotent heart progenitors

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Abstract

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 (SHF), 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 three heart lineages expressing endothelial, smooth muscle and cardiomyocyte markers. In multipotent cells, Tbx1 stimulates proliferation, explaining why $Tbx1^{-/-}$ embryos have reduced proliferation in the SHF. 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, while gain results in reduced differentiation *in vivo*. We found that Tbx1 binds Serum Response Factor (Srf), 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.

Keywords

cardiac progenitor cells (CPCs); cardiac differentiation; T-box transcription factors; Serum Response Factor (Srf)

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Disclosures
None.

Introduction

T-box transcription factors have important roles in development, and their mutation is associated with developmental disorders in humans and mice[1]. In particular, several members of this family are critical for heart development and are implicated in congenital heart disease[2]. 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[3]. *Tbx1* is expressed in several tissues but its mesodermal domain (but not cardiac tissue), is critical for heart development [4, 5], suggesting that the major role of 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) [4, 5]. 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 E9.5 in the mouse [6–9]. The expression of *Tbx1* in this migratory population was confirmed by cell fate mapping using a Cre-*lox*P strategy [10, 11]. Not only it is unknown how *Tbx1* functions within the SHF, but also it is unclear what mechanisms regulate the SHF function in general. In particular, is unclear how this cell population is maintained "active", i.e. capable of proliferating and providing differentiating cells to the heart, over several days of embryonic development, although it appears that FGF and BMP signals have a role in this process [12–14].

Recent data have uncovered that different cell types populating the heart (e.g. cardiomyocytes, endothelial cells, smooth muscle cells) may derive from a single progenitor [15–17]. 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 *ex vivo* or from reprogrammed cells. In addition, the use of multipotent progenitors in cardiac regeneration would have the theoretical advantage of regenerating several types of damaged cells.

Materials and Methods

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

Gene targeting

The allele $Tbx1^{Egfp}$ was generated by homologous recombination in AB2.2 mouse embryonic stem (ES) cells, as shown in Fig. 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 $Tbx1^{Lacz}$ [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 USA Public Health Service Policy on Humane Care and Use of Laboratory Animals. The following mouse mutant lines have been described previously:

Tbx1$^{LacZ/+}$ (also indicated as *Tbx1*$^{+/-}$) [18], *COET*[19], and *Mef2c-Cre*[20]. 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. 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 bacteriological Petri dishes and cultured for additional 4–7.5 days in suspension.

We performed flow cytometricanalysis using a two-laser instrument, FACScan(Beckton Dickinson). We carried out flow sorting of *in vitro* differentiated *Tbx1*$^{Egfp/+}$ cells 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, and cultured them for 2–3 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 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.

Transfection and cell cycle analysis

For cell cycle analysis, early passages clones were cultured to 80% confluency. Then cells were starved for 8 hours for synchronization, and transfected with a *Tbx1*-expressing plasmid for 24 hrs. Then, the growth media 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, 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.

Co-Immunoprecipitation and western blotting

C2C12 cells were transfected with *Tbx1-c-myc* cDNA plasmid and lysed in immunoprecipitation buffer. For immunoprecipitation assays we used the ProFound Mammalian Co-Immunoprecipitation kit (Pierce, 23605) following manufacturer instructions. C2C12 cells were transfected with the *Tbx1*-c-myc expressing plasmid or empty vector for 24 hours, followed by MG132 treatment for 2 hrs. Then cells were cultured in fresh media for another 4 hrs. Cells were trypsinized, protein 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 *WT* or *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, 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, 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 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). Sections were dehydrated, counter-stained, mounted and examined under a Zeiss light microscope.

Results

Tbx1 is expressed in multipotent progenitor cells

Cre-*lox*P-based fate mapping of *Tbx1*-expressing cells showed contribution to multiple tissue types of the heart, i.e. myocardium, endothelium, and smooth muscle [11]. With this method, however, it is not possible to establish whether individual *Tbx1* expressing cells have multi-lineage potential or whether the expression of *Tbx1* occurs in different lineages. To clarify this issue, we have carried out clonal assays of individual *Tbx1*-expressing cells. To this end, we have generated a $Tbx1^{Egfp}$ knock-in allele in mouse embryonic stem (ES) cells (Fig. 1A). A $Tbx1^{Egfp/+}$ clone (named D5) was subjected to *in vitro* differentiation, and we established that the wild type *Tbx1* allele, as well as the EGFP reporter allele, is turned on at day 6 of the "hanging drop" differentiation protocol (Fig. 1B). This result was confirmed by flow cytometry, which indicated the appearance of GFP+ cells at day 6.5, and detected the highest percentage of GFP+ cells (16%) at day 8.5 (Fig. 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 (Supplementary Fig. 1). Out of 288 cells seeded (one cell per well), 86 proliferated and formed clones. These clones were expanded and stocked at early passages (P3) (Fig. 2A). Next, we subjected these clones to spontaneous differentiation (Fig. 2). After 7–14 days of culture we tested markers of cardiac muscle, endothelial, and smooth muscle differentiation. Results showed that out of 86 clones tested, 47 (55%) were positive for the endothelial marker Pecam1, 18 (21%) were positive for the cardiomyocyte specific marker cardiac Troponin T (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) (Fig. 2B). None of these clones expressed Tbx1 by RT-PCR (Fig. 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 three 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 three markers (24%). Subsequently, we carried out a retrospective analysis of a subset of these clones prior to differentiation. We evaluated mRNA expression of the cardiac progenitor markers *NK2 transcription factor related, locus 5 (Nkx2.5)*, *Islet LIM homeobox 1 (Isl1)*, *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, while a Type I clone (capable of differentiating into endothelial cells) was negative for all these markers (Fig. 2C). All types of clones, at this level of differentiation, expressed Tbx1, as expected (Fig. 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, i.e. 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 over-expression 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 two clones expressed *Tbx1* and cardiac progenitor markers but did not express differentiation markers such as *Pecam1*, *cTnT*, and *SM-MHC* (Fig. 3A). Results showed an increased number of mitotic cells compared to cells transfected with an empty vector (Fig. 3B–C). Consistent results were obtained in three repeated experiments and with two 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 named *COET*[19]. We crossed the *COET* line with an SHF Cre driver, the *Mef2c-Cre* transgenic line [20] and evaluated cell proliferation in the SHF, compared with controls, $Tbx1^{+/-}$ and $Tbx1^{-/-}$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 (Supplementary Fig. 2).

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 and other regions of the heart [9]. Immunostaining of Tbx1 on mouse embryos at different stages (5–22 somites) showed overlap with the SHF marker Isl1 (Fig. 4D, H, and L). However, Isl1 immunostaining appeared much more extensive than Tbx1 immunostaining, as it was clearly visible also in the myocardial layer of the OFT (Fig. 4C, G, and K). In contrast, Tbx1 appeared restricted to the SHF, especially at 22 somites (Fig. 4F and J). To confirm this finding, we co-stained embryos at 16 and 22 somites with anti-Tbx1 and anti α-SMA (alpha-Smooth Muscle Actin, as differentiation marker) antibodies. Results showed that there is essentially no overlap between the two markers at both stages (Fig. 5A–F), 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 two markers was extended dorsally-posteriorly to encroach into the SHF anatomical region (Fig. 5G–P), consistent with recently reported data[22]. Next, we tested whether expansion of Tbx1 expression in the SHF could cause the opposite effect, i.e. 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 (Fig. 5Q–Z), 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 outflow tract defects such as ventricular septal defects, double outlet right ventricle (DORV), or truncus arteriosus (in 4 mutants analyzed, Supplementary Fig. 3).

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[23, 24], we tested whether the expression of Srf might also be extended posteriorly in $Tbx1^{-/-}$ embryos. Immunohistochemistry results showed that this is indeed the case (Fig. 6A–B). Conversely, in the *Tbx1* gain of function mutant *Mef2c-Cre; COET*, Srf

expression receded ventrally (Fig. 6C–D), 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 (Fig. 6E). In contrast, *Srf* mRNA level was not affected by *Tbx1* expression (Fig. 6F), indicating that the reduced level of the protein is not due 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 (Supplementary Fig. 4), thus confirming that Tbx1 does not regulate, directly or indirectly, *Srf* gene expression. Therefore, we tested whether Tbx1 and Srf proteins may interact. Co-Immunoprecipitation (Co-IP) experiments in Tbx1-transfected C2C12 cells demonstrated that indeed the two proteins co-immunoprecipitate, suggesting that they form a complex (not shown). To confirm this observation *in vivo*, we carried out Co-IP of the endogenous proteins from tissues of *WT* and $Tbx1^{-/-}$ (negative control) embryos at E9.5. Nuclear extracts from embryo tissues were immunoprecipitated using an anti-Srf antibody and revealed using an anti-Tbx1 antibody. Results clearly showed that Tbx1 and Srf are co-immunoprecipitated (Fig. 6G). A possible consequence of this interaction might be reduced stability of the proteins. Therefore, we transfected *Tbx1* into C2C12 cells with or without treatment with the proteasome inhibitor MG132 [25]. Results showed that in the presence of MG132, Tbx1 was unable to reduce the level of the Srf protein (Fig. 6H), suggesting that the negative regulation of Srf by Tbx1 may be due to higher rate of proteasome-mediated degradation.

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. In order 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 outflow tract 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 outflow tract of the heart. *Fgf8*, for example, is expressed early in the mesoderm of the SHF but not (or very little) in the OFT [12, 26]. Reduced dosage of *Fgf8* in the mesoderm leads to OFT defects typical of impaired SHF function [27]. 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 [28, 29]. 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 beta-galactosidase (β-gal) activity from a $Tbx1^{LacZ}$ reporter [30], thus probably biased by the stability of the β-gal 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 (Fig. 7). Because Tbx1 can regulate *Fgf8* expression in the mesodermal region that includes the SHF [4, 5, 31–33], 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 [22] while 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 co-immunoprecipitated *in vivo* suggests that the formation of the complex might reduce Srf protein stability.

Furthermore, we show that *Tbx1* identifies three-potent heart progenitors, suggesting that such cells are present in the SHF (Fig. 7A). This is consistent with the identification of common progenitors of at least some of the different cell types populating the heart [15–17, 34]. Our data indicate that Tbx1, at least in the SHF population of heart progenitors, regulates the balance between proliferation and differentiation (Fig. 7B).

Finally, our data beg 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 disregulation 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Non-standard Abbreviations and Acronyms

α-SMA	alpha-smooth muscle actin
β-gal	beta-galactosidase
BMP	bone morphogenetic protein
CPC	Cardiac progenitor cell
cTnT	cardiac troponin T
EBs	embryoid bodies
ES cells	embryonic stem cells
FBS	fetal bovine serum
FGF	Fibroblast growth factor
Foxa2	forkhead box A2
GATA4	GATA binding protein 4
Isl1	islet LIM homeobox 1
Nkx2.5	NK2 transcription factor related, locus *5*

OFT	outflow tract
Pecam1	platelet/endothelial cell adhesion molecule 1
SHF	second heart field
SM-MHC	smooth muscle-myosin heavy chain
Srf	Serum Response Factor
Tbx1	T-box transcription factor 1

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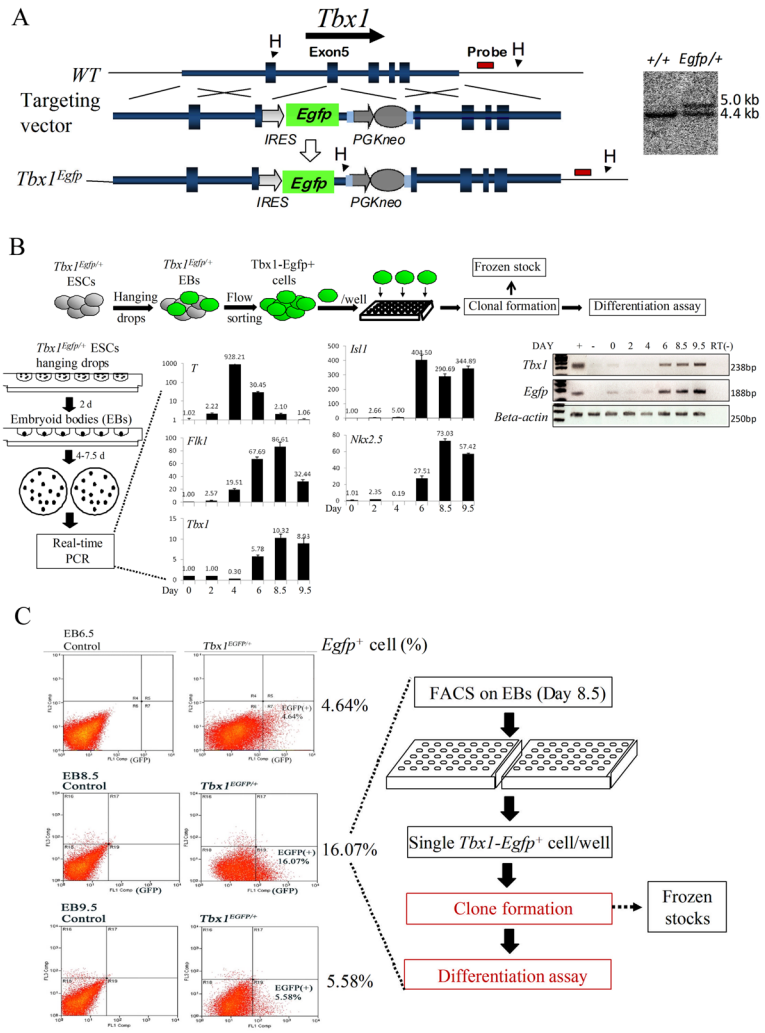

Figure 1. Generation of the *Tbx1*^{Egfp} allele and isolation *Tbx1*^{Egfp}+ ES cells

(A) Targeting strategy to generate the *Tbx1*^{Egfp} knock-in allele. An *Ires-Egfp* cassette was knocked into exon 5 of the *Tbx1* locus. Southern blotting analysis confirmed homologous recombination. *WT* allele: 4.4kb; mutant allele: 5.0kb. (B) For embryoid bodies (EBs) differentiation, ES cells were cultured in hanging drops for 2 days, followed by culture in bacteriological 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 *EGFP* and *Tbx1* primers in ES cells mRNA shows that the expressions of the two alleles are very similar to each other. (+) indicates positive control; (−) indicates negative control. (C) Flow cytometric analysis of *Tbx1*$^{Egfp/+}$ cells at day 6.5, 8.5 and 9.5 of differentiation. Left panels are *WT* controls (parental ES cell line) and right panels are *Tbx1*$^{Egfp/+}$ disaggregated EB cells; numbers on the left indicate the percentage of Egfp+ cells at day 6.5, 8.5 or 9.5 EBs. Egfp+ cells, sorted from day 8.5 EBs, were seeded individually into gelatin-coated 96-well plates for clonal assays.

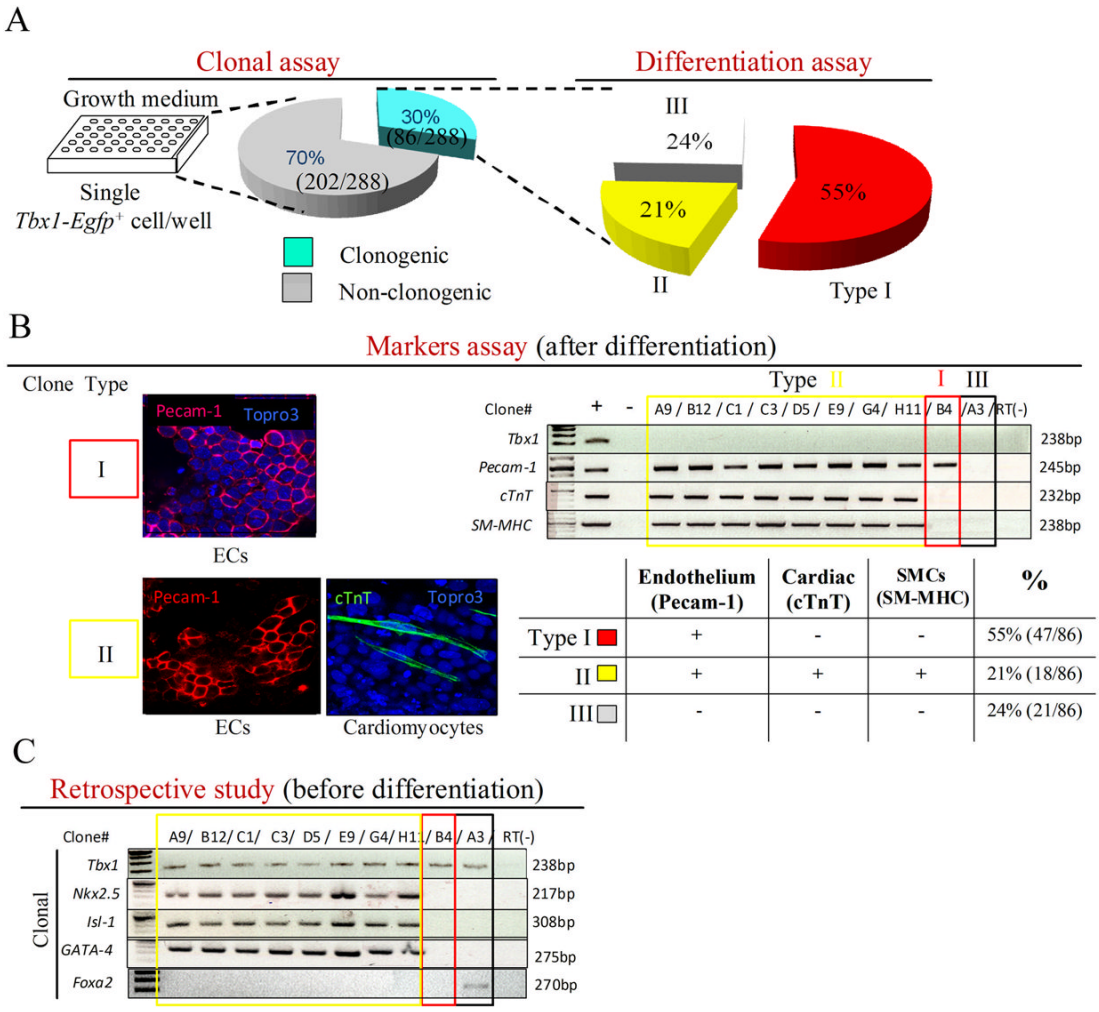

Figure 2. Clonal assay of *Tbx1*^{Egfp}+ cells

(A) Schematic procedure of clonal assay and differentiation analysis. About 30% (86/288) of single *Tbx1*^{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), cardiac troponin T (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*^{Egfp}+ cells. 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; (−) indicates negative control. Bottom-right panel: 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.

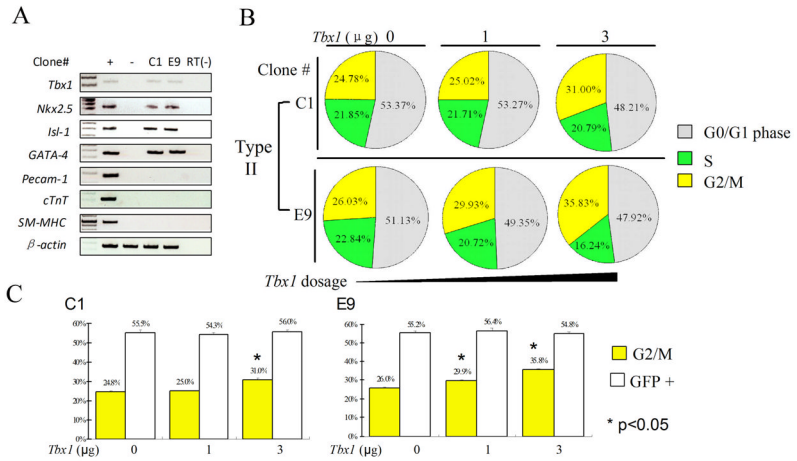

Figure 3. *Tbx1* enhances the mitotic activity of multipotent (Type II) heart progenitor clones

(A) RT-PCR-based expression analysis of 2 independent Type II clones C1 and E9 (before mitotic activity analysis) for the genes indicated. (B) Cell cycle analysis by flow cytometry of clones C1 and E9 after transfection with a *Tbx1*-expressing plasmid (0, 1, 3 μg). (C) Histograms show the percentage of cells G2/M phase (columns in yellow) from the above experiments: the asterisks indicate statistically significant differences compared to controls ($p<0.05$, t-test). Columns in white indicate transfection efficiency at each experimental point.

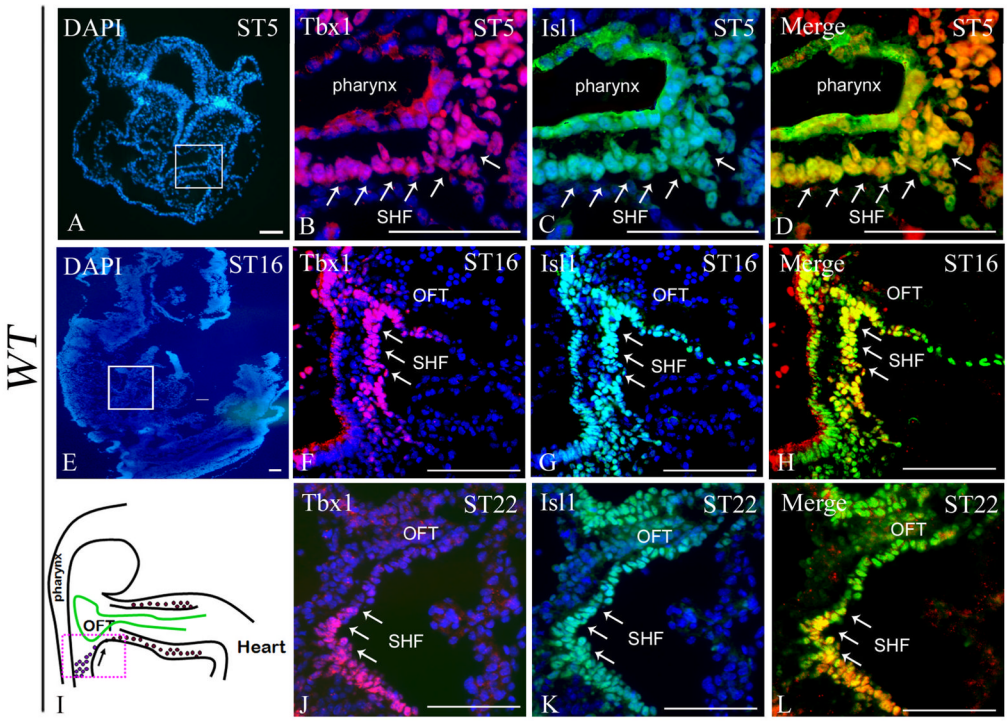

Figure 4. Tbx1 overlaps with Isl1 expression, but only in the SHF

(A–L) Confocal images of sections from E8.0-9.5 *WT* embryos double-stained with anti-Isl1 and anti-Tbx1 antibodies. Isl1 is expressed within the pharyngeal endoderm and splanchnic mesoderm at E8.0 (ST5) (C, D), the SHF and the outflow tract (OFT) proper at E9.0-9.5 (G, H, K, and L). *Tbx1* is expressed within the pharyngeal endoderm and the splanchnic mesoderm at E8.0 (ST5) (B, D), in the SHF but not in the OFT at E9.0-9.5 (F, H, J, L). PE: pharyngeal endoderm; OFT: outflow tract; SHF: second heart field; CA: common atrium. Scale bar: 100 μm.

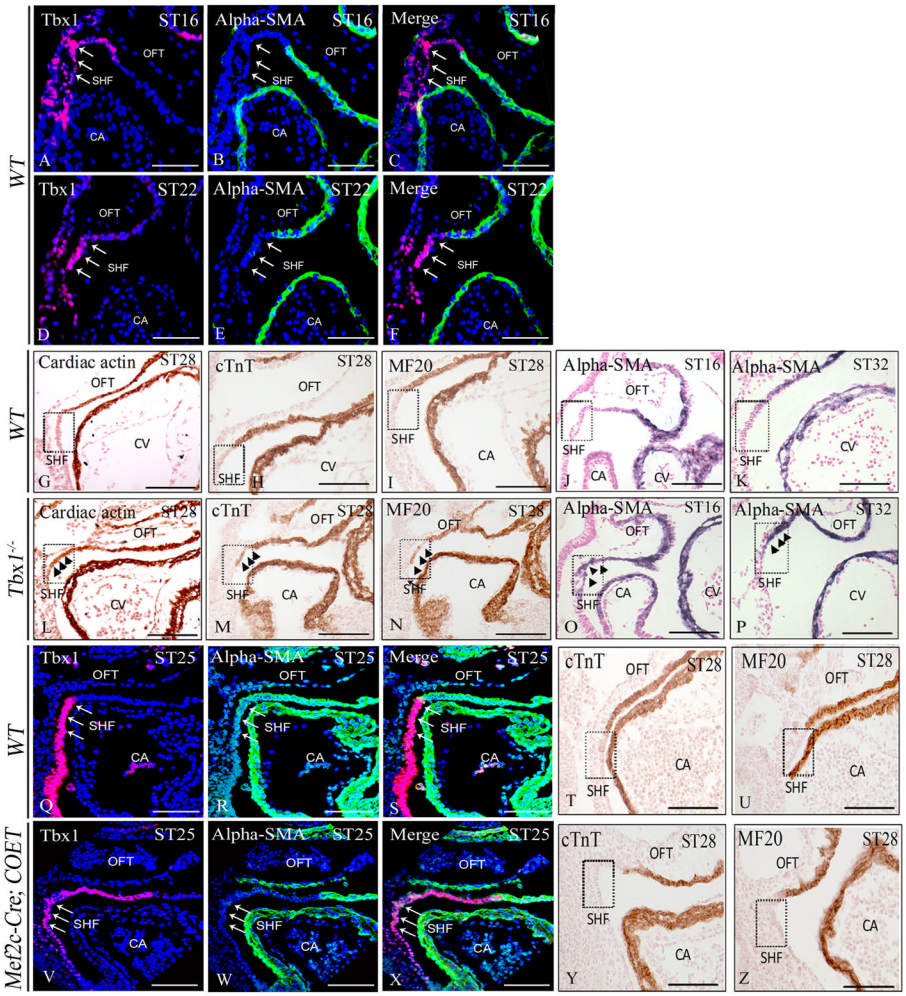

Figure 5. Tbx1 negatively regulates differentiation in the SHF

(A–F) Confocal images of sections from E9.0 (ST16) and E9.5 (ST22) *WT* embryos double-stained with anti-α-SMA and anti-Tbx1 antibodies. Tbx1 is expressed in the SHF but not in α-SMA^{+} cardiomyocytes of the OFT in both stages. (G–P) Immunohistochemistry of differentiation markers including cardiac sarcomeric actin, cardiac troponin T (cTnT), MF20 and α-smooth muscle actin (α-SMA) on E9.0-10.0 *WT* embryos (G–K), showing expression in the OFT proper but not in the SHF. However, in *Tbx1*$^{-/-}$ embryos (L–P), the expression of these markers extended ectopically into the SHF. The ectopic expression is more prominent at E10 (K, P). (Q–Z) Ectopic expression of Tbx1 in the OFT of *Mef2c-Cre; COET* embryos caused reduced expression of α-SMA (R, S, W, X), cTnT (T, Y) and MF20 (U, Z) in the OFT at E9.5. OFT: outflow tract; SHF: second heart field; CA: common atrium; CV: common ventricle. Scale bar: 100 μm.

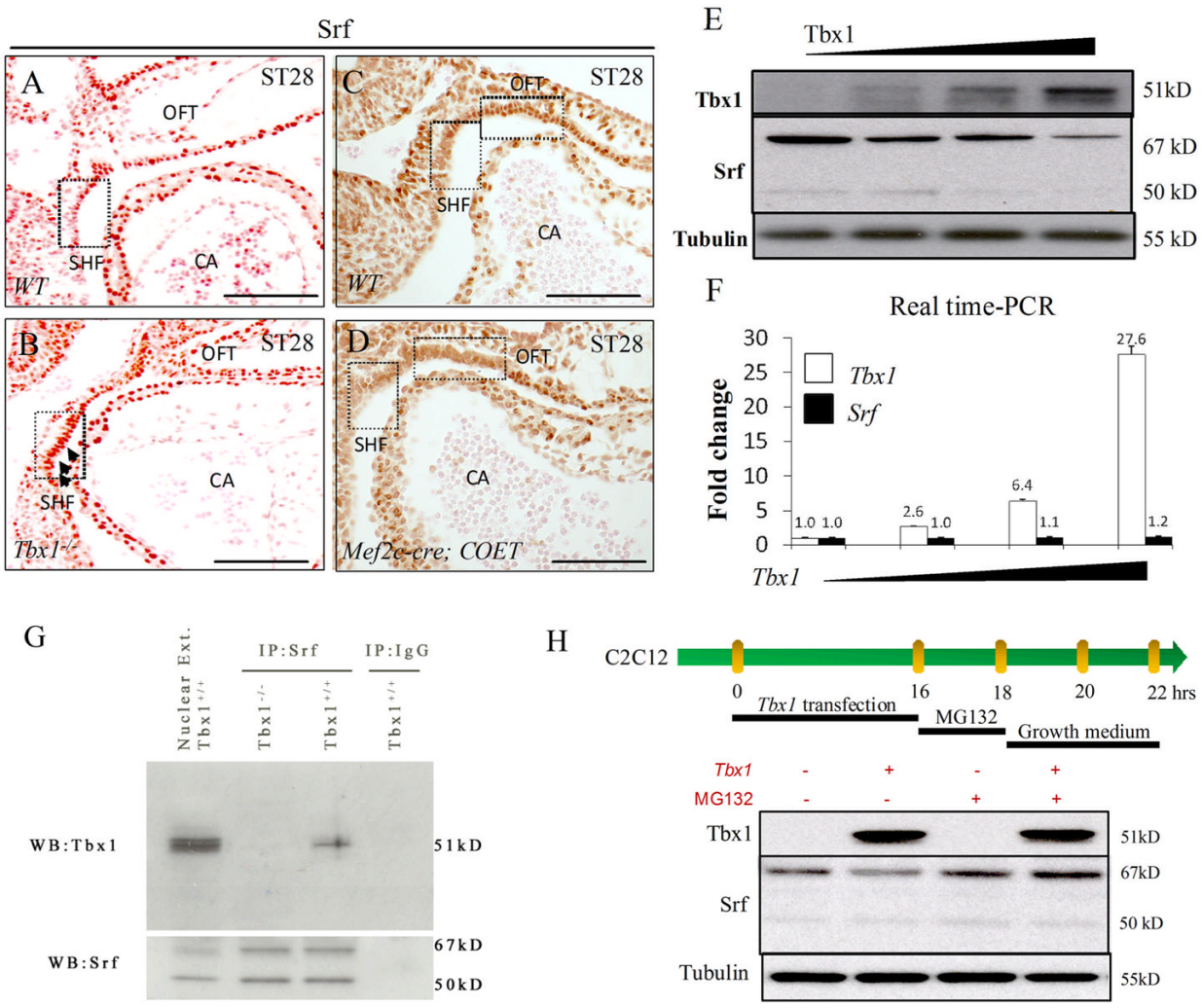

Figure 6. Tbx1 regulates and interacts with Srf

(A–B) Immunohistochemistry showed extended Srf expression in *Tbx1*$^{-/-}$ embryos at E9.5; (C–D) conversely, there is reduced expression in the OFT of the gain of function mutant *Mef2c-Cre; COET* embryos at E9.5. (E) 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 co-immunoprecipitates with Srf in *WT* embryos. (H) The proteasome inhibitor MG132 abolishes the Tbx1-induced reduction of Srf level in Tbx1-transfected C2C12 cells. OFT: outflow tract; SHF: second heart field; CA: common atrium; Srf: Serum response factor. Scale bar: 100 μm.

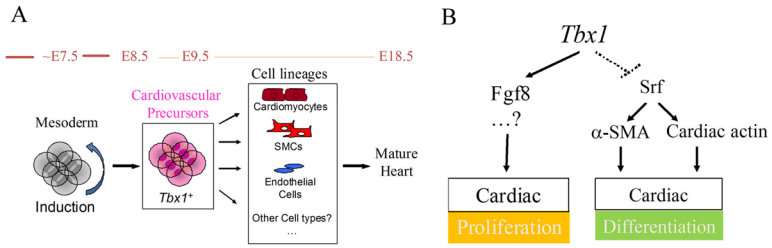

Figure 7.
Schematic model of the role of Tbx1 in regulating proliferation and differentiation of heart progenitors.

Table 1

PCR primer pairs used for gene expression analysis.

Genes	Sequence of forward primer	Sequence of reverse primer
Isl1	5′-GCC TCA GTC CCA GAG TCA TC-3′	5′-AGA GCC TGG TCC TCC TTC TG-3′
Nkx2-5	5′-CAG TGG AGC TGG ACA AAG CC-3′	5′-TAG CGA CGG TTC TGG AAC CA-3′
GATA-4	5′-CTG TCA TCT CAC TAT GGG CA-3′	5′-CCA AGT CCG AGC AGG AAT TT-3′
Foxa2	5′-CCC GGG ACT TAA CTG TAA CG-3′	5′-GCG CCC ACA TAG GAT GAC-3′
PECAM-1	5′-TGC AGG AGT CCT TCT CCA CT-3′	5′-ACG GTT TGA TTC CAC TTT GC-3′
SM-MHC	5′-AAG CTG CGG CTA GAG GTC A-3′	5′-CCC TCC CTT TGA TGG CTG AG-3′
cTnT	5′-CTG AGA CAG AGG AGG CCA AC-3′	5′-TTC TCG AAG TGA GCC TCG AT-3′
GFP	5′-GGA CGT GGT TTT CCT TTG AA-3′	5′-GAA CTT CAG GGT CAG CTT GC-3′
β-actin	5′-GGG ACG ACA TGG AGA AGA T-3′	5′-GTG TGG GTG ACC CCG TCT-3′