Journal of Molecular and Cellular Cardiology 97 (2016) 140–149

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Misexpression of *Tbx18* in cardiac chambers of fetal mice interferes with chamber-specific developmental programs but does not induce a pacemaker-like gene signature

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ARTICLE INFO

Article history:
Received 9 March 2016
Received in revised form 13 April 2016
Accepted 9 May 2016
Available online 11 May 2016

Keywords:
Sinoatrial node
Pacemaker
Working myocardium
Tbx18
Tbx3
Microarray
Conditional misexpression

ABSTRACT

Initiation of cardiac excitation depends on a specialized group of cardiomyocytes at the venous pole of the heart, the sinoatrial node (SAN). The T-box transcription factor gene *Tbx18* is expressed in the SAN myocardium and is required for formation of a large portion of the pacemaker. Previous studies suggested that *Tbx18* is also sufficient to reprogram ventricular cardiomyocytes into SAN cells in rat, guinea-pig and pig hearts. To evaluate the consequences of misexpression of *Tbx18* for imposing a nodal phenotype onto chamber myocardial cells in fetal mice, we used two independent conditional approaches with chamber-specific *cre* driver lines and an $Hprt^{Tbx18}$ misexpression allele. *Myh6-Cre*/+;$Hprt^{Tbx18/y}$ mice developed dilated atria with thickened walls, reduced right ventricles and septal defects that resulted in reduced embryonic and post-natal survival. *Tagln-Cre*/+;$Hprt^{Tbx18/y}$ mice exhibited slightly smaller hearts with rounded trabeculae that supported normal embryonic survival. Molecular analyses showed that the SAN gap junction and ion channel profile was not ectopically induced in chamber myocardium but the working myocardial gene program was partially inhibited in atria and ventricles of both misexpression models. Left atrial expression of *Pitx2* was strongly repressed in *Myh6-Cre*/+;$Hprt^{Tbx18/y}$ embryos. We conclude that exclusion of *Tbx18* expression from the developing atria and (right) ventricle is important to achieve normal cardiac left-right patterning and myocardial differentiation, and that Tbx18 is not sufficient to induce full SAN differentiation of chamber cardiomyocytes in fetal mice.

1. Introduction

The sequential and rhythmic contraction of the cardiac chambers is initiated and coordinated by electric signals from the cardiac conduction system [1]. Primary and dominant impulse generator is the sinoatrial node (SAN), a small group of cells located at the entry of the right superior caval vein into the right atrium. Although cells of the SAN and the other components of the cardiac conduction system are muscle cells, they differ from the surrounding chamber or working myocardium in many parameters. First, they show low contractibility due to reduced expression of contractile proteins [2]. Second, they exhibit poor electrical coupling due to presence of slow-propagating connexins, Cx30.2 (Gjd3) and Cx45 (Gjc1), whilst Cx40 (Gja5) and Cx43 (Gja1), which are essential for fast propagation of the electrical impulse in the working myocardium, are absent [3,4]. Third and most importantly, they express a unique profile of ion channels including hyperpolarization-activated cyclic nucleotide gated (Hcn) channels (strongly Hcn4, more weakly Hcn1 and Hcn2), the L-type Ca^{2+}-channel Cav1.3 (Cacna1d), and the T-type Ca^{2+}-channels Cav3.1 (Cacna1g) and Cav3.2 (Cacna1h) and

Abbreviations: ASD, atrial septal defects; AP, action potential; AVN, atrioventricular node; ChIP-Seq, chromatin immunoprecipitation-sequencing; Cx, Connexin; DAPI, 4,6-diamidino-2-phenylindol; E, embryonic day; FC, fold change; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; Gjc, gap junction protein, gamma; Gjd, gap junction protein, delta; IRES, internal ribosomal entry site; Hcn, hyperpolarization-activated cyclic nucleotide gated channel; Hprt, hypoxanthine guanine phosphoribosyl transferase; Kcnd, potassium voltage-gated channel Shal-related family; Kcnj, potassium inwardly-rectifying channel subfamily J; Lbh, limb-bud and heart; Mlc2a (also known as Myh7), myosin, heavy polypeptide 7 cardiac muscle beta; Mlc2a (also known as Myl2), myosin, light polypeptide 2 regulatory cardiac slo; Nppa, natriuretic peptide type A; P, postnatal day; PCR, polymerase chain reaction; Pitx2, paired-like homeodomain transcription factor 2; Postn, periostin; SAN, sinoatrial node; Scn5a, sodium channel voltage-gated typeV alpha; Shox2, short stature homeobox 2; Tagln, transgelin; T-box, Tbx; Tnni3, troponin I cardiac 3; VSD, ventricular septal defect; Wm, Working myocardium.

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http://dx.doi.org/10.1016/j.yjmcc.2016.05.004
0022-2828/

others that together allow the cyclical generation of action potentials (APs). Pacemaker cells lack expression of *Scn5a* that encodes the voltage-gated sodium channel Nav1.5 responsible for fast AP upstroke in working myocardium [5,6].

Genetic or acquired dysfunction of the pacemaker tissues results in severe arrhythmias. So far, patients are treated by implantation of artificial pacemakers that are occasionally afflicted with infection or damage. Furthermore, size-mismatch develops in children. Biological pacemaker tissues may overcome these shortcomings and may even allow autonomous regulation of contraction rates. Efforts of generating biological pacemaker tissues have initially aimed to evoke automaticity by targeted expression of SAN ion channel genes such as *Hcn4* that generate the pacemaker current [7,8]. Since such an approach does not account for the complexity of ionic currents required for pacemaker activity, efforts have been undertaken to use transcriptional regulators of SAN differentiation, namely the T-box transcription factors Tbx3 and Tbx18, to reprogram working into pacemaker-like cardiomyocytes (for reviews see [9,10]).

Tbx3 encodes an evolutionary conserved transcriptional repressor that is specifically expressed in all components of the conduction system except the Purkinje fibres in the developing and mature heart [11]. Loss-of-function studies have shown that *Tbx3* is required to suppress differentiation into working myocardium of nodal tissue, allowing these cells to acquire the pacemaker phenotype [12]. Ectopic expression of Tbx3 in embryonic atrial myocytes *in vivo* resulted in suppression of *Gja5* and *Gja1*, induction of *Hcn4* expression, and the formation of ectopic pacemaker sites within the atrial myocardium [12]. However, *Tbx3* failed to completely reprogram terminally differentiated chamber cardiomyocytes into pacemaker cells since expression of *Hcn4* was not induced in adult atria or in neonatal rat ventricular myocytes after overexpression of *Tbx3* [13]. This suggests that fetal atrial cardiomyocytes have additional epigenetic and/or genetic features that allow re-specification into nodal cells.

Tbx18 is expressed in the sinus horns and the SAN during cardiac development [14]. *Tbx18*-null mice display severe hypoplasia of the SAN "head" whereas the SAN "tail" region develops normally [15]. More recently, it was shown that Tbx18 is sufficient to repress *Gja1*, and to induce pacemaker-like morphological and electrophysiological features in neonatal rat ventricular myocytes, in guinea pig hearts, and in a porcine model of heart block arguing for a possible therapeutic application of Tbx18 for reprogramming cardiomyocytes into pacemaker-(like) cells *in vivo* [16–18].

Here, we investigate the functional consequences of misexpression of *Tbx18* in the chamber myocardium of fetal mouse hearts in order to evaluate the potential of Tbx18 in SAN development and reprogramming of working myocardial cells. We show that *Tbx18* represses the patterning gene *Pitx2* and a set of working myocardial genes but is not sufficient to induce an SAN gene signature in chamber myocardium.

2. Methods

2.1. Mice and genotyping

For conditional misexpression of *Tbx18*, we used an *Hprt* allele with knock-in of a *Tbx18-IRES-GFP* expression cassette ($Hprt^{tm3(CAG\text{-}Tbx18,\text{-}Venus)Aki}$, synonym: $Hprt^{Tbx18}$) [19]. Transgenic mice expressing the *Cre* recombinase under the control of the *Myh6* promotor (*Tg*(*Myh6-Cre*)*2182Mds*, synonym: *Myh6-Cre*) or the *Tagln* promotor (*Tg*(*Tagln-Cre*)*1Her*, synonym: *Tagln-Cre*) were used as *Cre* driver lines for myocardial recombination [20,21]. As a reporter line we used $Gt(ROSA)26Sor^{tm4(ACTB\text{-}tdTomato\text{-}EGFP)Luo}$ (synonym: $R26^{mTmG}$) [22]. All mice were maintained on an outbred (NMRI) background. For timed pregnancies, vaginal plugs were checked on the morning after mating and noon of this day was defined as embryonic day (E) 0.5. Female mice were sacrificed by cervical dislocation, uteri were harvested in PBS and embryos decapitated. Tissues were fixed in 4% paraformaldehyde overnight and stored in 100% methanol at −20 °C. Genomic DNA prepared from yolk sacs or tail biopsies was used for genotyping by PCR.

All animal work conducted for this study was performed according to European and German legislation. The breeding of mice lines was approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (Permit Number: AZ33.12-42502-04-13/1356).

2.2. Histological analysis

Transverse sections of embryos or frontal sections of neonatal hearts were paraffin-embedded and sectioned to 5 μm. Hematoxylin and eosin staining was performed according to standard procedures.

2.3. In situ hybridization analysis

Non-radioactive *in situ* hybridization analysis with digoxigenin-labeled antisense riboprobes was performed as described [23]. At least three embryos of each genotype were used for each analysis.

2.4. RNA isolation and reverse transcription (RT)-PCR

Total RNA was extracted from left and right atria of E18.5 wild-type and *Myh6-Cre*/+;$Hprt^{Tbx18/y}$ embryos (ten per pool) or right ventricles of E14.5 wild-type and *Tagln-Cre*/+;$Hprt^{Tbx18/y}$ embryos (ten per pool) using the RNeasy Microarray Tissue Mini Kit (Qiagen, 73304). For RT-PCR experiments RNA was treated with DNaseI (Roche, #776785) and transcribed with oligo-$(dT)_{18}$ primer using the RevertAid TM Reverse Transcriptase (Fermentas, #EP0442). *Gja5*, *Gja1* transcripts were amplified with intron-spanning primers in semi-quantitative RT-PCR. Primers for the amplification of *Hcn1*, *Hcn4*, *Kcnj2*, *Kcnj3* and *Scn5a* transcripts were previously published [13]. For quantification, lane intensity was measured with ImageJ and normalized to *Gapdh* expression [24]. Wildtype expression levels were set to one, and the relative expression changes plotted as column. The error bars indicate the standard deviation between two experiments.

For microarray analysis, total RNA samples of male *Myh6-Cre*/+;$Hprt^{Tbx18/y}$ and control embryos (two pools each) were sent to the Agilent technology CSP ImaGenes (ImaGenes GmbH, Berlin) where RNA was Cy3-labeled and hybridized to Agilent SurePrintG3 Mouse GE 8x60K Microarrays. Total RNA of *Tagln-Cre*/+;$Hprt^{Tbx18/y}$ and control right ventricles was sent to the Research Core Unit Transcriptomics of Hannover Medical School where RNA was Cy3-labeled and hybridized to Agilent Whole Mouse Genome Oligo v2 (4x44K) Microarrays.

2.5. Microarray experiments and data analysis

To identify differentially expressed genes in microarrays, normalized expression data was filtered using Excel. Functional enrichment analysis was performed using DAVID and ToppFun software, and terms were selected based on *p*-value ($p < 0.05$) [25,26]. Heatmaps were generated using EXPANDER [27]. *p*-Values for the overlap of different gene sets were calculated using Fisher's exact test.

Tbx3 ChIP-seq data was analyzed with the peak-calling software OccuPeak [28,29]. Evolutionary conserved binding sites for T-box proteins (T-sites) were identified using oPOSSUM-3 [30]. Intersections of Tbx3 peaks and conserved regions were identified with Galaxy [31].

2.6. Immunofluorescent detection of proteins

For immunofluorescence analysis on 5 μm paraffin sections monoclonal rat-anti-Hcn4 (1:300, ab32675, Abcam) monoclonal mouse anti-GFP (1:200, Roche), polyclonal rabbit anti-Postn (1:300, ab14041, Abcam), polyclonal rabbit anti-Wt1 (1:200, CA1026-50UL, Calbiochem), polyclonal rabbit anti-GFP (1:200; sc-8334, Santa Cruz) or polyclonal goat anti-Tnni3 (1:300, 4 T21/2, HyTest) were used as primary