Tbx20 regulation of cardiac cell proliferation and lineage specialization during embryonic and fetal development in vivo

Santanu Chakraborty, Katherine E. Yutzey *

The Heart Institute, Cincinnati Children’s Medical Center, Cincinnati, OH 45229, USA

Abstract

Tbx20 gain-of-function mutations in humans are associated with congenital heart malformations and myocardial defects. However, the effects of increased Tbx20 function during cardiac chamber development and maturation have not been reported previously. CAG-CAT-Tbx20 transgenic mice were generated for Cre-dependent induction of Tbx20 in myocardial lineages in the developing heart. MHCCre-mediated overexpression of Tbx20 in fetal ventricular cardiomyocytes results in increased thickness of compact myocardium, induction of cardiomyocyte proliferation, and increased expression of Bmp10 and pSmad1/5/8 at embryonic day (E) 14.5. MHCCre-mediated Tbx20 overexpression also leads to increased expression of cardiac conduction system (CCS) genes Tbx5, Cx40, and Cx43 throughout the ventricular myocardium. In contrast, Nlk2.5Cre mediated overexpression of Tbx20 in the embryonic heart results in reduced cardiomyocyte proliferation, increased expression of a cell cycle inhibitor, p21, and decreased expression of Tbx2, Tbx5, and N-myc1 at E9.5, concomitant with decreased phospho-ERK1/2 expression. Together, these analyses demonstrate that Tbx20 differentially regulates cell proliferation and cardiac lineage specification in embryonic versus fetal cardiomyocytes. Induction of pSmad1/5/8 at E14.5 and inhibition of dpERK expression at E9.5 are consistent with selective Tbx20 regulation of these pathways in association with stage-specific effects on cardiomyocyte proliferation. Together, these in vivo data support distinct functions for Tbx20 in regulation of cardiomyocyte lineage maturation and cell proliferation at embryonic and fetal stages of heart development.

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Introduction

T-box (Tbx) transcription factors are critical regulators of heart development, and mutations in several Tbx genes in humans are associated with congenital cardiac anomalies (Plageman and Yutzey, 2005; Stennard and Harvey, 2005). Tbx20, a member of the Tbx1 subfamily of T-box genes, is expressed in multiple organs, including the developing heart (Plageman and Yutzey, 2004; Stennard et al., 2003; Takeuchi et al., 2003). Mutations in human Tbx20 that result in gain or loss of protein function are associated with a wide array of cardiac malformations, including septal defects, defects in valve development, and cardiomyopathy (Kirk et al., 2007; Posch et al., 2010). In mice, systemic loss of Tbx20 results in lethality by embryonic day (E) 10.5, associated with reduced myocardial proliferation and inhibition of chamber differentiation (Cai et al., 2005; Singh et al., 2005; Stennard et al., 2005; Takeuchi et al., 2005). Direct downstream target genes of Tbx20 in the primitive myocardium include Tbx2 and N-myc1, that function to regulate cardiomyocyte proliferation (Cai et al., 2005). In adult mice, heterozygous loss of Tbx20 leads to dilated cardiomyopathy (Stennard et al., 2005) and conditional homozygous loss of Tbx20 in cardiomyocytes results in severe cardiomyopathy with associated arrhythmias and death (Shen et al., 2011). However, the specific functions of Tbx20 in the developing myocardium after the initial stages of heart chamber specification have not been defined in vivo.

During embryonic and fetal development, multiple regulatory pathways control differential rates of cardiomyocyte proliferation necessary for proper cardiac chamber morphogenesis and function (Sedmera and Thompson, 2011). During embryonic stages of chamber morphogenesis (mouse E9.5-E11.5), Neuregulin1 (Nrg1), an endocardium-derived mitogen, promotes the initiation of trabecular myocardial outgrowth and cardiomyocyte proliferation via ErbB receptors and ERK/MAP-kinase activation (Lai et al., 2010; Stennard et al., 2005; Woldeyesus et al., 1999). During formation of the ventricular compact layer at fetal stages (E12.5–E17.5), Bmp10 signaling through pSmad1/5/8 activation is required for ventricular cardiomyocyte proliferation, and cardiomyocyte-specific loss of Smad4, necessary for BMP signaling, leads to myocardial hypoplasia with decreased expression of N-myc1, cyclinD1, and cyclinD2 (Chen et al., 2004; Song et al., 2007). A critical difference in regulation of
embryonic and fetal myocardial proliferation is the presence of the epicardium, which is a source of several mitogens, including IGF2 and FGF9, that promote fetal ventricular compact layer cell proliferation (Lavine et al., 2005; Li et al., 2011; Ssebu et al., 2009). Thus, multiple signaling pathways have been implicated in the regulation of cardiomyocyte proliferation in the developing heart. However, less is known about the contributions of specific transcription factors or the mechanisms of integration of these pathways in the control of differential rates of proliferation during cardiac organogenesis.

Transgenic mice were generated with Cre-dependent overexpression of Tbx20 in embryonic or fetal cardiomyocytes. Induction of Tbx20 expression in the fetal ventricles (E12.5–E17.5) with βMHCcre results in increased cell proliferation, with increased expression of N-myc1, Bmp10, and pSmad1/5/8, as well as increased expression of conduction system markers Tbx5, Cx40, and Cx43 throughout the ventricular myocardium. In contrast, induction of Tbx20 expression in the embryonic heart (E9.5) with Nkx2.5Cre results in a small heart with decreased cell proliferation, apparently normal induction of chamber maturation gene expression, and attenuated activation of ERK1/2 MAPK. Thus, Tbx20 overexpression has opposite effects on cardiomyocyte cell proliferation and lineage maturation in embryonic and fetal cardiomyocytes that is associated with differential regulation of ERK1/2 and Smad1/5/8 signaling in vivo.

Materials and methods

Generation of transgenic mice

The Cre-responsive transgene CAG-CAT-Tbx20 (CC-Tbx20) was constructed by modification of the CAG-CAT-Z construct, which contains a CMV enhancer and chicken β-actin gene (CAG) promoter, linked to the chloramphenicol acetyltransferase (CAT) gene, flanked by lox sites (Araki et al., 1995). The full-length murine Tbx20 coding sequence was isolated from pAC-CMV-Tbx20 (Plageman and Yutzey, 2004) and inserted into the BamHI site of CAG-CAT-Z in place of LacZ (Araki et al., 1995). The Tbx20 transgene consists of the Tbx20α isoform that includes both transactivation and transpression domains (Stennard et al., 2003). This isoform is preferentially expressed in the cardiac OFT region at E9.5–E12.5, although expression is detected throughout the primitive heart tube at E9.5–E10.5 (Takeuchi et al., 2005). Successful insertion of the full-length murine Tbx20 coding sequence was verified by direct sequencing of the CC-Tbx20 transgenic construct. The purified linearized construct was introduced by pronuclear microinjection of fertilized blastocysts of FVB mice. Transgenic founder lines were validated by genotyping using CAG-CAT (5′-TCA CTG CAT TCT AGT TGT GGT TTG-3′) and Tbx20 (5′-TTG GAC TGA TCA GGA TTC ACT CC-3′) specific primers. Transgene expression in multiple organs was confirmed by the presence of CAT protein detected by CAT-ELISA assay (Roche, #1163727001, used as per manufacturer’s protocols) in two independent founder lines. The founder line with robust expression of CAT protein in the heart was used for all subsequent in vivo transgenic analyses. Female CAG-CAT-Tbx20 mice were bred with βMHCcre (Parsons et al., 2004) or Nkx2.5Cre (Moses et al., 2001) males to generate double transgenic embryos, neonates, and adult offspring. Timed matings were established, with the morning of an observed copulation plug set at E0.5. All studies were performed on cohorts of βMHCcre/CC-Tbx20, or Nkx2.5Cre/CC-Tbx20, double transgenic (DTG) animals compared to single transgenic (STG) CC-Tbx20 littermate controls. Genotyping for βMHCcre (Parsons et al., 2004) and Nkx2.5Cre (Moses et al., 2001) was performed as described previously by PCR of genomic DNA isolated from embryonic yolk sacs or 3 weeks postnatal tail clips. All experiments involving animals were carried out with experimental protocols and procedures reviewed and approved by the Cincinnati Children’s Medical Center Institutional Biosafety Committee and Institutional Animal Care and Use Committee.

In situ hybridization (ISH) and immunohistochemistry

Whole embryos isolated at E9.5 or E14.5 and hearts isolated at E17.5 were collected and processed for either in situ hybridization (ISH) or immunohistochemistry as previously described (Chakraborty et al., 2008, 2010; Lincoln et al., 2006). The generation of antisense RNA probes and ISH was performed as previously described (Chakraborty et al., 2008). The plasmids for generation of Bmp10 (Chen et al., 2004), Nppa, Smox, Tbx2, Cx40, Cx43 (Christoffels et al., 2004), Tbx5 (Takeuchi et al., 2005), N-myc1 (Cai et al., 2005) and MC2v (Liberatori et al., 2000) antisense RNA probes were used as previously described. For histology, tissues were deparaffinized and dehydrated through a graded ethanol series, followed by staining with Hematoxylin and Eosin (H&E) as previously described (Hinton et al., 2006).

The following antibodies were used for immunofluorescence (IF) and/or colorimetric immunohistochemistry with dianamidine benzidine (DAB) detection: Phospho-Histone H3 (pH3) (1:100; Millipore, 06–570), MF20 (1:200; Developmental Studies Hybridoma Bank, University of Iowa), pSmad1/5/8 (1:100; Millipore, AB3848), Tbx20 (1:100; Orbigen, PAB 11248), p21WAF1 (BD Pharmingen, 556430), Diphosphorylated ERK-1/2 (dpERK) (1:10000; Sigma, M 8159) and Twist1 (1:50; Santa Cruz, SC 81417). IF and colorimetric detection methods were performed essentially as previously described (Chakraborty et al., 2010). For IF, Alexa Fluor 488 (green)- and/or 568 (red)-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (MoTL251, 1:100) were used with ToPro3 (Molecular Probes, 1:1000) for visualization of nuclei. Fluorescence was visualized using a Zeiss LSM 510 confocal microscope and LSM version 3.2 SP2 software. For colorimetric immunohistochemistry, primary antibodies were detected using the Ultra-sensitive ABC rabbit (anti-Tbx20) or mouse (anti-Twist1, dpERK) IgG staining kit according to manufacturer’s suggested guidelines (Thermo Scientific, 32054 and 32052). Antibody staining was visualized using the metal enhanced DAB substrate staining kit (Thermo Scientific, 34065).

β-galactosidase (β-gal) detection

β-gal expression was analyzed in whole (E12.5) and bisected hearts (E14.5 and E17.5) using X-gal detection of β-gal activity as previously described (Lincoln et al., 2004; Searcy et al., 1998). For histology, X-gal stained embryos (E12.5) or bisected hearts (E14.5 and E17.5) were dehydrated through a graded isopropanol/PBT series and paraffin wax embedded. Ten µm sections were cut, deparaffinized in two changes in xylene for 5 min each and mounted in Cytoseal 60 (Richard-Allen Scientific) for microscopy and imaging.

RNA isolation and real-time quantitative RT-PCR (qRT-PCR)

Hearts were isolated from E9.5 embryos, and ventricles were isolated from E14.5 and E18.5 embryos generated from multiple matings of CC-Tbx20 females and Cre (βMHC or Nkx2.5) males. After PCR genotyping, tissue from 4–6 early (E9.5 and E14.5) or 2–3 late (E18.5) embryos was pooled for RNA isolation, and real time qRT-PCR was performed as previously described (Chakraborty et al., 2008, 2010). Forward and reverse primer sequences designed for qRT-PCR are Nppa (5′-GTG AGA AGA CTG AGG AAG CA-3′ and 5′-TGC TTT TCA AGG GGG CAG AT-3′); Tbx5 (5′-ATG GTC CTT AGG TGG CAA AG-3′ and 5′-TTT CTT GGT CTT TCA CAG TG-3′); Cx40 (5′-CTT GGA TAC CCT GCA TGT CT-3′ and 5′-GCC TTC TAG GCT CGT TCT GC-3′); Cx43 (5′-GAA CAC GGC AAG GTG AAG AT-3′ and 5′-GA CGA GAC ACA AGG AC-3′); N-myc1 (5′-CAG CGT CAC GGC TTC TC-3′ and 5′-CAT GCA GTC TTC TG-3′); MLC2v (5′-CAC AGA CGT TGG CAA AAG TCA GCC-3′ and 5′-GAT CAA GCA GGT TCA-3′); Bmp10 (5′-ACC AGA CTG TGG CAA AAG TCA GCC-3′ and 5′-GAT CAT GCA GGA GTC TCA-3′); and Tbx2 (5′-TCA ACA CCA GGA TCA TCA AGG-3′ and 5′-GTA GCC AGT AGC AGT CAT-3′). The primer sequences for p21(1CP), p27(1DTPI) (Evans-Anderson et al., 2008), Cyclin A2 (Gurtner et al., 2000) and Cyclin D1 (Rahmati et al., 2000) were designed for qRT-PCR.
et al., 2008), Cyclin D1 (Kroger et al., 2007), Cyclin E1 (Xue et al., 2010), Mlc2v, Smpx (Lai et al., 2010) and Nrg1 (Kato et al., 2010) qRT-PCR were described previously. All the amplification reactions were performed with 34 cycles of 94 °C for 30 s; 55 °C for 45 s; and 72 °C for 30 s. A standard curve was generated for each primer pair using E9.5 or E14.5 whole embryo-derived cDNA, and all the values were normalized to ribosomal protein L7 expression (Hemmerich et al., 1993). For each gene, normalized expression in STG tissue is set to 1 and then the fold-change is calculated for corresponding DTG samples. qRT-PCR results represent three independent samples (biological 3X) performed in triplicate (technical 3X). Statistical significance of observed differences was determined by Student’s t-test (p<0.01).

Protein isolation and Western blotting

Protein lysates were isolated from E9.5, E12.5, E14.5, and E17.5 whole hearts using ice-cold Cellytic MT tissue lysis buffer (Sigma), containing protease inhibitor mixture (Pierce) and phosphatase inhibitor (Pierce) according to manufacturer’s guidelines. After PCR genotyping, hearts from 6–8 early (E9.5 and E12.5) or 2–3 late (E14.5 and E17.5) embryos were pooled for total protein isolation, and Western blots were performed as described previously (Chakraborty et al., 2010). Immunoblots were developed using chemiluminescent detection with the Vistra ECF reagent (Amersham Biosciences) and scanned using a Storm 860 phosphorimager (Amersham Biosciences). Signal intensities were quantified with ImageQuant 5.0 software (GE Healthcare). Tbx20 (1:500; Orbigen, MB-1248), pSmad 1/5/8 (1:500; Millipore, AB3848), ERK (1:1000; Cell Signaling, 9102) and p-ERK (1:1000; Cell Signaling, 9101) primary antibodies were used for immunoblot analysis. GAPDH (1:4000; Santa Cruz) antibody reactivity was used as a loading control. Statistical significance was determined by Student’s t-test (p<0.05).

Quantitative analyses of cell density, ventricular wall thickness, and cell proliferation

The average number of cell nuclei per area of compact myocardium and left ventricular wall thickness were determined using ImageJ software (NIH). Measurements were taken on H&E stained photomicrographs of 5–6 tissue sections per heart from at least 4 hearts per genotype. Proliferative indices of cardiomyocytes were calculated as the total number of pH3 nuclei in MF20-positive cardiomyocytes/total number of nuclei in MF20-positive cardiomyocytes. Likewise, the number of Tbx20 nuclei in MF20-positive cardiomyocytes/total number of nuclei in MF20-positive cardiomyocytes was determined as a quantitative measure of Tbx20 overexpression in Nkx2.5Cre mice. At least 250 cell nuclei were counted from each heart section showing LV or RV chamber myocardium. Proliferative indices and total cell counts were calculated in multiple sections of 4–6 hearts of each genotype using ImageJ software (NIH). All the average measurements are reported with ± standard error of mean (SEM) and statistical significance was determined by Student’s t-test (p<0.05).

Chromatin immunoprecipitation (ChIP) assay

Direct Tbx20 binding to chromosomal DNA in vivo was evaluated in individual mouse hearts dissected from βMHCCre;CC-Tbx20 (DTG) and littermate CC-Tbx20 (STG) controls at E17.5. DNA/protein complexes were cross-linked for 10 min in formaldehyde (Sigma) at a final concentration of 1%. The fixed cells were lysed and sonicated twice for 5 s each output 5 (Vinsonic 60; Virtis) and a 2 min refractory period. For immunoprecipitation, cell lysates were incubated with an antibody against Tbx20 (5 μg; Orbigen) and incubated overnight at 4 °C. Immunoprecipitation with normal rabbit IgG was used as a negative control. ChIP assays were performed according to the manufacturer’s instructions (EZChiP, Upstate), with the exception that protein A-agarose beads were used. The immunoprecipitated and input DNA were subjected to quantitative PCR using SYBR Green PCR reagents and MJ Research Opticon 2 machine. P-4030 and P-4330 primers were used to amplify the N-myc intron 1 promoter region, and P-813 and P-573 primers were used to amplify the Tbx2 promoter region as previously described (Cai et al., 2005). Fold enrichment relative to IgG antibody control (negative control) was calculated using the comparative C_{t} method (ΔΔC_{t}) as described previously (n = 3) (Mandel et al., 2010; Sengupta et al., 2011). Statistical significance was determined by Student’s t-test (p<0.05).

Results

βMHCCre-mediated Tbx20 overexpression in differentiated cardiomyocytes results in thickening of ventricular myocardium

In order to determine the effects of Tbx20 gain-of-function in the developing heart, mice were generated to express a Cre-inducible CAG-CAT-Tbx20 (CC-Tbx20) transgene (Fig. 1A). The CAG-CAT-Tbx20 transgene consists of a CAG regulatory element, that includes a CMV enhancer/β-actin promoter driving the ubiquitous expression of CAT, flanked by loxp sites, linked to Tbx20. CAT expression from the transgene was detected by ELISA in multiple organs (heart, brain and lung) in founder lines (data not shown). In the presence of Cre, the floxed CAT gene is deleted and Tbx20 is expressed. Therefore, conditional overexpression of Tbx20 is achieved in both a spatially and temporally regulated manner in double transgenic (DTG) animals expressing Cre from βMHCCre or Nkx2.5 promoters in the presence of the CC-Tbx20 transgene.

In order to achieve increased Tbx20 expression in differentiated cardiomyocytes, CC-Tbx20 transgenic mice were mated with βMHCCre mice (Parsons et al., 2004). βMHCCre-mediated overexpression of Tbx20 was confirmed by Tbx20 specific antibody staining in E14.5 chamber myocardium. βMHCCre activity in the developing murine heart is apparent throughout the myocardium at E12.5, E14.5, and E17.5 as indicated by Rosada26R reporter expression analysis (Supplemental Fig. S1). Tbx20 immunoreactivity, is increased both in the interventricular septum (IVS in Fig. 1C) and left ventricular (LV in Fig. 1E) myocardium of the βMHCCre;CC-Tbx20 double transgenic (DTG) animals compared to the littermate CC-Tbx20 single transgenic (STG) control (Fig. 1D). Tbx20 protein localization, as detected by immunostaining, is predominantly nuclear in both STG and DTG animals. Quantitative Western blot analysis reveals increased expression of Tbx20 in βMHCCre;CC-Tbx20 DTG hearts beginning at E12.5, with an approximately 3-fold increase in Tbx20 protein expression at E14.5 and E17.5, compared to endogenous Tbx20 levels (Fig. 1F,G). During development and after birth, βMHCCre;CC-Tbx20 DTG animals were obtained at the expected Mendelian ratio, and no embryonic or adult morbidity or mortality was observed (Table 1).

Histological analysis of E14.5 DTG chamber myocardium overexpressing Tbx20 reveals fewer trabeculae in the ventricular myocardium (asterisks, Fig. 1H,I) and an increase in the average number of nuclei per area of the compact myocardium (Fig. 1J), compared to littermate STG controls. In addition, the thickness of the compact myocardium also is significantly increased in the E17.5 DTG hearts as compared to littermate controls (Fig. 1K–M). Thus, βMHCCre-mediated Tbx20 overexpression results in increased cell number and thickness of the developing compact myocardium at E14.5–E17.5.

βMHCCre-mediated Tbx20 overexpression promotes cardiomyocyte proliferation with increased binding to the N-myc1 promoter region in fetal hearts in vivo

Cardiomyocyte proliferation was examined in the hearts of βMHCCre; CC-Tbx20 DTG embryos relative to littermate controls. Consistent with the
increased number of cells in ventricular myocardium with Tbx20 overexpression, the number of phospho-histone H3 (pHH3) positive nuclei in MF20 positive cardiomyocytes is increased in DTG embryos, compared to littermate STG controls at E14.5 (arrowheads, Fig. 2A,B). Quantitation of these results demonstrates that 20.7% of the nuclei of MF20 positive cardiomyocytes are also positive for pHH3 in DTG myocardium overexpressing Tbx20, compared to 9.3% of the cardiomyocyte nuclei in STG littermates (Fig. 2C) at E14.5. Thus increased Tbx20 expression promotes myocardial cell proliferation at E14.5.

N-myc1 is a downstream target gene of Tbx20 in cardiomyocytes and is a positive regulator of cellular proliferation (Cai et al., 2005; Davis and Bradley, 1993). N-myc1 expression is increased in
βMHCCre;CC-Tbx20 myocardium with Tbx20 overexpression as detected by ISH (arrows, Fig. 2D,E). Expression of N-myc1 mRNA is increased by 3.8-fold in E14.5 DTG hearts overexpressing Tbx20, relative to controls, as determined by qRT-PCR (Fig. 2F). In cardiomyocytes, Tbx20 promotes cell proliferation through activation and binding to regulatory elements of N-myc1 and Tbx2 genes (Cai et al., 2005). Therefore, ChIP assays were performed to determine if Tbx20 binding to the promoter region of N-myc1 is increased in association with increased gene expression in fetal cardiomyocytes overexpressing Tbx20. In E17.5 DTG hearts, Tbx20 binds to the promoter region of N-myc1 with 14.5-fold enrichment over IgG controls, compared to 3-fold enrichment in STG littermates (Fig. 2G). Interestingly, no difference in Tbx20 binding to the Tbx2 promoter region was observed by ChIP analysis, consistent with no observed change in gene expression in DTG versus STG hearts (data not shown). Together, these data suggest that Tbx20 directly binds and induces N-myc1 expression while also promoting cardiomyocyte proliferation in βMHCCre;CC-Tbx20 hearts in vivo.

Bmp10 expression and pSmad1/5/8 signaling is increased in differentiated fetal cardiomyocytes overexpressing Tbx20

Cardiomyocyte proliferation during heart chamber maturation is dependent on Bmp10 (Chen et al., 2004). Therefore, Bmp10 mRNA expression was evaluated in DTG hearts overexpressing Tbx20. Expression of Bmp10 transcripts is increased in DTG myocardium overexpressing Tbx20, as detected by ISH, compared to littermate

### Table 1

<table>
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βMHCCre;CC-Tbx20 DTG myocardium with Tbx20 overexpression as detected by ISH (arrows, Fig. 2D,E). Expression of N-myc1 mRNA is increased by 3.8-fold in E14.5 DTG hearts overexpressing Tbx20,

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** βMHCCre-mediated Tbx20 overexpression promotes cardiomyocyte proliferation with increased binding to the N-myc1 promoter region in βMHCCre;CC-Tbx20 fetal hearts in vivo. (A–B) Cardiomyocyte cell proliferation was detected by phospho-histone H3 (pHH3) nuclear staining in MF20 positive cells at E14.5 in βMHCCre;CC-Tbx20 (DTG) myocardium (arrowheads in B) compared to littermate controls (arrowheads in A). (C) Quantitation of these results demonstrates a significant increase in proliferative indices of cardiomyocytes overexpressing Tbx20 compared to littermate controls (n = 4). (D–F) Increased expression of N-myc1, a known downstream target of Tbx20 in cardiomyocytes, is apparent by ISH in DTG myocardium overexpressing Tbx20 (compare D and E). (F) Observed differences in N-myc1 gene expression were further confirmed by qRT-PCR analysis of mRNA levels in E14.5 DTG hearts relative to STG controls, set to 1.0 (n = 3). (G) ChIP assays performed at E17.5 demonstrate increased Tbx20 binding (14.5-fold) to a previously identified T-box consensus site within intron 1 of the N-myc1 promoter region in DTG hearts compared to littermate STG controls (3-fold). Graphs represent fold enrichment relative to the IgG antibody control as determined by qPCR (n = 3). Statistical significance was determined by Student’s t test, where * denotes p < 0.05.
control (Fig. 3A,B). Likewise, Bmp10 mRNA expression is increased by 2.9-fold in E14.5 DTG hearts relative to littermate STG controls, as detected by qRT-PCR (Fig. 3C). Operated differences in gene expression were confirmed by qRT-PCR analysis of Bmp10 mRNA levels in E14.5 DTG hearts relative to levels in STG controls, which were set to 1.0 (n = 3). (D–G) The presence of pSmad1/5/8 was assessed by immunofluorescence and confocal microscopy. pSmad1/5/8 immunoreactivity (green nuclei) is increased in MF20 positive cardiomyocytes (red) and throughout the ventricles overexpressing Tbx20 in both E14.5 (arrowheads in E) and E17.5 (arrowheads in G) DTG hearts, compared to the littermate STG controls (arrowheads in D and F, respectively). (H–I) Quantitation of pSmad1/5/8 protein by Western blot (n = 6) demonstrates a 2.1-fold up-regulation in E14.5 DTG hearts, compared to littermate STG controls. Statistical significance was determined by Student's t test, where * denotes p < 0.05.

![Fig. 3. Bmp10 expression and pSmad1/5/8 signaling are increased in βMHCre;CC-Tbx20 fetal hearts overexpressing Tbx20. (A–B) Increased expression of Bmp10 transcripts is detected in the DTG myocardium overexpressing Tbx20 by ISH (arrows in A, B). (C) Observed differences in gene expression were confirmed by qRT-PCR analysis of Bmp10 mRNA levels in E14.5 DTG hearts relative to levels in STG controls, which were set to 1.0 (n = 3). (D–G) The presence of pSmad1/5/8 was assessed by immunofluorescence and confocal microscopy. pSmad1/5/8 immunoreactivity (green nuclei) is increased in MF20 positive cardiomyocytes (red) and throughout the ventricles overexpressing Tbx20 in both E14.5 (arrowheads in E) and E17.5 (arrowheads in G) DTG hearts, compared to the littermate STG controls (arrowheads in D and F, respectively). (H–I) Quantitation of pSmad1/5/8 protein by Western blot (n = 6) demonstrates a 2.1-fold up-regulation in E14.5 DTG hearts, compared to littermate STG controls. Statistical significance was determined by Student's t test, where * denotes p < 0.05.

βMHCre-mediated Tbx20 overexpression represses Tbx2, while promoting Tbx5, connexin 40 (Cx40) and connexin 43 (Cx43), expression in ventricular cardiomyocytes

Localized expression of Tbx2 and Tbx5 was examined in hearts with Tbx20 overexpression. In βMHCre;CC-Tbx20 DTG hearts, the expression of Tbx2 is reduced in the AVC myocardium at E14.5, compared to normal expression in the littermate control heart, as detected by ISH (Fig. 4A,B). In contrast, Tbx5 expression is induced throughout the septum (IVS) and in the ventricles in DTG hearts compared to localized expression in the littermate control heart at E18.5 (Fig. 4C–F). Consistent with previous reports (Hatcher et al., 2000), Tbx5 expression is detected at the epicardial surface of E18.5 STG hearts, and decreased epicardial expression of Tbx5 was noted in multiple E18.5 DTG hearts (Fig. 4E,F). Thus Tbx20 overexpression leads to decreased Tbx2 expression in the AVC and increased expression of Tbx5 throughout the developing ventricles, but not epicardium, of βMHCre;CC-Tbx20 DTG fetuses in vivo.

Gene expression of T-box downstream target genes Cx40 and Cx43, expressed in the cardiac conduction system (CCS), was examined in βMHCre;CC-Tbx20 DTG hearts at E18.5 (Christoffels et al., 2010). At this stage, Cx40 expression is normally restricted to the ventricular conduction tissues, and Cx43 is expressed in the trabecular and, to a lesser extent, the compact myocardium (Coppen et al., 2003). In βMHCre;CC-Tbx20 DTG hearts, the expression of both the Cx40 and Cx43 transcripts is increased throughout the full thickness of the ventricular walls, including the interventricular septum (Fig. 4G–N). In contrast, expression of Nppa, which is regulated by multiple Tbx factors including Tbx5 and Tbx2 (Habets et al., 2002), is apparently unaltered in the DTG trabecular myocardium, compared to a littermate
Gene expression changes detected by ISH were further confirmed by qRT-PCR (Fig. 4Q). Since altered expression of Connexin genes would be expected to affect the CCS, AV conduction system function was evaluated in adult hearts overexpressing Tbx20 by surface electrocardiography (ECG). However no significant changes in AV conduction system amplitudes or intervals were detected, at least at the baseline level, in Tbx20 DTG adult animals compared to littermate controls (data not shown). Thus Tbx20 overexpression in ventricular cardiomyocytes at fetal stages results in decreased expression of Tbx2 and increased expression of Tbx5, with widespread increased expression of Connexins (Cx40 and Cx43) beyond the CCS.

Nkx2.5Cre-mediated Tbx20 overexpression results in a smaller heart with decreased expression of Tbx5, but apparently normal expression levels of chamber maturation genes

Nkx2.5Cre;CC-Tbx20 DTG mice were generated in order to determine effects of increased Tbx20 function in embryonic cardiomyocytes during the early stages of heart chamber formation (Moses et al., 2001). Nkx2.5Cre;CC-Tbx20 DTG embryos exhibit growth retardation and obvious lethality by E10.5 (Table 1), therefore all the experimental analyses were performed at E9.5. Nkx2.5Cre-mediated overexpression of Tbx20 in embryonic cardiomyocytes results in a smaller heart size in E9.5 DTG embryos (black arrowheads in Fig. 5A,B), compared to littermate control embryos (white arrowheads in Fig. 5A,B). In heart tissue sections of the DTG and the littermate STG control embryos, cardiomyocyte differentiation is apparent by MF20 immunoreactivity (Fig. 5D,G), but the ventricles are thin with decreased trabeculation in Nkx2.5Cre;CC-Tbx20 embryos at E9.5 (Fig. 5C,F). In addition, endocardial cushion formation seems to be unaffected in E9.5 DTG hearts as evidenced by similar Twist1-antibody reactivity in cushion mesenchymal cells compared to littermate control hearts (Fig. 5E,H). Tbx20 overexpression in DTG embryos was confirmed by increased Tbx20 antibody reactive nuclei in the DTG ventricular myocardium, including LV, RV and OFT regions compared to a littermate control heart (Fig. 5J,L). Direct cell counts reveal that approximately 38.8% MF20-positive cardiomyocytes are also positive for Tbx20, compared to 16% Tbx20 and MF20 positive cardiomyocytes in littermate controls (Fig. 5M). Quantitative Western blot analysis reveals a 3.4-fold overexpression of Tbx20 protein in E9.5 DTG hearts, compared to littermate STG controls (Fig. 5N,O). Thus induced expression of Tbx20 in the primitive heart tube leads to reduced cardiac growth, which is in contrast to the increased cardiac growth observed in the ventricular myocardium of βMHCre;CC-Tbx20 DTG embryos at E14.5.

Mice lacking Tbx20 fail to activate chamber lineage maturation genes (Cai et al., 2005; Singh et al., 2005; Stennard et al., 2005). Therefore expression of indicators of chamber differentiation was

Fig. 4. βMHCre-mediated Tbx20 overexpression represses Tbx2, while inducing expression of Tbx5, connexin40, and connexin43 in differentiated cardiomyocytes. (A–F) Expression of Tbx2 and Tbx5 transcripts was evaluated in the βMHCre;CC-Tbx20 (DTG) myocardium overexpressing Tbx20 by ISH. In E14.5 DTG hearts, the expression of Tbx2 is reduced along the AV canal myocardium (B, arrow) compared to a littermate STG control (A, arrow). In E18.5 DTG hearts, Tbx5 expression is increased throughout the interventricular septum (IVS) and in the ventricles (D, F, arrows) compared to restricted expression in the littermate control heart (C, E, arrows). Expression of Cx40 (H, J) and Cx43 (L, N) is increased in the DTG myocardium (arrows) compared to expression in STG littermate controls (G, I and K, M). In contrast, Nppa expression is unchanged in the DTG trabecular myocardium compared to the littermate control heart (compare O and P, arrows). (Q) Observed differences in gene expression were further validated by qRT-PCR in DTG hearts relative to STG controls (n = 3). Statistical significance was determined by Student’s t test, where * denotes p < 0.05.
assessed in the Nkx2.5Cre;CC-Tbx20 DTG myocardium by ISH. The expression of Tbx2 is decreased in the E9.5 AVC myocardium overexpressing Tbx20, compared to somite-matched STG embryos (white arrowheads in A and B), displayed in both left (A) and right (B) lateral views. Histological (H&E) and MF20 antibody staining also reveal the smaller heart size in Nkx2.5Cre;CC-Tbx20 (DTG) embryos (compare C versus F and D versus G). The generation of mesenchymal cells and expression of Twist1, indicative of EMT, is apparently normal in the DTG endocardial cushion (arrowhead in H) compared to littermate STG control (arrowhead in E). Tbx20-specific antibody staining reveals Tbx20 overexpression as indicated by positive green nuclei (arrowheads in I), throughout LV, RV and OFT regions of the DTG myocardium compared to the littermate control (arrowheads in J), as detected by immunofluorescence and confocal imaging. (M) Direct cell counts reveal that 38.8% of MF20 positive cardiomyocytes are also positive for Tbx20 in DTG ventricular chamber myocardium compared to 16% in littermate controls. (N, O) Quantitative Western blot analysis reveals a 3.4-fold increase of Tbx20 protein expression in E9.5 DTG hearts, compared to littermate STG controls (n = 6–8). Statistical significance is determined by Student’s t test, where * denotes p < 0.05.
Nkx2.5Cre-mediated overexpression of Tbx20 in embryonic cardiomyocytes results in decreased $Tbx5$ expression, but does not affect expression of markers of chamber myocardial maturation.

Nkx2.5Cre-mediated Tbx20 overexpression leads to decreased proliferation with induction of p21CIP1 in cardiomyocytes

Cardiomyocyte proliferation and expression of cell cycle regulatory genes were examined in Nkx2.5Cre;CC-Tbx20 DTG hearts. Co-labeling of pH3 and MCM20 demonstrates reduced cardiomyocyte proliferation in E9.5 DTG embryos, compared to littermate STG controls (Fig. 7A,B). Quantitation of these results demonstrates that 18.5% of MCM20 positive cardiomyocyte nuclei are also positive for pH3 in DTG myocardium, compared to 36.6% MCM20 and pH3 positive nuclei in STG controls (Fig. 7C). In contrast, the percent of nuclei with pH3 immunoreactivity in the endocardial endothelial cell layer is unchanged in DTG versus STG hearts (data not shown). Consistent with reduced cardiomyocyte proliferation, expression of the cyclin dependent kinase (CDK) inhibitor p21CIP1 is increased in DTG myocardium overexpressing Tbx20, compared to the littermate control myocardium as detected by immunostaining and qRT-PCR (Fig. 7D–F). Expression of additional cell cycle regulatory genes that promote proliferation, including cyclin A2, cyclin D1, and cyclin E1, is also reduced in Nkx2.5Cre;CC-Tbx20 as detected by qRT-PCR. However mRNA expression of another CDK inhibitor (p27KIP1) remains unchanged in E9.5 DTG hearts compared to controls (Fig. 7I). In contrast to βMHCcre;CC-Tbx20 DTG ventricular myocardium as detected by ISH and qRT-PCR (Fig. 7G–I), consistent with reduced cardiomyocyte proliferation in these embryos.

Tbx20 overexpression inhibits activation of ERK1/2 MAPK in Nkx2.5Cre;CC-Tbx20 embryonic cardiomyocytes in vivo

In order to further characterize the differential effects of Tbx20 on embryonic versus fetal cardiomyocyte proliferation, pERK1/2 expression was evaluated in E9.5 Nkx2.5Cre;CC-Tbx20 DTG hearts compared to littermate controls by antibody staining (A–D) and Western blot (E–F) analyses. At E9.5, dpERK is normally active in the myocardium and has been implicated as a downstream mediator of Neuregulin signaling (Lai et al., 2010). In Nkx2.5Cre;CC-Tbx20 DTG hearts, dpERK expression is reduced throughout the ventricular chamber myocardium, compared to control hearts (Fig. 8A–D). Quantitation of these results by Western blot analysis demonstrates that the ratio of dpERK/total ERK expression is reduced by 42% in Nkx2.5Cre;CC-Tbx20 hearts, relative to control hearts, and that total ERK protein levels are unchanged (Fig. 8E,F). Together, these data indicate that ERK activation is decreased with Tbx20 overexpression at E9.5. However, the expression of Tgfβ, an mRNA predominant in endothelial lineage, is apparently unaffected by Tbx20 overexpression as detected by qRT-PCR and IHC (data not shown). In contrast to the decreased expression in Nkx2.5Cre;CC-Tbx20 DTG hearts, dpERK activation is not decreased in βMHCcre;CC-Tbx20 ventricular cardiomyocytes, compared to littermate STG controls, consistent with the observed...
stage-specific effects on cell proliferation (Supplemental Fig. S2E–H). Together, these data demonstrate that increased Tbx20 protein levels inhibit dpERK activation in embryonic (E9.5), but not fetal (17.5), cardiomyocytes in vivo.

**Discussion**

Here we demonstrate that Tbx20 overexpression in the primitive heart results in decreased cardiomyocyte cell proliferation with decreased N-myc1 expression at E9.5. Previous studies reported that mice with loss of Tbx20 also exhibit reduced cardiomyocyte proliferation and decreased expression of N-myc1 at E10.5 (Cai et al., 2005; Singh et al., 2005; Stennard et al., 2005). Thus, increased or decreased Tbx20 expression similarly affects cardiomyocyte proliferation and N-myc1 gene expression during the initial stages of heart chamber maturation. Nkx2.5Cre-mediated overexpression of Tbx20 also results in reduced expression of multiple cyclin genes (A2, D1 and E1), as well as increased expression of the cell cycle inhibitor p21CIP1, consistent with the observed reduction in cardiomyocyte proliferation. Neuregulin signaling has been implicated in chamber-specific gene regulation and in ventricular trabeculation through dpERK activation (Lai et al., 2010; Stennard et al., 2005; Woldeyesus et al., 1999). Similar to loss of Nrg1 function, Tbx20 overexpression leads to decreased dpERK expression and decreased cell proliferation. However, unlike embryos with Nrg1 or Tbx20 loss-of-function, markers of chamber myocardial maturation are normally induced with Tbx20.
indicative of stage-specific cardiomyocytes that are distinct in their responses to Tbx20 overexpression, which may be due to their increased expression of Tbx20 in fetal ventricular cardiomyocytes (Brown et al., 2005; Hatcher et al., 2001). Together, these data provide evidence for a feedforward regulatory mechanism of Tbx20 and Bmp10 in endocardial cushion cells supporting a similar feedforward mechanism in endocardial cushion development (Cai et al., 2011; Shelton and Yutzey, 2007). Additional feedback regulatory mechanisms in BMP/Smad signaling occur at the protein level since Tbx20 interacts with Smad1/5 proteins to attenuate their transcriptional regulatory activity (Singh et al., 2009). Together, these studies demonstrate that Tbx20 interacts with BMP signaling in multiple ways to regulate diverse aspects of heart formation, including myocardial cell proliferation and endocardial cushion development.

Several T-box transcription factors, including Tbx2, Tbx3, and Tbx5, coordinate regulatory networks in the CCS, and Nkx2.5 is a direct downstream target gene of Tbx5 (Aanhaanen et al., 2011; Bakker et al., 2008; Bruneau et al., 2001; Moskowitz et al., 2004). However, Tbx20 function in CCS development has not previously been reported. Tbx20 overexpression in fetal ventricular cardiomyocytes leads to increased expression of Tbx5, Cx40, and Cx43 throughout the ventricular myocardium. Since Cx40 and Cx43 are regulated by multiple T-box transcription factors, it is likely that increased expression of Tbx20 disrupts the balance of repressive and activating T-box factors on these gene regulatory elements leading to their increased expression in the ventricular myocardium. However, the dysregulation of connexin gene expression is apparently insufficient to cause cardiac arrhythmias in adult animals (data not shown). It is striking to note that expression of Nppa, which is responsive to Tbx5, Tbx2, and Tbx20, is apparently unaffected by Tbx20 overexpression in fetal ventricular cardiomyocytes (Habets et al., 2002; Plageman and Yutzey, 2004; Sennard and Harvey, 2005). Thus, T-box regulation of cardiac gene expression is more sensitive to increased Tbx20 than is the expression of chamber myocardium genes, such as Nppa, in the fetal myocardium. Together, these results demonstrate that ventricular cardiomyocytes with increased Tbx20 expression exhibit enhanced molecular features of the CCS, including increased expression of Tbx5, Cx40, and Cx43.

In human patients, Tbx20 gain- and loss-of-function mutations are associated with a wide spectrum of cardiovascular abnormalities, including cardiomyopathies and congenital valvuloseptal defects (Kirk et al., 2007; Posch et al., 2010). Defects in prenatal heart chamber growth can occur in individuals with a Tbx20 loss-of-function mutation, consistent with the importance of Tbx20 function in cardiac growth during development (Kirk et al., 2007). Gain- and loss-of-function mutations in Tbx20 also are associated with dilated cardiomyopathy in adult and pediatric patients (Kirk et al., 2007; Posch et al., 2010). Arrhythmias and sudden death have been reported in patients with a Tbx20 mutation, and conditional loss of Tbx20 in adult mice results in severe cardiomyopathy associated with arrhythmias and death (Qian et al., 2008; Shen et al., 2011). Here, we provide evidence that increased levels of Tbx20 can affect cardiac conduction system gene expression during development, further supporting Tbx20 as a candidate gene in hereditary conduction system anomalies. The combination of human genetic analyses and studies in animal model systems has linked Tbx20 function to congenital cardiac malformations, conduction system overexpression. Thus, the level of Tbx20 expression is a critical determinant in cardiomyocyte cell proliferation and lineage maturation in embryonic cardiomyocytes.

In contrast to embryonic cardiomyocytes, βMHCCre-mediated Tbx20 overexpression in fetal ventricular cardiomyocytes (E12.5-17.5) promotes cell proliferation. Tbx20 overexpression at fetal stages leads to increased expression of N-myc1, Bmp10, pSmad1/5/8, and Tbx5, all of which promote cardiomyocyte cell proliferation (Brown et al., 2005; Cai et al., 2005; Chen et al., 2004; Hatcher et al., 2001). N-myc1 has been identified as a direct downstream target of Tbx20, and increased Tbx20 binding to T-box responsive elements in the N-myc1 promoter region is observed in fetal cardiomyocytes, supporting a direct transcriptional activation mechanism (Cai et al., 2005). However, N-myc1 gene expression is not induced in embryonic cardiomyocytes with Tbx20 overexpression, providing evidence for a more complex regulatory mechanism. Likewise Bmp10 expression and pSmad1/5/8 activation, required for fetal cardiomyocyte cell proliferation (Chen et al., 2004, 2006), are increased in response to Tbx20 overexpression at fetal, but not embryonic, stages. Tbx5 also promotes cell proliferation in cardiomyocytes, and the observed increase in Tbx5 expression with Tbx20 overexpression at E14.5, but not E9.5, could contribute to the stage-specific increase in cell proliferation of fetal cardiomyocytes (Brown et al., 2005; Hatcher et al., 2001). Together, these analyses demonstrate that embryonic and fetal cardiomyocytes are distinct in their responses to Tbx20 overexpression, which may be indicative of stage-specific differential regulation of cofactors or intersecting signaling pathways. However, further studies are necessary to fully characterize these complex regulatory mechanisms.

Tbx20 has been implicated as both an upstream regulator and downstream target of BMP signaling. Here, we demonstrate that Tbx20 overexpression in fetal cardiomyocytes leads to increased Bmp10 expression and pSmad1/5/8 activation. In addition, Bmp10 is necessary and sufficient to activate Tbx20 expression in the fetal myocardium, and a conserved Smad binding site mediates Bmp10 induction of Tbx20 proximal promoter sequences in cell culture studies (Zhang et al., 2011). Together, these data provide evidence for a feedforward regulatory mechanism of Tbx20 and Bmp10 induction of Tbx20 specifically from the atrioventricular canal (AVC) myocardium results in downregulation of Bmp2, and Bmp2 induces Tbx20 expression in endocardial cushion cells supporting a similar feedforward mechanism in endocardial cushion development (Cai et al., 2011; Shelton and Yutzey, 2007). Additional feedback regulatory mechanisms in BMP/Smad signaling occur at the protein level since Tbx20 interacts with Smad1/5 proteins to attenuate their transcriptional regulatory activity (Singh et al., 2009). Together, these studies demonstrate that Tbx20 interacts with BMP signaling in multiple ways to regulate diverse aspects of heart formation, including myocardial cell proliferation and endocardial cushion development.

### Fig. 8. Nkx2.5Cre-mediated overexpression of Tbx20 results in decreased dpERK expression in embryonic cardiomyocytes.

(A–D) Expression of the diphosphorylated form of ERK1/2 (dpERK) in E9.5 chamber myocardium is detected by dpERK-specific antibody reactivity visualized by DAB staining. Panels C and D represent higher magnified views (40× objective) of the LV region from panels A and B (20× objective), respectively. Reduced dpERK reactivity (brown/black nuclei) is apparent in the ventricular cardiomyocytes (arrowheads in D) of a DTG embryo compared to the STG littermate control (arrowheads in C). (E–F) dpERK expression was quantified by Western blot analysis as the ratio of dpERK to total ERK protein in E9.5 DTG hearts compared to littermate STG controls (n=6–8). Statistical significance is determined by Student’s t test, where * denotes p<0.05.
anomalies, and adult cardiomyopathy. Therefore manipulation of Tbx20 function or intersecting regulatory pathways could be exploited therapeutically. However these studies should proceed with caution because increased or decreased Tbx20 function in cardiomyocytes can lead to pleiotropic downstream effects on cell proliferation, lineage matura-

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Appendix A Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2011.12.034.

References


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