Congenital Heart Block Maternal Sera Autoantibodies Target an Extracellular Epitope on the $\alpha_{1G}$ T-Type Calcium Channel in Human Fetal Hearts

Linn S. Strandberg$^{1,9}$, Xuezhi Cui$^{1,9}$, Arianna Rath$^{2}$, Jie Liu$^{3}$, Earl D. Silverman$^{1}$, Xiaoru Liu$^{1}$, Vinayakumar Siragam$^{1}$, Cameron Ackerley$^{1}$, Brenda Bin Su$^{1}$, Jane Yuqing Yan$^{4}$, Marco Capecchi$^{4}$, Luca Biavati$^{4}$, Alice Accorroni$^{4}$, William Yuen$^{1}$, Filippo Quattrone$^{4}$, Kalvin Lung$^{1}$, Edgar T. Jaeggi$^{1}$, Peter H. Backx$^{3,5}$, Charles M. Deber$^{2,6}$, Robert M. Hamilton$^{1*}$

1 Department of Physiology and Experimental Medicine, The Hospital for Sick Children, Toronto, Ontario, Canada, 2 Division of Molecular Structure and Function, The Hospital for Sick Children, Toronto, Ontario, Canada, 3 Departments of Physiology and Medicine, University of Toronto, Toronto, Ontario, Canada, 4 Scuola Superiore Sant’Anna, Pisa, Italy, 5 Division of Cardiology, University Health Network, Toronto, Ontario, Canada, 6 Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada

Abstract

Background: Congenital heart block (CHB) is a transplacentally acquired autoimmune disease associated with anti-Ro/SSA and anti-La/SSB maternal autoantibodies and is characterized primarily by atrioventricular (AV) block of the fetal heart. This study aims to investigate whether the T-type calcium channel subunit $\alpha_{1G}$ may be a fetal target of maternal sera autoantibodies in CHB.

Methodology/Principal Findings: We demonstrate differential mRNA expression of the T-type calcium channel CACNA1G ($\alpha_{1G}$ gene) in the AV junction of human fetal hearts compared to the apex (18–22.6 weeks gestation). Using human fetal hearts (20–22 wks gestation), our immunoprecipitation (IP), Western blot analysis and immunofluorescence (IF) staining results, taken together, demonstrate accessibility of the $\alpha_{1G}$ epitope on the surfaces of cardiomyocytes as well as reactivity of maternal serum from CHB affected pregnancies to the $\alpha_{1G}$ protein. By ELISA we demonstrated maternal sera reactivity to $\alpha_{1G}$ was significantly higher in CHB maternal sera compared to controls, and reactivity was epitope mapped to a peptide designated as p305 (corresponding to aa305–319 of the extracellular loop linking transmembrane segments S5–S6 in $\alpha_{1G}$ repeat I). Maternal sera from CHB affected pregnancies also reacted more weakly to the homologous region (7/15 amino acids conserved) of the $\alpha_{1H}$ channel. Electrophysiology experiments with single-cell patch-clamp also demonstrated effects of CHB maternal sera on T-type current in mouse sinoatrial node (SAN) cells.

Conclusions/Significance: Taken together, these results indicate that CHB maternal sera antibodies readily target an extracellular epitope of $\alpha_{1G}$ T-type calcium channels in human fetal cardiomyocytes. CHB maternal sera also show reactivity for $\alpha_{1H}$, suggesting that autoantibodies can target multiple fetal targets.

Citation: Strandberg LS, Cui X, Rath A, Liu J, Silverman ED, et al. (2013) Congenital Heart Block Maternal Sera Autoantibodies Target an Extracellular Epitope on the $\alpha_{1G}$ T-Type Calcium Channel in Human Fetal Hearts. PLoS ONE 8(9): e72668. doi:10.1371/journal.pone.0072668

Editor: Thomas Berger, Medical University Innsbruck, Austria

Received April 4, 2012; Accepted July 17, 2013; Published September 9, 2013

Copyright: © 2013 Strandberg et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by CIHR Operating Grant 225107 and 160797 to RM Hamilton. Portions of the work were performed by a postdoctoral fellow, supported by the Swedish Research Council. Additionally, this research was supported by a Gina M. Finzi Memorial Student Summer Fellowship to William Yuen from the Lupus Foundation of America, Inc. This work was also supported, in part, by a grant to CM Deber from the Canadian Institutes of Health Research (CIHR FRN-5810), and by a generous donation from the Caitlin Morris Memorial Fund of the Hospital for Sick Children Foundation to RM Hamilton. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: Robert.hamilton@sickkids.ca

$^9$ These authors contributed equally to this manuscript.

Introduction

Congenital heart block (CHB) is a passively acquired autoimmune disease that occurs in pregnancies of rheumatic mothers, but also in healthy mothers, and has been associated with maternal anti-Ro/SSA and anti-La/SSB antibodies. The disease is characterized by atrioventricular (AV) block, which can be detected in the developing fetus between 16–25 weeks gestation [1,2]. In the absence of congenital structural abnormalities in the offspring, maternal autoantibodies are usually present, and it is generally accepted that maternal antibodies cross the placenta and induce fetal injury in the AV node. More generalized effects on the heart, associated with anti-Ro/SSA and anti-La/SSB, have also been suggested in the past decade such as sinus bradycardia, myocardial inflammation, QTc prolongation, endocardial fibroelastosis and dilated cardiomyopathy [3–9]. In most studies, untreated autoimmune CHB has been associated with high fetal/neonatal mortality rates (14%–34%) [4,10–16].
Understanding the pathology of CHB, and predicting outcome in pregnancies, have been complicated by low incidence and recurrence rates. In a population of women with anti-Ro/SSA and anti-La/SSB autoantibodies, the incidence of CHB is approximately 1–2% [17], yet the recurrence rate in these mothers is approximately 10% [1,10,19], despite persisting antibodies [20], indicating that additional factor(s) contribute to the fetal susceptibility of CHB.

A link between maternal anti-Ro52 antibodies and CHB is supported by a number of studies [20–31], whereas maternal seroreactivity to the La protein tends to be associated with dermatologic neonatal lupus erythematosus (NLE) [32]. Nevertheless, both anti-Ro60 and anti-La autoantibodies have been suggested to amplify the immune insult occurring in fetal hearts after development of CHB [33].

CHB pathology reports have shown that the disease is associated with deposition of IgG and with the presence of inflammatory cells in the AV node of fetal hearts, as well as AV node fibrosis and calcification [34–36]. Effects on the sinoatrial (SA) node of the fetal heart have also been reported, with sinus bradycardia demonstrated in patients [3,37,38], as well as in vitro [39] and in vivo animal models [40,41]. Mothers are not affected by AV block, which could be due to developmental expression of the target in the AV node, or unique vulnerability of the fetal heart.

Maternal-fetal antibody transfer as the event precipitating CHB was initially proposed in 1977 [42]. A single protein target for these antibodies, however, has not emerged. Instead, maternal sera reactivity to several proteins has been demonstrated in previous studies, including the serotoninergic 5-HT4 receptor [43,44], and two voltage-dependent L-type calcium channel subunits: α1C/Cav1.2 (α1C), and α1D/Cav1.3 (α1D) [39,45,46]. Sera from mothers with CHB affected pregnancies (CHB+ sera) were shown by patch clamp to affect currents mediated by recombinantly expressed α1G in Xenopus oocytes [47], and by α1G in a transformed embryonal human kidney cell line (sa201) [48]. Contribution of L-type channels to CHB was also demonstrated in a mouse model where α1G transgenic and α1G knock out mice were immunized with Ro52, Ro60 and La [19], but these channels did not account for all of the effects of CHB. Results from the Xenopus system also indicated that currents through the recombinant voltage-dependent T-type calcium channel subunit α1H/Cav3.2 (α1H) were decreased in the presence of CHB+ serum [47]. Since multiple targets of CHB maternal autoantibodies have been identified, and the spectrum of symptoms in affected offspring are not limited to AV block alone, it remains possible that the specific pathogenic autoantibody in anti-Ro/La pregnancies recognizes an epitope that is at least partially common to several ion channels and receptors.

The voltage-dependent T-type calcium channel subunit α1G/Cav3.1 (α1G) is a developmentally regulated channel that is thought to participate along with α1H/Cav3.1 (α1H) regulating cardiac conduction through the AV-node [50,51]. It is known that α1G is highly expressed in the compact node (CN) of the AV axis in human hearts [52], and homozynous transgenic mice lacking α1G exhibit first-degree AV block and bradycardia [53] – a phenotype consistent with extant reproducible rodent models of CHB [29,54–56].

The voltage-dependent T-type calcium channel subunit α1G/Cav3.1 (α1G) is a developmentally regulated channel that is thought to participate along with α1H/Cav3.1 (α1H) regulating cardiac conduction through the AV-node [50,51]. It is known that α1G is highly expressed in the compact node (CN) of the AV axis in human hearts [52], and homozynous transgenic mice lacking α1G exhibit first-degree AV block and bradycardia [53] – a phenotype consistent with extant reproducible rodent models of CHB [29,54–56].

The suggestion of a role for α1G in cardiac development, and the comparable electrophysiology of the α1G knock out and CHB mouse models, led us to hypothesize that α1G might represent an additional cross-reactive target of maternal serum antibodies in CHB. In the present work, we found that α1G (mRNA and protein) is expressed in human fetal hearts and that CHB-affected maternal sera contains antibodies reacting to the α1G protein. Accessibility of the epitope by serum antibodies is further demonstrated with confocal microscopy and α1G affinity-purified serum. Antibody reactivity of maternal sera maps to peptide sequences found in the extracellular S5–S6 portion of repeat I of α1G (aa305–319), and a similar sequence is found in the α1H T-type channel subunit, but not in the L-type channel subunits α1C or α1D. Single-cell patch-clamp studies demonstrated that CHB serum irreversibly decreases T-type calcium channel current in mouse SAN cells. In summary, our results support the conclusion that maternal autoantibodies in CHB pregnancies bind to the T-type calcium channel α1G, and can decrease T-type currents. These antibodies may also bind to the α1H channel, but binding to the native protein remains to be verified in human fetal hearts. These results provide further support for the contention that more than one target may be involved in the complex pathology of the disorder.

Results

Ion channel blockers demonstrate preferential inhibition of newborn heart AV conduction likely conferred by α1G.

Since CHB affects the fetal heart, but not the maternal heart, we wanted to identify ion channels that may be preferentially affected by autoantibodies in fetal hearts. To accomplish this objective we applied selective calcium channel blockers and pacemaker current (I\textsubscript{f}) blockers to newborn and adult rabbit Langendorff hearts and measured Wenckebach cycle length (WBCL) by examining the effects of decremental atrial pacing on the P-R interval and AV-conduction block. We have used this method previously to demonstrate that WBCL prolongation occurs with perfusion of sera from mothers of children with CHB, but not in the presence of sera from mothers of unaffected children [57]. We have also used this method previously to establish a greater sensitivity to AV block in newborn versus adult rabbit Langendorff hearts upon perfusion of sera from mothers of children with CHB (RM Hamilton, unpublished observations). Once baseline WBCL was established, hearts were perfused with either L-type calcium channel blockers (Diltiazem or Verapamil), the pacemaker current (I\textsubscript{f}) blocker (ZD7288), or, a relatively selective T-type calcium channel blocker (Mibebradil) and 15 minutes after equilibration, WBCL was recorded every five minutes for 15 minutes (Figure 1A-D). The ion channel blocker concentration range was optimized in a pilot study to cause at least double prolongation of WBCL in neonatal rabbit hearts. Baseline WBCLs did not differ significantly in any of the treatment groups (data not shown). Although both newborn and adult hearts are affected by channel blockers, there was no significant difference in WBCL between newborn and adult hearts when they were perfused with L-type calcium channel blockers (Diltiazem, Figure 1A; or Verapamil, Figure 1B), or with the I\textsubscript{f} blocker ZD7288 (Figure 1C). However, newborn hearts were more sensitive to T-type calcium channel blockade (Mibebradil, Figure 1D) compared to adults. Moreover, the percent increase of WBCL in newborn compared to adult hearts during atrial pacing was only increased (p<0.001) with Mibebradil. In order to evaluate whether Mibebradil-induced AV Wenckebach block resulted from blockade of α1G or α1H, we also measured WBCL during perfusion with increasing concentrations of nickel chloride (NiCl\textsubscript{2}), because α1G and α1H have different sensitivities to Ni\textsuperscript{2+} block (IC\textsubscript{50} = 13 μM for α1H and IC\textsubscript{50} = 250 μM for α1G) [58]. Since WBCL was unaffected by Ni\textsuperscript{2+} up to concentrations of 300 μM (Figure 1E) in 5/6 hearts, we conclude that α1G is
primarily responsible for the Mibefradil effects on WBCL prolongation in newborn vs. adult hearts.

\( \alpha_{1G} \) expression in the AV node of human fetal hearts

Since the expression of \( \alpha_{1G} \) in the AV node of the human fetal heart is a prerequisite for any potential involvement of this protein in the AV block characteristic of CHB, we performed anatomical dissection, as previously described [59], and immunohistochemistry (IHC) studies to demonstrate presence of \( \alpha_{1G} \) in the AV node. Sections from a 21-week human fetal heart with Masson’s trichrome stain demonstrates morphology of the AVJ region, clearly showing the AV node and AV bundle, which coincides with appropriate anatomical regions identified by collagen (green) and myocardium (light pink) (Figure 2A). The NF-160 antibody (green), which identifies cardiac conducting tissue [60], clearly shows the AV node and AV bundle (Figure 2B). We observed dense staining with \( \alpha_{1G} \) antibodies (red) in the region of the AV node and AV bundle with more diffuse staining in surrounding regions corresponding to ventricular myocardium identified as regions without NF-160 staining (Figure 2B). Due to lack of suitable antibodies, we were not able to perform IHC to investigate expression in the AV node of the other T-type channel subunit expressed in the heart, \( \alpha_{1H} \).

\( \alpha_{1G} \) gene expression is enriched vs. \( \alpha_{1H} \) in the atrioventricular junction

To further explore the expression of \( \alpha_{1G} \) channels in fetal human hearts, we anatomically dissected [59] atrioventricular junction (AVJ) and the ventricular apex from three fetuses between 18–22.6 weeks gestation (18 weeks, 18.6 weeks, 22.6 weeks),...
coinciding with the time point when CHB is diagnosed in utero. Real time PCR measurements revealed that the expression of the α1G genes (CACNA1G), normalized to the level of expression in the apex, is higher at 18 weeks compared to later time points (Figure 3A). Although the availability of only a single sample per gestational week does not permit us to assess the reproducibility of these expression patterns, pooling of the results supports the conclusion that the relative expression of CACNA1G is greater (p<0.05) in the AVJ compared to the ventricular apex (Figure 3B). For comparison, we also measured the mRNA expression levels of the α1H gene, CACNA1H, and could not detect higher expression in the AVJ versus the ventricular apex, suggesting low expression of α1H in the AVJ.

Maternal sera autoantibodies from CHB-affected pregnancies are immunoreactive to the α1G protein in human fetal hearts

In order to investigate reactivity of CHB maternal sera to α1G, we performed Western blot and immunoprecipitation experiments with lysates from fetal heart ventricle. We first confirmed presence of α1G protein in human fetal hearts by Western blot with lysates from a 20.4-week-old human fetal heart. A commercial α1G/Cav3.1 antibody (specific for aa1–22 of α1G) identified a band ~180–200 kDa in size (Figure 3C, lane 1) which was eliminated by pre-incubation with a peptide corresponding to aa1–22 of rat α1G. Blotting with anti-Ro/La positive CHB maternal sera (titer >100 IU for both anti-Ro and anti-La) also identified a similar band (~180–200 kDa, Figure 3C, lane 3) that was eliminated by pre-incubation with peptide p305 (aa305–319 of α1G, see below). Serum obtained from a mother positive for anti-Ro/La autoantibodies (anti-Ro titer = 60 IU and anti-La titer >100 IU), but with an unaffected fetus, did not yield a band with the expected molecular weight of α1G (Figure 3G, lane 3, no bands). We also show in Figure 3C (lane 6) that a commercial antibody to α1H revealed a band, but the predicted molecular weight was much smaller than α1G (180–200 kDa). Note further that proper loading in all the wells was ensured using control anti-calnexin (CNX) antibody (Figure 3C, bottom panel).

The presence of α1G in human fetal heart lysates, and verification of immune reactivity by CHB maternal sera, was next assessed via immunoprecipitation (IP) experiments. IP was performed with the commercial α1G/Cav3.1 antibody (specific for aa1–22 of α1G) on lysates prepared from three dissected regions of human fetal hearts (AVJ, ventricle, apex). Immunoblots of IP samples from the AVJ, ventricle and apex, yielded α1G bands when probed with commercial α1G/Cav3.1 antibody and maternal serum from an anti-Ro/La positive pregnancy with CHB outcome (CHB serum, titer >100 IU for both anti-Ro/La), but not with maternal serum from an anti-Ro/La negative healthy pregnancy (Normal serum) (Figure 3D). These blots were subsequently stripped and re-probed with a second commercial α1G/Cav3.1 antibody recognizing a different α1G epitope (specific for aa6–50), demonstrating specificity of the α1G IP. We were unable to find appropriate conditions, or antibodies, that yielded evidence for the presence of α1H protein levels with IP; and, although Western blot did demonstrate a band for α1H, the size of this band differed from that recognized by CHB maternal sera.

Accessibility of α1G T-type calcium channel epitope on cardiomyocytes

Because our RT-PCR and Western Blotting studies showed convincingly the expression of α1G in the ventricles, we elected to explore whether α1G affinity-purified CHB sera identified surface targets in the myocardium using immunofluorescence and confocal microscopy. Although CHB is primarily characterized by atrioventricular block affecting the AV node, effects of anti-Ro/La autoantibodies have also been suggested on the whole heart [3–9,61], thus expression of targets in regions such as the ventricle are also of interest. Immunofluorescence co-staining of human fetal heart (21.6 weeks) with anti-α1G (Red) and with anti-cTnT
antibodies (Green) exhibited preferential staining of a1G localized predominantly to the cell surface, with some perinuclear staining observed by confocal microscopy (Figure 4A). Differential interference contrast (DIC) microscopy images of cardiomyocytes used for staining experiments demonstrate typical morphology of ventricular cardiomyocytes (Figure 4A, right panel).

Maternal CHB sera (titer 100 IU for both anti-Ro and anti-La), that had been affinity-purified towards the a1G peptide p305 (aa305–319, see below), preferentially stained surface regions of the ventricular cardiomyocytes (yellow, Figure 4B, right panel). Interestingly, some surface regions only showed a1G staining with the commercial antibody, and not co-staining with the affinity-purified sera, suggesting that the a1G epitope recognized by the maternal sera may be masked (or even absent) in some membrane areas.

CHB maternal sera profile screening of a1G peptides

In order to better understand the patterns of antibody reactivity to a1G described above, we undertook to delineate the a1G epitope(s) recognized by CHB maternal sera. The a1G protein is comprised of 24 transmembrane segments organized into four homologous domains (termed repeats I–IV); consequently, the protein has many extracellular (12) and intracellular sequences (13) [62]. The first 4 transmembrane segments of each internal repeat (S1–S4) form the voltage-sensors, whereas the S5–S6, and the intervening extracellular loop, form the pore [62].

Given that CHB-derived antibodies stained surface-expressed a1G in cardiomyocytes, it seems reasonable to conclude that this sera recognizes extracellular epitopes of the a1G protein. Accordingly, a PEPscreen (Sigma) custom peptide library consisting of 15aa-long overlapping peptides was generated that encompassed selected regions of the a1G protein [aa130–380 (Figure 5A, B)]; II [aa774–963 (Figure 5C, D)]; and, III [aa1308–1536 (Figure 5E, F)] corresponding to the largest extracellular loops formed between S5 and S6 of repeat domains I, II and III. Sera from three mothers with CHB affected pregnancies, and three patients with pregnancies unaffected by CHB (denoted CHB+ and CHB−, respectively), were subsequently screened for reactivity to the peptide library (see Figure 5 for representative data). We identified two peptides in repeat I, zero peptides in repeat II, and six peptides in repeat III with levels of reactivity that exceeded background (Figure 5, A–C); no reactivity was observed with a1H.
The magnitude of response of CHB sera differed between peptides based on repeat I and repeat III. For example, all the peptides derived from repeat I exceeded background by 80–100%, whereas the most reactive repeat III peptide read out was 20% above background (compare Figure 5A and Figure 5C). We therefore chose to continue our epitope mapping to the region of repeat I encompassed by the two highly reactive peptides. These peptides, denoted p305 (aa305–319, Figure 5A, light grey bar) and p315 (aa315–325, Figure 5A, dark grey bar), are comprised of sequences predicted to reside between S5 and S6 in the third extracellular loop of repeat I.

Epitope mapping of the repeat I peptides was refined in enzyme-linked immunosorbent assay (ELISA) experiments that utilized four overlapping 15aa peptides, and one 20aa peptide, with sequences corresponding to this protein region, as follows: p300 (aa300–314); p305 (aa305–319); p310 (aa310–324); p315 (aa315–329); and, p305/310 peptide (aa305–324).
eters were developed using healthy control sera as a negative control, with the positive control provided by serum from a patient clinically identified as anti-Ro/La positive and confirmed as immunoreactive to a1G by immunoblotting (Figure 3C, lane 1). Using these controls, a reproducible ELISA screen for each peptide was established, and the reactivity of small cohorts of maternal CHB+ and CHB+2 serum samples to each peptide was investigated (Figure 6A–E).

Maternal sera reactivity from CHB+ or CHB+2 pregnancies was different towards peptides p300 and p315 (Figure 6A and Figure 6D, respectively), which were also the slowest-reacting peptides (read at 2 h 30 min). However, CHB+ sera were far more reactive to p305, p310, and p305/310 than CHB+2 sera (Figure 6B, C, E) with the greatest reactivity (Figure 6E, p<0.05) seen for the p305/310 peptide. Moreover, pre-incubation of the CHB+ maternal sera with p305 peptide reduced reactivity on ELISA, indicating specificity (Figure 6F). These results support the conclusion that p305 and p305/310 contained the predominant epitope(s) of a1G recognized by CHB+ maternal sera.

Protein structure and identity in S5–S6 extracellular loop regions in T- and L-type calcium channels

Inspection of the patterns of the CHB+ maternal sera reactivity to the peptides revealed that the sequence NTTCVNWNQY, herein referred to as the ‘core sequence’, was common to all the reactive peptides (Figure 7A). An alignment of the a1G and a1H proteins revealed that a1H contains a similar peptide sequence in the S5–S6 loop in repeat I with sequence identity for NWNQY, as well as nearby amino acids, with the conserved Cys residue being implicated previously in channel gating activation, inactivation, and deactivation [63].

By contrast, alignments of a1G with the L-type calcium channel a1C and a1D sequences did not uncover evidence of sequence similarity (data not shown). It is important to note that examination of the peptide sequences of the Ro52, Ro60 and La autoantigens, despite their association with CHB, do not have comparable sequences in a1G, a1H, a1C or a1D. However, only 2% of Ro/La positive pregnancies are affected by CHB, supporting the conclusion that these antibodies are not specific to the CHB outcome alone.

To evaluate whether the core sequence has the potential to form a protruding epitope for antibody binding, we employed the ElliPro Epitope Modeling Analysis Tool (http://tools.immuneepitope.org) which predicts linear and discontinuous antibody epitopes based on a protein antigen’s 3D structure [64]. We attempted to avoid bias towards the core sequence by submitting the entire sequence of the S5–S6 extracellular loop of a1G repeat I (aa235–370, UniProt) to this server for epitope prediction. Six potential linear epitopes were identified; one of these had the sequence SSSNTTCVNWNQYY, which obviously encompasses the core sequence, and is nearly identical in sequence to p305 (see Figure 8A). We also submitted the same region to the Kolaskar & Tongaonker Antigenicity prediction
and found that the eight amino acids NTTCVNWN, present in our "core sequence", were predicted to be an antigenic peptide based on a method predicting antigenicity from the expression of certain hydrophobic amino acids present on the surface of a molecule [65].

CHB+ Maternal sera are immunoreactive to peptides derived from the S5–S6 extracellular loops of α1G and α1H repeat I

Based on the results above, we hypothesized that CHB+ maternal sera might react with the S5–S6 segment in repeat I of α1H. A peptide corresponding to the aligned region of α1H (p330, aa330–343, Figure 7B) was accordingly utilized to screen a group of 23 CHB+ maternal sera, and the reactivity profile compared to that of p305. The pattern of reactivity obtained with each peptide was qualitatively similar (Figure 7B), and the levels of α1H reactivity observed among the sera were significantly correlated to those of α1G (Spearman correlation, r = 0.89, p = 0.0001). The parallel reactivity of CHB+ sera towards p305 and p330 led us to hypothesize that p330 might also provide a means to distinguish between CHB+ and CHB- sera. Screening of maternal sera with p305 (α1G) and with p330 (α1H) indicated a tendency for the CHB+ and CHB- groups to have differential reactivity for the peptides, although these results were of marginal significance (p = 0.075 and 0.184 for p305/α1G and p330/α1H, respectively). Each peptide was nevertheless capable of distinguishing between the CHB+ and healthy control (HC) groups (p < 0.05 for both peptides, Figure 7C). These results support the conclusion that CHB+ sera can also react with α1H, as well as α1G, consistent with previous studies [66]. This conclusion is consistent with our RT-PCR and Western blot studies (Figure 3) establishing expression of α1H in human fetal hearts.

Figure 6. Reactivity in CHB pregnancies is specific for the p305 peptide of α1G and can be blocked. Four overlapping 15aa peptides, and one 20aa peptide were selected to further characterize maternal sera reactivity to α1G in mothers with CHB pregnancies (CHB+) compared to mothers with unaffected pregnancies (CHB-). Reactivity to (A) p300 (aa300–314), (B) p305 (aa305–319), (C) p310 (aa310–324), (D) p315 (aa315–329) demonstrate that sera from mothers with pregnancies affected by CHB (CHB+), have significantly higher p305 antibody levels compared to unaffected (CHB-) pregnancies (p<0.05). Although p305 (aa305–319) had the highest reactivity, a longer peptide combining p305 and p310 designated p305/310 (aa305–24) demonstrated a significant difference between CHB+ and CHB- maternal serum (E). Pre-incubation of one CHB+ maternal sera, and one healthy control (HC) sera with increasing concentration (0, 20, 40 μg/ml) of peptide p305 (aa305–319) demonstrates that the reactivity of sera is specific for this peptide and can be blocked (F). Error bars indicate mean ± SE.

doi:10.1371/journal.pone.0072668.g006
Electrophysiological recordings of T-type current in SAN cells

To evaluate a functional effect of CHB maternal serum on T-type Ca\(^{2+}\) currents in SAN cells, we isolated sinoatrial node (SAN) cells from mice. Single-cell patch-clamp experiments were performed on adult mouse heart SAN cells to isolate Ca\(^{2+}\) currents since protein analysis demonstrated expression of \(\alpha_{1G}\) in both neonatal and adult mouse (Figure 8A). As expected from previous reports [67,68], the Ca\(^{2+}\) current began activating at voltages negative to \(-80\) mV, which became one component at holding potentials of \(-40\) mV. Previous studies have established that the first component activated at the more negative potentials (between \(-60\) mV and \(-40\) mV) is a signature of T-type calcium currents in SAN pacemaker myocytes [69]. Importantly, the application of CHB\(^*\) serum irreversibly and preferentially decreased (\(p = 0.0513\)) Ca\(^{2+}\) current at voltages between \(-60\) and \(-20\) mV, precisely where T-type calcium currents are differentially activated (Figure 8B, D). At \(-50\) mV, the CHB\(^*\) serum reduced the Ca\(^{2+}\) current from \(-1.23 \pm 0.34\) pA/pF to \(-0.48 \pm 0.15\) pA/pF (\(n = 6\)) while no significant change (\(p = 0.8772\)) was observed when normal serum was added (baseline: \(-1.32 \pm 0.90\) pA/pF, control serum: \(-1.22 \pm 0.81\) pA/pF; Figure 8C, E; \(n = 6\)). CHB\(^*\) serum also decreased (\(p = 0.0438\)) current at \(-40\) mV from \(-2.25 \pm 0.53\) pA/pF to \(-1.32 \pm 0.24\) pA/pF (\(n = 6\)). At more positive potentials (i.e., above \(-20\) mV) the relative effects of the serum were far less (and the changes did not reach significance) because L-type Ca\(^{2+}\) currents are the dominant currents at these voltages [69].

Discussion

Anti-Ro/SSA and anti-La/SSB autoantibodies have been associated with CHB for decades, but the precise targets for these autoantibodies remain incompletely understood. For example, maternal sera from CHB pregnancies were reported to affect both L- and T-type currents (\(\dot{i}_{Ca,T}\)) [47]. Arguments can be made for the involvement of both of these Ca\(^{2+}\) currents in CHB. Indeed, mice lacking \(\alpha_{1D}\) (Cav1.3\(^{-/-}\)) or \(\alpha_{1G}\) (Cav3.1\(^{-/-}\)) display heart block [53,70] as well as altered Wenckebach cycle lengths (WBCL). On the other hand, mice lacking the \(\alpha_{1H}\) T-type calcium channel (Cav3.2\(^{-/-}\)) have shown no ECG alterations [71]. These latter findings led us to hypothesize that the \(\alpha_{1G}\) T-type calcium channel might be a target of CHB autoantibodies.

Consistent with our hypothesis, IHC staining demonstrated that \(\alpha_{1G}\) is expressed in the AV node and AV bundle of the fetal human heart (Figure 2). In addition, our immunofluorescence and confocal results demonstrate that \(\alpha_{1G}\) is expressed on the surface of ventricular cardiomyocytes (Figure 4). Protein (Western Blotting and IPs) and mRNA expression results (Figure 3) further support
the presence of \(\alpha_{1G}\) in the fetal myocardium (AVJ, ventricle). However, the expression of \(\alpha_{1G}\) in the fetal heart does not establish a functional role. In this regard, previous studies in rabbit sinoatrial cells have established that T-type Ca\(^{2+}\) current is blocked by Mibefradil, a T-type calcium channel blocker at low concentrations \([72,73]\). Consistent with these functional effects on T-type current, we found that Mibefradil strongly affected the WBCL in newborn, but not adult, rabbit hearts. On the other hand, the T-type calcium channel blocker NiCl\(_2\), had no effect on WBCLs in newborn rabbit hearts at concentrations up to 300 \(\mu\)M.

These pharmacological observations support the conclusion that the \(\alpha_{1G}\) (Cav3.1)-dependent T-type Ca\(^{2+}\) current, rather than the \(\alpha_{1H}\)-dependent T-type Ca\(^{2+}\) current, is the dominant current in the AV node in newborns since the Ni\(^{2+}\) sensitivity of \(\alpha_{1H}\) channels (IC\(_{50} = 13 \mu\)M) is much lower than \(\alpha_{1G}\) channels (IC\(_{50} = 250 \mu\)M). This contention is consistent with results showing that mice lacking \(\alpha_{1G}\) channels (Cav3.1\(^{-/-}\)) have decreased T-type currents (\(I_{Ca,T}\)) in both sinoatrial (SAN) and AV nodal (AVN) cardiomyocytes, as well as slowed AV conduction \([53]\), while mice lacking \(\alpha_{1H}\) channels (Cav3.2\(^{-/-}\)) show no such abnormalities \([67]\). Further, the differential WBCL response in adult and newborn rabbits matches observations in mouse models showing that \(\alpha_{1G}\) and \(\alpha_{1H}\) are differentially regulated during development, with expression reductions occurring with aging \([74]\), a trend that we observe in our quantitative PCR studies (decrease in \(\alpha_{1G}\) and slight decrease in \(\alpha_{1H}\), Figure 3A). However, reports differ on developmental expression of \(\alpha_{1G}\) and \(\alpha_{1H}\) \([75]\). Human studies have demonstrated expression of both \(\alpha_{1G}\) and \(\alpha_{1H}\) channel transcripts in adult human heart \([51,76]\), and \(\alpha_{1G}\) protein in adult heart sinus node \([77]\), but ours is the first report of \(\alpha_{1G}\) protein expression in the AV node of human fetal hearts. A limitation of our mRNA measurements, however, was sample numbers of human fetal
hearts (one per time point). Clearly, further investigation of the temporal expression of α1G in human fetal hearts will be necessary in order to understand developmental expression. We nevertheless demonstrate expression of α1G protein in the AV node of 21-week-old fetal hearts, indicating that α1G is present during the time period when CHB usually occurs in the fetus.

Involvement of α1G in CHB is supported by a number of our present observations. Firstly, CHB sera recognized α1G protein from human fetal hearts in Western blot. Further, the reactivity of CHB sera was epitope mapped to the α1G p305 peptide (aa305–319), where we have identified an essential “core” signature sequence. However, although our ELISA screen identified clear patterns of reactivity/binding consistent with the need for a core sequence of 10 amino acids for recognition, a significant difference in reactivity of CHB vs. CHB sera was observed only for the 305/310 peptide, with considerable overlap between the two sera types. These observations suggest that maternal antibody reactivity to an additional target(s) may be involved in the disease.

Additional evidence for functional involvement of T-type currents in CHB was obtained by applying our CHB sera to adult mouse SAN cardiomyocytes, cells previously demonstrated to exhibit relatively high, and therefore easily measured, T-type Ca2+ currents [78]. Moreover, the T-type Ca2+ current in the adult mouse heart is primarily comprised of α1G-dependent channels [53]. Consistent with these previous studies, we observed expression of α1G (Cav3.1) in the neonatal and adult mouse heart. CHB sera also recognized a protein with the same molecular weight in neonates and in adults, and was able to preferentially block Ca2+ currents activating at more negative voltages (from −60 to −30 mV) – known to correspond with activation of T-type channels at a more negative voltage [78]. Additionally, this block by CHB sera was not reversed on washout, consistent with high affinity binding, and no block was seen when CHB- sera was added.

While collectively our results firmly support the conclusion that α1G channels play a role in CHB in newborns, we cannot rule out the possible involvement of other channels. Indeed, maternal sera from CHB pregnancies have been shown to effect both L-type Ca2+ currents and α1H-based T-type Ca2+ currents [47]. Our peptide mapping studies of α1G identified a short sequence located in the S5–S6 extracellular (P-loop) segment in repeat I as a likely potential antigenic epitope for CHB sera. Sequence alignment revealed that the other cardiac T-type calcium channel subunit, α1H, has a sequence with high similarity with the α1G channel protein in this region, which also was recognized by CHB sera. Potential involvement of α1H-dependent T-type calcium channels in CHB is further supported by our observation that α1H mRNA is expressed in fetal human hearts. Surface expression of α1G in human fetal cardiomyocytes has not been demonstrated in this study. Accessibility of the α1G extracellular epitope on ventricular cardiomyocytes was demonstrated by CHB maternal sera antibodies affinity-purified towards the p305 α1G peptide surface staining non-permeabilized ventricular cardiomyocytes (Figure 4). After permeabilization, and staining for α1G, co-staining could be seen with a portion of α1G present on the surface. Further studies are needed to determine the accessibility of the α1H epitope for antibody binding in human fetal hearts and its functional significance. CHB sera has also been shown previously to interact with a recombinant protein derived from the S5–S6 extracellular region in repeat I of the L-type calcium channel alpha subunit, α1D [48]. Although sequence alignments did not reveal a similar “core sequence”, it seems possible that the S5–S6 extracellular loop region in repeat I of both T-type and L-type calcium channels may have a conserved structure or, alternatively, this region may have a strong propensity for promoting immune responses, consistent with identification of this region of α1G protein by ElliPro Epitope Prediction and the Kolaskar & Tongaonker Antigenicity as having a high likelihood of antigenicity. The possibility of multiple protein targeting in CHB requires further investigation, and characterization of sera antibody specificity with respect to anti-Ro52, anti-Ro60 and anti-La will be necessary to determine the fine specificity of the antibodies cross-reacting with these channels. In this regard, we wish to note that expansion beyond CHB of the clinical spectrum for mothers with anti-Ro/SSA and anti-La/SSB antibodies [3–9,61] might be tied to the existence of a common epitope on several protein targets.

In summary, the data presented here implicate α1G as one specific target of CHB maternal sera antibodies, and suggest the potential for involvement of α1H, although we note that further functional studies with human cardiomyocytes are necessary to determine this conclusively. Investigations into specific channel isoforms and modifications, that may impact antibody binding to the native human proteins, will be important in understanding which targets bound by antibodies are important for development of the human disease.

Materials and Methods

Patients

The study included sera from 28 women who were followed at the Hospital for Sick Children, Toronto, Canada. Seventeen of the samples were from mothers with anti-Ro/La autoantibodies and CHB affected pregnancies with AVB III (CHB). Ten samples were from mothers with anti-Ro/La antibodies who were at risk for CHB, and seen at the rheumatology clinic, but with normal fetal heart rate (CHB-) outcome, evaluated by fetal echocardiography (ECD) during pregnancy, and 10 anti-Ro/La negative samples were used as healthy controls (HC). The Hospital for Sick Children Research Institute Research Ethics Board (REB) approved the study under protocol 0019970143 and written consent was obtained from mothers for use of the sera.

Human fetal heart tissue

Human fetal hearts (17- to 23-week gestation) were obtained through the Heart Centre Biobank Program from elective terminations of normal pregnancies. The use of human fetal tissue was approved by the Hospital for Sick Children Research Institute REB under protocol 1000029263. Written consent was obtained in the clinic, and portions of fetal hearts from fetal terminations are sent under a material transfer agreement to undergo banking within the Heart Centre Biobank Registry program under REB protocol number 1000011232.

Fetal hearts were dissected by an experienced cardiologist following an established method [59]. In brief, hearts are immobilized in a dissecting dish, right ventricle uppermost, which is slit open to visualize the tricuspid inlet below the interatrial septum. The AV junction is identified using anatomical landmarks of the septal cusp of the tricuspid valve inferiorly, the coronary sinus posteriorly, the tendon of Todaro superiorly. Tissue was either dissociated for immunofluorescence, flash frozen for immunoprecipitation experiments, or quickly submerged in RNA stabilizing reagents for immediate RNA preparation.

Preparation and perfusion of the Langendorff rabbit CHB model

The Animal Care Committee at the Hospital for Sick Children has approved the use of neonatal and adult rabbits as well as the experimental design of this study under protocol number 3250.
The animals were housed in the Animal Care Facility at the Hospital for Sick Children and maintained in accordance with hospital regulations and CGAC guidelines. Rabbit hearts were prepared for perfusion as previously described [57]. Briefly, following loss of the pain reflex, the rabbit sternum was incised and removed. The beating heart was dissected, and perfused with fresh oxygenated modified Krebs-Henseleit solution. The flow rate was constant at 9 ml/min for neonates and 36 ml/min for adults, the temperature was kept at 37°C and the left ventricle was left unloaded. The sinus node at the level of the superior vena cava-right atrial junction was excised until junctional rhythm was obtained, following which the hearts were allowed to equilibrate for 15 min.

Electrophysiologic recording for WBCL analysis

In order to compare calcium channel blocker effects on newborn versus mature AV nodal conduction, the rabbit Langendorff heart model was modified by atrial pacing the preparation at sequentially shorter cycle lengths until the AV nodal Wenckebach cycle length (WBCL) was identified, as described previously [57]. At baseline and 15 minutes following equilibration of each concentration of channel blocker, the atrium was paced at incrementally faster rates to determine WBCL. Finally, the hearts were perfused with fresh modified Krebs-Henseleit solution for 15 min and paced to determine WBCL in the washout state. The WBCL was defined as the slowest atrial paced rate with any failure of AV conduction within 8 consecutive seconds of pacing.

Calcium channel blockers

Prior to the comparative study, we assessed the effect of each blocker on WBCL prolongation in neonatal rabbit Langendorff preparations. We chose the concentration of each drug to cause at least double prolongation of WBCL in newborn rabbits. Adults (n = 7) and newborn (n = 8) hearts were exposed to two concentrations of either I$_f$ blocker (ZD7288; 10, 20 μM), I$_{Ca-L}$ blockers (Verapamil; 0.2, 0.4 μM, Diltiazem; 0.4, 0.8 μM) or I$_{Ca-T}$ blocker (Mibebradil; 0.4,0.8 μM). Increasing concentrations of a single drug were added in the perfusate in a sequential cumulative pattern. Newborn hearts were also assessed for WBCL prolongation upon exposure to a specific blocker of the type-I calcium channel α1H subunit (Nickel Chloride; 1, 10, 100 and 300 μM).

Histology and Immunohistochemistry

AVJ was dissected and fixed in 4% paraformaldehyde. After fixation, AVJ tissue was preserved as paraffin-embedded blocks. Slides were made from the blocks and underwent Masson’s trichrome stain and were immunolabelled for α1G and NF-160. The slides were dewaxed, rehydrated and rinsed 2× 5 min in double distilled water. The slides were then incubated with Target Retrieval Solution (DAKO Canada) at 95°C for 40 min and then room temperature 20 min. After rinsing 2× with PBS, sections were blocked with blocking buffer (PBS with 3% fish gelatin, 2 mg/ml BSA and 2% Tween-20) for one hour, and incubated with rabbit-anti-α1G (Novus Biologicals Canada) at a dilution of 1:400 and mouse anti-NF-160 (1:500, US Biological) for 2 hours. After rinsing 3× with PBS, slides were then incubated in the dark with donkey-anti-rabbit Cy3 (1:1000, Jackson Immunolabs) and donkey anti-mouse Alexa488 (1:1000, Invitrogen) for 40 min, DAPI was then added in the last 5 min, slides were washed 3×5 min in PBS and mounted with a coverslip. Slides were examined using a Zeiss Epiphelourescence microscope.

Real time PCR

Total RNA was prepared according to the protocol RNasy Fibrous Tissue Mini Kit (Qiagen). Homogenization was performed with a Tissue Tearor. RNA concentration and purity was determined and purified total RNA was converted to cDNA with Superscript III reverse transcriptase (Invitrogen). Power SYBR Green PCR Master Mix (Applied Biosystems) was used to perform relative quantification of target sequences CACNA1H (forward primer: CAAACCTTGTGGCCCTTGTGT, reverse primer: GTTGAGCTCTCGGTTCACAGT) and CACNA1I (forward primer: ATACACCAAGGAAGTCGCTG, reverse primer: CAGGACCATGAAAGAGGCG) compared to the housekeeping gene GAPDH (forward primer: CCTGGTTCGACACGTCCACGCGCATC, reverse primer: GGTGACAGGCGCCCCAATACG). Assay was optimized for the cDNA amount as well as PCR conditions to validate the primers and ascertain equal efficiency of primers. Samples were prepared in triplicates and subjected to default conditions for the 7000 Sequence Detection System by ABI (annealing at 60°C for 1 minute).

Immunoprecipitation, Western blot

Fresh tissue from 20–22 week-old fetal human hearts were homogenized in NP40 lysis buffer (1% NP40, 0.15M NaCl, 0.01M Na$_3$PO$_4$ pH 7.2, 2.5 mM Na$_2$O$_2$P$_2$, 2 mM EDTA, 1 mM Na$_2$VO$_4$, 1 mM PMSF) with proteinase inhibitor cocktail (Roche, Penzberg, Germany). Homogenized lysates were prepared at room temperature, incubated for 30 min and then centrifuged at 12,000 rpm for 15 min. Protein concentration was determined with the Bradford protein assay (Bio-Rad, Hercules, CA, USA) and 1 mg of total protein lysates were used per immunoprecipitation sample. Immunoprecipitation of α1G protein was achieved with Protein G Sepharose beads and 2 μg of α1G antibody (Cav3.1 antibody, Alomone Labs). Samples were incubated at 70°C for 5 min in Laemmli loading buffer. Samples were loaded onto 10% Tris-glycine sodium dodecyl sulfate polyacrylamide gels electrophoresed at 120 V for 2.5 h, and transferred overnight at room temperature (20 V) to nitrocellulose membranes. Membranes were blocked with 5% milk in Tris-buffered saline/0.05% Tween-20 (TBS-T), then probed with either human sera (1:100), anti-Cav3.1 antibody (1:200 dilution, Santa Cruz for Western blot probing after IP, 1:100 dilution, Sigma for western probing), or anti-Cav3.2 antibody (1:200 dilution, Sigma) for 2 h at room temperature. For pre-incubation to demonstrate specificity of commercial antibody for α1G, a peptide corresponding to aa1–22 of rat α1G was added at the recommended concentration of 1:1 with the antibody prior to addition to the blot. Membranes were washed between each step 3×15 min with TBS-T. Membranes were incubated 1 h with anti-human IgG-HRP (1:10,000 dilution, Jackson Immunolabs) or goat anti-rabbit IgG-HRP (1:5,000 dilution, Jackson Immunolabs). Anti-calcinexin (CNX) antibody was used as loading control for Western blot. Membranes were developed with ECL substrate (Santa Cruz Biotechnology) and film exposed for visualization of bands. Anti-Ro and anti-La titers given for maternal sera used in immunoassays are from routine clinical screening based on an ELISA using recombinant human anti-Ro60-kD and anti-Ro52-kD, and anti-La 48-kD protein (Phadlia GmbH, Freiburg, Germany).

Immunofluorescence

Dissected regions of fetal heart tissue was washed in Hank’s solution (HBSS) and incubated with gentle shaking overnight in Hank’s solution supplemented with 5 mM BDM and 1 mg/ml collagenase. Heart pieces were minced and incubated in fresh mincing solution (HBSS with 10 mM taurine, 0.1 mM EGTA, 10 mM BDM, 1 mg/ml BSA, 1 mg/ml collagenase) gently

PLOS ONE | www.plosone.org 12 September 2013 | Volume 8 | Issue 9 | e72668
spinning with a small magnet at 37 °C to harvest cells for 5 min. This procedure was repeated 3 times. Cells were washed then fixed with 2% paraformaldehyde for 10 min, shaking slowly for subsequent immunofluorescence labeling.

For detection of α1G T-type calcium channel, rabbit anti-human Cav3.1 antibody was used at a dilution of 1:1,500 (Novus Biologicals Canada), and for detection of cardiomyocyte markers mouse anti-human cardiac Troponin-T was used at a dilution of 1:500 (Thermo Scientific). Cells were resuspended in blocking buffer (PBS with 3% fish gelatin, 2 mg/ml BSA, 0.05% Tween-20) at room temperature for 1 h. Primary antibodies were added overnight at 4 °C, shaking gently. After 2×15 min washes in PBS, cells were incubated in the dark for 4 h at 4 °C with secondary antibodies: donkey anti-rabbit-IgG-Cy3 (1:5,000 Jackson ImmunoResearch) and donkey anti-mouse-IgG-Alexa488 (1:1,000 Green Invitrogen). DAPI stain was used to visualize nucleus (0.1 μg/ml). Cells were washed 2×15 min in PBS and mounted on glass for confocal microscopy (60× magnification).

For the surface staining with affinity-purified sera, non-permeabilized cells were blocked (PBS with 3% fish gelatin, 2 mg/ml BSA) at room temperature for 1 h before incubation with Cav3.1 antibody. Affinity purified sera was diluted in blocking buffer (without detergent) 1:2 (as already diluted from elution/neutralization buffers) for 2 h at room temperature. Cardiomyocytes were washed and secondary antibody (rabbit anti-human-IgG-Cy3 at 1:1,000, Jackson Immunolabs) was added, and cells were incubated for 1 h at room temperature in the dark. Subsequent staining for the α1G, T-type calcium channel followed the procedure described above, except that the secondary antibody used was donkey anti-rabbit-IgG-Dylight488 (1:1,000 Jackson ImmunoResearch).

Affinity Purification of Antibodies from Maternal Sera Specific for the p305 Peptide
Serum was affinity purified towards the α1G p305 peptide (aa305–319 of α1G) for use in immunofluorescence experiments as follows: A streptavidin resin filled column was equilibrated and washed with PBS. Serum was pre-incubated overnight with biotinylated p305 peptide. Free biotin was then added to the serum solution to block all non-specific biotin binding sites, after which the solution was applied to the streptavidin column. The column was then washed 3 times with PBS to remove antibodies not specific for peptide, and antibodies of interest were then eluted with 0.1 M glycine, pH 2.5 and immediately transferred into 1M Tris, pH 8.0 in a 1:10 ratio.

Peptides and ELISA
ELISA epitope mapping was performed using α1G peptide PEPScreen Custom peptide libraries (Sigma, St. Louis, MO, USA). We investigated fifty overlapping 15aa peptides corresponding to aa130–385. Thirty-six 15aa peptides covering aa1308–1536 were studied. Peptides were dissolved (80% DMSO/20% water) and 96-well ELISA plates were coated with antigen (20 μg/ml) in carbonate coating buffer pH 9.6 (0.03 M Na2CO3, 0.07 M NaHCO3, 0.1% NaN3), and incubated overnight at 4°C. Plates were washed and blocked with 5% BSA for 2 h and incubated for 2 h with sera at room temperature. Plates were washed 3× with washing buffer and incubated for 1 h with goat anti-human IgG AP-conjugate (Sigma, St. Louis, MO, USA). Plates were then developed with AP substrate buffer (R&D systems) and read at OD 405 nm minus that of the reagent blank. All samples were run in duplicate. Controls were from ten anti-Ro/La positive mothers who were at-risk for CHB and followed in the rheumatology clinic, but with healthy pregnancy outcomes, as well as control sera (anti-Ro/La negative) for assay development. Cut-offs for epitope mapping were determined by calculation of HG average+3× standard deviation.

The time at which ELISA experiments were read by spectrophotometer OD (405 nm) was optimized for each peptide using two criteria: (i) OD405<0.3 in healthy control samples, and (ii) OD405 as high as possible among test samples within spectrophotometric limits (OD405<2.0). OD values shown in Figures 7 and 8 were accordingly obtained at various incubation times. Peptide ELISA reading times were as follows: 50 min for p305; 2 h 30 min for p300 and p313; 1 hr 30 min for p310 and p305/p310.

Comparison of α1G and α1H peptides was accomplished with peptide amines modified at their N- and C-termini with lysine residues to enhance water solubility [79] (α1G, sequence H2N-KKKYNSSSSNTTCVNWNQYKKK-NH2; α1H, H2N-KKKVGAARNAICWNQYKKK-NH2). These peptides were dissolved in water and coated at 1 μg/ml and blocked with 1% fish serum for 2 h before incubating with primary sera for 2 h. Otherwise the procedure was identical to the above.

Electrophysiological recordings of T-type calcium current
Mice were cared for and housed in the Animal Care Facility at University of Toronto and maintained in accordance with university regulations and Canadian Council on Animal Care (CCAC) guidelines. The use of mice for electrophysiology studies was approved by The Animal Care Committee of University of Toronto under Protocol 20009885.

After heparinized mouse (C57BL/6, 8 weeks old, male, Charles River Inc. Canada) was anesthetized, heart was quickly removed and retrogradely perfused through aorta with 37°C Ca2+-free Tyrode’s solution, containing (in mM): 137 NaCl, 5.4 KCl, 1.0 MgCl2, 0.33 NaH2PO4, 22 D-glucose, 10 HEPES, pH 7.3, for 8–10 min. Then, collagenase (1.0 mg/ml, Worthington, type II) and elastase (0.15 mg/ml, Worthington) were added. Heart was digested for 35–50 min. SAN region (as described in [80]) was cut and cells were dissociated and kept in solution which contained (in mM): 120 potassium glutamate, 20 KCl, 20 HEPES, 1.0 MgCl2, 10 D-glucose, 0.5 K-EGTA, and 0.1% bovine serum albumin, pH 7.35 with KOH.

Ca2+ current was introduced by 400 ms test pulse from −70 mV to +30 mV with 10 mV step from holding potential at −80 mV or −40 mV. Cells were bathed in solution containing (in mM): 140 CsCl, 1 MgCl2, 1.8 CaCl2, 10 HEPES, 10 D-glucose, pH 7.35 with CsOH. SAN cells were picked as described previously [68]. Whole-cell patch clamp recording (Axopatch 200B and Clampex 8 software, Axon Instrument, CA, USA) was performed with 3.0–4.0 MΩ pipette when filled with solution containing (in mM): 135 CsCl, 5 TEA, 1 MgCl2, 4 MgATP, 10 HEPES, 10 EGTA, 0.3 Na3GTP, pH 7.2 with CsOH. Serial resistance was 80%–85% compensated. Data were analyzed with Clampfit 10 (Axon Instrument, CA, USA).

In order to evaluate the effect of serum on T-type calcium current, positive (CHB+) or control serum (normal serum) was added into external solution (1:50 dilution) and kept for 5 min for full reaction, then serum was fully washed out to remove Na+ from external solution.

Statistical analysis
For ELISA assay, Mann-Whitney U-test or Kruskall-Wallis ANOVA were used for statistical analysis. Mann-Whitney analysis was used for gene expression comparison. For analysis of
electrophysiology data a paired T-test was used. The level of significance was set at p<0.05.

Acknowledgments
The authors wish to acknowledge the assistance of Shusmita Rahman for her technical support.

References
1. Buyon JP, Hibbert R, Copel J, Craft J, Friedman D, et al. (1998) Autoimmune-associated congenital heart block: demographics, mortality, morbidity and recurrence rates obtained from a national neonatal lupus registry. The Journal of the American College of Cardiology 31: 1636–1656.
2. Friedman DM, Rupel A, Buyon JP (2007) Epidemiology, etiology, detection, and treatment of autoantibody-associated congenital heart block in neonatal lupus. Curr Rheumatol Rep 9: 101–108.
3. Chokalingam P, Jaeggi ET, Rammelo OA, Haak MC, Adama van Scheltema PN, et al. (2011) Persistent fetal sinüs bradyarrhythmia associated with maternal anti-SSA/Ro and anti-SSB/La antibodies. J Rheumatol 38: 2682–2685.
4. Jaeggi ET, Hamilton RM, Silverman ED, Zumora SA, Hornbaker LR (2002) Outcome of children with fetal, neonatal or childhood diagnosis of isolated congenital atrioventricular block. A single institution's experience of 30 years. J Am Coll Cardiol 39: 130–137.
5. Bronen R, Heikila P, Terano K (2001) Congenital complete heart block in the fetus: hemodynamic features, antenatal treatment, and outcome in six cases. Pediatr Cardiol 22: 385–392.
6. Taylor-Albert E, Reichlin M, Toews WH, Overholt ED, Lee LA (1997) Delayed dilated cardiomyopathy as a manifestation of neonatal lupus: case reports, autoantibody analysis, and management. Pediatrics 99: 733–735.
7. Nield LE, Silverman ED, Taylor GP, Smallhorn JF, Mullen JB, et al. (2002) Maternal anti-Ro and anti-La antibody-associated endocardial fibroelastosis. Circulation 105: 943–948.
8. Nield LE, Silverman ED, Smallhorn JF, Taylor GP, Mullen JB, et al. (2002) Endocardial fibroelastosis associated with maternal anti-Ro and anti-La antibodies in the absence of atrioventricular block. J Am Coll Cardiol 40: 796–802.
9. Lazzarin PE, Caporelli PL, Arcarona M, Morozzi G, Bellisai F, et al. (2011) Anti-Ro/SSA-associated QTc interval prolongation in the mothers: the role of antibody level and Specificity. Arthritis Care Res (Hoboken).
10. Buyon JP, Hibbert R, Copel J, Craft J, Friedman D, et al. (1998) Autoimmune-associated congenital heart block: demographics, mortality, morbidity and recurrence rates obtained from a national neonatal lupus registry. J Am Coll Cardiol 31: 1638–1666.
11. Finkeinstein Y, Adler Y, Harel L, Nussinovitch M, Younou P (1997) Anti-Ro (SSA) and anti-La (SSB) antibodies and complete congenital heart block. 140: 295–298.
12. Groves AM, Allan LD, Rosenthal E (1996) Outcome of isolated congenital complete heart block. British Heart Journal 60: 512–515.
13. Schmidt KG, Ulmer HE, Silverman NH, Kleinman CS, Copel JA (1991) Isolated congenital complete heart block and relation to maternal anti-Ro antibodies. The American Journal of Cardiology 58: 291–294.
14. Reeth KH, Kraft K, Heikila R, Izumori K, Itoh H, et al. (1997) Isolated congenital heart block - Long-term outcome of mothers and characterization of the immune response to SSA/A-Ro and to SS-B/La. Arthritis Rheum 40: 1538–1598.
15. Julkunen H, Kaaja R, Siren MK, Mack G, McCready S, et al. (1998) Immune-mediated congenital heart block (CHB): identifying and counseling patients at risk for having children with CHB. Semin Arthritis Rheum 28: 97–106.
16. Salomonsson S, Doerner T, Thander E, Bremme K, Larsson P, et al. (2002) A serologic marker for fetal risk of congenital heart block. Arthritis and Rheumatism 46: 1233–1241.
17. Buyon JP, Hiebert R, Craft J, Friedman D, et al. (1998) Autoimmune-associated congenital heart block: demographics, mortality, morbidity and recurrence rates obtained from a national neonatal lupus registry. J Am Coll Cardiol 35: 2758–2784.
18. Silverman ED, Buyon J, Laxer RM, Hamilton R, Bini P, et al. (1995) Autoantibody response to the Ro/La particle may predict outcome in neonatal lupus erythematosus. Clinical and Experimental Immunology 100: 499–505.
19. Salomonsson S, Dorner T, Thander E, Bremme K, Larsson P, et al. (2002) A serologic marker for fetal risk of congenital heart block. Arthritis and Rheumatism 46: 1382–1383.
20. Salomonsson S, Sonesson SE, Otteson L, Mahbub S, Olsson T, et al. (2005) Ro/SSA autoantibodies directly bind cardiomyocytes, disturb calcium homeo-stasis, and mediate congenital heart block. J Exp Med 201:11–17.
21. Buyon JP, Hibbert R, Copel J, Craft J, Friedman D, et al. (1998) Autoimmune-associated congenital heart block: demographics, mortality, morbidity and recurrence rates obtained from a national neonatal lupus registry. J Am Coll Cardiol 31: 1638–1666.
22. Lazzarin PE, Caporelli PL, Arcarona M, Morozzi G, Bellisai F, et al. (2011) Anti-Ro/SSA-associated QTc interval prolongation in the mothers: the role of antibody level and Specificity. Arthritis Care Res (Hoboken).
23. Brucato A, Frassi M, Franceschini F, Cinraz M, Faden D, et al. (2001) Risk of congenital complete heart block in newborns of mothers with anti-Ro/SSA antibodies detected by counterimmunoelectrophoresis: a prospective study of 100 women. Arthritis Rheum 44:1832–1833.
24. Silverman ED, Buyon J, Laxer RM, Hamilton R, Bini P, et al. (1995) Autoantibody response to the Ro/La particle may predict outcome in neonatal lupus erythematosus. Circulation 105: 1483–1490.
25. Salomonsson S, Sonesson SE, Otteson L, Mahbub S, Olsson T, et al. (2005) Ro/SSA autoantibodies directly bind cardiomyocytes, disturb calcium homeo-stasis, and mediate congenital heart block. J Exp Med 201:11–17.
26. Struland B, Winquist O, Sonesson SE, Molren S, Salomonsson S, et al. (2008) Antibodies to amino acid 200–239 (p200) of Ro52 as serological markers for the risk of developing congenital heart block. Circulation 116:30–37.
27. Finkelstein Y, Adler Y, Harel L, Nussinovitch M, Younou P (1997) Anti-Ro (SSA) and anti-La (SSB) antibodies and complete congenital heart block. 140: 295–298.
28. Groves AM, Allan LD, Rosenthal E (1996) Outcome of isolated congenital complete heart block diagnosed in utero. Heart 75: 190–194.
29. Reeth KH, Kraft K, Heikila R, Izumori K, Itoh H, et al. (1997) Isolated congenital heart block - Long-term outcome of mothers and characterization of the immune response to SSA/A-Ro and to SS-B/La. Arthritis Rheum 40: 1538–1598.
30. Brucato A, Frassi M, Franceschini F, Cinraz M, Faden D, et al. (2001) Risk of congenital complete heart block in newborns of mothers with anti-Ro/SSA antibodies detected by counterimmunoelectrophoresis: a prospective study of 100 women. Arthritis Rheum 44:1832–1833.
31. Salomonsson S, Sonesson SE, Otteson L, Mahbub S, Olsson T, et al. (2005) Ro/SSA autoantibodies directly bind cardiomyocytes, disturb calcium homeo-stasis, and mediate congenital heart block. J Exp Med 201:11–17.
32. Reeth KH, Kraft K, Heikila R, Izumori K, Itoh H, et al. (1997) Isolated congenital heart block - Long-term outcome of mothers and characterization of the immune response to SSA/A-Ro and to SS-B/La. Arthritis Rheum 40: 1538–1598.
33. Brucato A, Frassi M, Franceschini F, Cinraz M, Faden D, et al. (2001) Risk of congenital complete heart block in newborns of mothers with anti-Ro/SSA antibodies detected by counterimmunoelectrophoresis: a prospective study of 100 women. Arthritis Rheum 44:1832–1833.
34. Brucato A, Frassi M, Franceschini F, Cinraz M, Faden D, et al. (2001) Risk of congenital complete heart block in newborns of mothers with anti-Ro/SSA antibodies detected by counterimmunoelectrophoresis: a prospective study of 100 women. Arthritis Rheum 44:1832–1833.
50. Mizuta E, Shirai M, Arakawa K, Hidaka K, Miake J, et al. (2010) Different
48. Karnaibi E, Qu Y, Wadgaonkar R, Manzarella S, Yue Y, et al. (2010)
46. Qu Y, Xiao GQ, Chen L, Boutjdir M (2001) Autoantibodies from mothers of
45. Qu Y, Baroudi G, Yue Y, Boutjdir M (2005) Novel molecular mechanism
44. Karnaibi E, Qu Y, Wadgaonkar R, Manzarella S, Yue Y, et al. (2010)
43. Monteil A, Chemin J, Bourinet E, Mennessier G, Lory P, et al. (2000) Molecular
42. Greener ID, Monfredi O, Inada S, Chandler NJ, Tellez JO, et al. (2011)
41. Hancox JC, Levi AJ, Lee CO, Heap P (1999) Nickel block of three
40. Lee JH, Gomora JC, Cribbs LL, Perez-Reyes E (1999) Nickel block of three
39. Hamilton RM, Lee PM, Kruger K, Silverman ED (1998) Investigative methods of congenital complete heart block. Journal of Electrocardiology 30 Suppl: 69–74.
38. Lee JH, Gomora JC, Cribbs LL, Perez-Reyes E (1999) Nickel block of three cloned T-type calcium channels: low concentrations selectively block alpha1H. Biophys J 77: 3034–3042.
37. Hancox JC, Levi AJ, Lee CO, Heap P (1999) A method for isolating rabbit atrioventricular node myocytes which retain normal morphology and function. The American Journal of Physiology 265: H733–766.
36. Rothenberg F, Elinosir IR (2006) Three-dimensional anatomy of the conduction system of the early embryonic rabbit heart. Anat Rec A Discov Mol Cell Evol Biol 285: 3–7.
35. Jaeggi ET, Hornberger LB, Smallborn JF, Fournon JC (2005) Prenatal diagnosis of complete atrioventricular block associated with structural heart disease: combined experience of two tertiary care centers and review of the literature. Ultrasound Obstet Gynecol 26: 16–21.
34. Swartz KJ (2008) Sensing voltage across lipid membranes. Nature 456: 891–897.
33. Karmazinova M, Bely S, Stary-Weinzinger A, Suwattanasophon C, Klugbauer N, et al. (2010) Cysteines in the loop between BS5 and the pore helix of Cav(V)3.1 are essential for channel gating. Pflugers Arch 460: 1015–1028.
32. Ponomarenko J, Bui HH, Li W, Fusseder N, Bourne PE, et al. (2008) ElliPro: a new structure-based tool for the prediction of antibody epitopes. BMC Bioinformatics 9: 514.
31. Kolaskar AS, Tongaonkar PC (1990) A semi-empirical method for prediction of antigenic determinants on protein antigens. FERS Lett 276: 172–174.
30. Xiao GQ, Hu K, Boutjdir M (2001) Direct inhibition of expressed cardiac L- and t-type calcium channels by IgG from mothers whose children have congenital heart block. Circulation 103: 1599–1604.
29. Hagiwara N, Inooka H, Kameyama M (1998) Contribution of two types of calcium currents to the pacemaker potentials of rabbit sino-atrial node cells. J Physiol 395: 235–253.
28. Mangoni ME, Nargeot J (2001) Properties of the hyperpolarization-activated current (I(f)) in isolated mouse sino-atrial cells. Cardiovasc Res 52: 51–64.
27. Catterall WA (2000) Structure and regulation of voltage-gated Ca2+ channels. Annu Rev Cell Dev Biol 16: 521–555.
26. Zhang Z, He Y, Tuteja D, Xu D, Timofeyev V, et al. (2005) Functional roles of Cav1.3 (alpha1D) calcium channels in atria: insights gained from gene-targeted null mutant mice. Circulation 112: 1936–1944.
25. Chen CC, Lamping KG, Nuno DW, Barresi R, Prouty SJ, et al. (2003) Abnormal coronary function in mice deficient in alpha1H T-type Ca2+ channels. Science 302: 1416–1418.
24. Masumiya H, Kase J, Tanaka Y, Tanaka H, Shigenobu K (1999) Effects of mibebradil, a selective T-type Ca2+ channel antagonist, on sino-atrial node and ventricular myocardia. Res Commun Mol Pathol Pharmacol 104: 321–329.
23. Mishra SK, Hermanneyer K (1994) Selective inhibition of T-type Ca2+ channels by Ro 49–5067. Circ Res 75: 144–148.
22. Cribbs LL, Martin BI, Schroder EA, Keller BB, Dohle BP, et al. (2001) Identification of the t-type calcium channel (Cav3.3, delta1) in developing mouse heart. Circ Res 88: 403–407.
21. Nica N, Yasui K, Optflo T, Takenaka H, Shimizu A, et al. (2004) Cav3.2 subunit underlies the functional T-type Ca2+ channel in murine hearts during the embryonic period. Am J Physiol Heart Circ Physiol 286: H2257–2263.
20. Cribbs LL, Lee JH, Yang J, Sain J, Zhang Y, et al. (1998) Cloning and characterization of alpha1H from human heart, a member of the T-type Ca2+ channel gene family. Circ Res 83: 103–109.
19. Chandler NJ, Greener ID, Tellez JO, Inada S, Musa H, et al. (2009) Molecular architecture of the human specialised atrioventricular conduction axis. J Mol Cell Cardiol 42: 69–80.
18. Mangoni ME, Traboulsie A, Leoni AL, Couette B, Marger L, et al. (2006) MHC-Encoded Genes Determine Susceptibility in Congenital Heart Block. Pediatr Res 57: 557–562.
17. Strandberg LS, Ambrosi A, Jagodic M, Drukaite V, Janson P, et al. (2008) Maternal MHC Regulates Generation of Pathogenic Antibodies and Fetal MHC-Encoded Genes Determine Susceptibility in Congenital Heart Block. J Immunol 175: 3574–3582.
16. Ambrosi A, Drukaite V, Park J, Strandberg L, Kuchroo VK, et al. (2012) Anti-Ro52 monoclonal antibodies specific for amino acid 200–239, but not other Ro52 epitopes, induce congenital heart block in a rat model. Ann Rheum Dis 71: 448–454.
15. Hamilton RM, Lee PM, Kruger K, Silverman ED (1998) Investigative methods of congenital complete heart block. Journal of Electrocardiology 30 Suppl: 69–74.
14. Zhang Z, He Y, Tuteja D, Xu D, Timofeyev V, et al. (2005) Functional roles of Cav1.3 (alpha1D) calcium channels in atria: insights gained from gene-targeted null mutant mice. Circulation 112: 1936–1944.
13. Chen CC, Lamping KG, Nuno DW, Barresi R, Prouty SJ, et al. (2003) Abnormal coronary function in mice deficient in alpha1H T-type Ca2+ channels. Science 302: 1416–1418.
12. Masumiya H, Kase J, Tanaka Y, Tanaka H, Shigenobu K (1999) Effects of mibebradil, a selective T-type Ca2+ channel antagonist, on sino-atrial node and ventricular myocardia. Res Commun Mol Pathol Pharmacol 104: 321–329.
11. Mishra SK, Hermanneyer K (1994) Selective inhibition of T-type Ca2+ channels by Ro 49–5067. Circ Res 75: 144–148.
10. Cribbs LL, Martin BI, Schroder EA, Keller BB, Dohle BP, et al. (2001) Identification of the t-type calcium channel (Cav3.3, delta1) in developing mouse heart. Circ Res 88: 403–407.
9. Nica N, Yasui K, Optflo T, Takenaka H, Shimizu A, et al. (2004) Cav3.2 subunit underlies the functional T-type Ca2+ channel in murine hearts during the embryonic period. Am J Physiol Heart Circ Physiol 286: H2257–2263.
8. Cribbs LL, Lee JH, Yang J, Sain J, Zhang Y, et al. (1998) Cloning and characterization of alpha1H from human heart, a member of the T-type Ca2+ channel gene family. Circ Res 83: 103–109.
7. Chandler NJ, Greener ID, Tellez JO, Inada S, Musa H, et al. (2009) Molecular architecture of the human sinus node: insights into the function of the cardiac pacemaker. Circulation 119: 1562–1575.
6. Ono K, Iijima T (2010) Cardiac T-type Ca2+ channels in the heart. J Mol Cell Cardiol 48: 65–70.
5. Melnyk RA, Partridge AW, Yip J, Wu Y, Goto NK, et al. (2003) Polar residue tagging of transmembrane peptides. Biopolymers 71: 675–685.
4. Mangoni ME, Couette B, Marger L, Bournet E, Stary-Weinzinger A, Suwattanasophon C, Klugbauer N, et al. (2010) Cysteines in the loop between BS5 and the pore helix of Cav(V)3.1 are essential for channel gating. Pflugers Arch 460: 1015–1028.
3. Ponomarenko J, Bui HH, Li W, Fusseder N, Bourne PE, et al. (2008) ElliPro: a new structure-based tool for the prediction of antibody epitopes. BMC Bioinformatics 9: 514.
2. Kolaskar AS, Tongaonkar PC (1990) A semi-empirical method for prediction of antigenic determinants on protein antigens. FERS Lett 276: 172–174.
1. Xiao GQ, Hu K, Boutjdir M (2001) Direct inhibition of expressed cardiac L- and t-type calcium channels by IgG from mothers whose children have congenital heart block. Circulation 103: 1599–1604.