Cooperative and antagonistic roles for Irx3 and Irx5 in cardiac morphogenesis and postnatal physiology

Nathalie Gaborit1, Rui Sakuma2, John N. Wylie1, Kyoung-Han Kim2, Shan-Shan Zhang1, Chi-Chung Hui2,3 and Benoit G. Bruneau1,4,5,*

SUMMARY
The Iroquois homeobox (Irx) homeodomain transcription factors are important for several aspects of embryonic development. In the developing heart, individual Irx genes are important for certain postnatal cardiac functions, including cardiac repolarization (Irx5) and rapid ventricular conduction (Irx3). Irx genes are expressed in dynamic and partially overlapping patterns in the developing heart. Here we show in mice that Irx3 and Irx5 have redundant function in the endocardium to regulate atrioventricular canal morphogenesis and outflow tract formation. Our data suggest that direct transcriptional repression of Bmp10 by Irx3 and Irx5 in the endocardium is required for ventricular septation. A postnatal deletion of Irx3 and Irx5 in the myocardium leads to prolongation of atrioventricular conduction, due in part to activation of expression of the Na+ channel protein Nav1.5. Surprisingly, combined postnatal loss of Irx3 and Irx5 results in a restoration of the repolarization gradient that is altered in Irx5 mutant hearts, suggesting that postnatal Irx3 activity can be repressed by Irx5. Our results have uncovered complex genetic interactions between Irx3 and Irx5 in embryonic cardiac development and postnatal physiology.

KEY WORDS: Transcription factors, Heart development, Electrophysiology, Mouse

INTRODUCTION
Heart development is governed by a complex network of transcription factors that precisely regulates cardiac gene expression (Olson, 2006; Srivastava, 2006; Evans et al., 2010). Many transcription factors are specifically expressed in cardiogenic tissue early in development and are required for early steps in cardiogenesis. These factors are also relevant to human diseases, as mutations in these genes are associated with human congenital structural heart defects, including defects in chamber septation and outflow tract formation (Bruneau, 2008). No single transcription factor has been shown to be essential for all aspects of cardiogenesis, which is likely to reflect considerable functional redundancy between transcription factors in the cardiac lineage.

Transcription factors of the Iroquois homeobox (Irx) family are expressed in the heart and have conserved roles during embryonic development in metazoans (Christoffels et al., 2000; Cavodeassi et al., 2001; Mummenhoff et al., 2001). In mouse, five out of the six Irx members have been individually deleted, and deletions of three result in defects of cardiac function. Irx4-deficient mice develop adult mild cardiomyopathy and abnormal ventricular gene expression (Bruneau et al., 2001). Deletions of Irx3 or Irx5 lead to specific defects in adult ventricular conduction or repolarization, respectively (Costantini et al., 2005; Zhang, S. S. et al., 2011). The Irx genes encode proteins of similar structure, containing a highly conserved DNA-binding homeodomain, followed by an acidic activation domain and the IRO (Iroquois) box, a conserved motif of unknown function (Burglin, 1997). Irx genes provide a rare example of genetic redundancy in Drosophila (Gomez-Skarmeta et al., 1996; Cavodeassi et al., 2001) and are highly redundant in zebrafish (Itoh et al., 2002). Murine Irx genes are expressed in partially overlapping patterns in most developing tissues and organs, including the heart (Christoffels et al., 2000; Houweling et al., 2001; Mummenhoff et al., 2001). Although Irx3 or Irx5 deletion does not lead to abnormalities in embryonic cardiac morphogenesis (Costantini et al., 2005; Zhang, S. S. et al., 2011), the Fused toes mouse, in which three Irx genes (Irx3, Irx5 and Irx6) are deleted, displays cardiac abnormalities in utero (Peters et al., 2002). Along with the protein homology of the Irx3 and Irx5 transcription factors and their overlapping expression patterns, these data suggest that they function redundantly in pre-natal cardiac development as well as postnatal cardiac function.

To investigate the potential functional redundancy between Irx3 and Irx5, we generated mice bearing a deletion of both genes. In contrast to the single knockouts, mice lacking both Irx3 and Irx5 die in utero and have severe defects of the outflow tract (OFT) and atrioventricular (AV) canal due to specific requirement for Irx3 and Irx5 in the endocardium. Several BMPs are upregulated in the embryonic double-knockout (DKO) mice, Bmp10 being a direct downstream target of repression by Irx3 and Irx5. In addition, we analyzed the postnatal redundancy of Irx3 and Irx5 in the cardiac conduction system by generating a postnatal deletion of both genes in the cardiac tissue. The spectrum of electrophysiological defects in these mice suggests redundant and unique roles for Irx3 and Irx5 in the conduction of the ventricular electrical influx. These results reveal new and partially redundant roles for Irx3 and Irx5 during heart development and postnatal function.

MATERIALS AND METHODS
Gene targeting and mice
Generation of the Irx3flox/Irx5EGFP/Irx3flox/Irx5EGFP mice was achieved by sequential gene targeting in embryonic stem cells (R.S. and C.-C.H., unpublished). Briefly, a conditional allele for Irx3 was generated by

1Gladstone Institute of Cardiovascular Disease, San Francisco, CA 94158, USA.
2Program in Developmental & Stem Cell Biology, The Hospital for Sick Children, Toronto, ON M5G 1X8, Canada.
3Department of Molecular Genetics, University of Toronto, Toronto, ON M5S 3E2, Canada.
4Cardiovascular Research Institute, University of California, San Francisco, San Francisco, CA 94158, USA.
5Department of Pediatrics, University of California, San Francisco, San Francisco, CA 94143, USA.

*Author for correspondence (bbruneau@gladstone.ucsf.edu)

Accepted 31 July 2012
flanking exons 2 to 4 with loxP sites. This line was then retargeted to replace Irx3 exon 1 by EGFp cDNA, followed by a polyadenylation signal sequence. A PKG-puro cassette was also introduced in place of Irx5 exon 2, thereby deleting the homeobox domain. Genotyping was carried out using the following primers (5'-3'): Irx3fllox allele, CAAGAGG-GGTGATGAGTGCTGGCCG and GGAGGGGAACCCAGCG- CGAAGAAGGCCCTA; Irx3fl allele, CTCGGATACCAGTACCATCG- CCCCCTCTACA and GAGAGGGGAACCAGCGCAGAAGCGCTCA; Irx5fllox allele, GGCTCCCAGGAGGCGCAGATGAAATTGCGG, GAATTCTCCGGTACCGGGGTCCCATATA and CCGGGTGGATGTCGAGGCC.

Complete deletion of Irx3 was obtained by crossing with Mef2cAHF::Cre female mice, which have germ line Cre activity (Verzi et al., 2005). Irx3flloxflloxZ/+, Wnt1::Cre, Mef2cAHF::Cre, Nkx2.5::Cre, Tie2::Cre, Isl1::Cre, Myh6::MerCreMer and ca-Bmpnr1a have been described (Brault et al., 2001; Kisanuki et al., 2001; Sohal et al., 2001; McFadden et al., 2004; Verzi et al., 2005; Yang et al., 2006; Kamiya et al., 2008; Zhang, S. S. et al., 2011). Animals were cared for in accordance with national and institutional requirements.

Histology
Embryos were harvested from timed matings and fixed in 4% paraformaldehyde (PFA) overnight, followed by embedding in paraffin and staining with Hematoxylin and Eosin (H&E). Optical projection tomography (OPT) imaging of hearts was performed as described (Sharpe et al., 2002; Licker et al., 2004).

Protein expression analysis
For immunofluorescence analysis, 8 µm cryosections were stained with primary antibodies against β-galactosidase (Cappel), green fluorescent protein (GFP) (Abcam), phospho-Smad1/5/8 (Cell Signaling Technology), Cx40 (Alpha Diagnostic), Nav1.5 (Alomone) and Kv4.2 (Abcam). Secondary antibody staining was performed using Alexa 488- and Alexa 594-conjugated antibodies (Invitrogen). Vectashield Mounting Media with DAPI was used for DNA counterstaining and mounting (Vector Laboratories).

Gene expression analysis
For microarray analysis, ventricles and OFT were dissected from E12.5 hearts and digested with 0.2 mg/ml trypsin (Invitrogen) and 50 U/ml type II collagenase (Worthington) in calcium- and magnesium-free Hanks’ buffer with Hepes at 37°C. Irx5EGFP-positive cells were isolated using FACSDiva. RNA was isolated using the PicoPure RNA Isolation Kit (Arcturus Bioscience). For each sample, 1 ng RNA was amplified using the FFPE Kit (NuGEN) and hybridized on Mouse Gene ST 1.0 microarrays (Affymetrix). For quantitative (q) real-time RT-PCR, 20 ng amplified cDNA was used. Primers (TaqMan assay, Applied Biosystems) for Tgfr3, Runx1t1, Isl1, Fgfr10, Fgfr2, Bmp2, Bmp5, Bmp10 and Irx3 were as follows: Mm00803358_m1, Mm00486771_m1, Mm00627860_m1, Mm00433275_m1, Mm01275521_m1, Mm01340178_m1, Mm00432091_m1, Mm01183889_m1 and Mm00500463_m1. β-actin (Mm00607939_s1) was used as internal control (Applied Biosystems). In situ hybridization was performed according to a standard protocol using 10 µm paraffin sections.

Luciferase assays, co-immunoprecipitation and chromatin immunoprecipitation
Transfections and luciferase assays were performed using published methods (Bruneau et al., 2001). Cos7 cells were transfected with 250 ng of each plasmid using Fugene (Roche). Co-immunoprecipitation was performed using the ExactaCruz Kit (Santa Cruz) following the manufacturer’s instructions. Primary antibody against Irx5 (Sigma WH00010265M1) was used for immunoprecipitation and antibody against Irx3 (Abcam AB25703) was used for immunoblot analysis. For chromatin immunoprecipitation, chromatin was isolated from wild-type E12.5 and E14.5 hearts, sonicated and incubated with antisera against Irx3 (Abcam AB25703) or Irx5 (Sigma WH00010265M1). DNA fragments were analyzed by custom TaqMan assay for the Bmp10 promoter: 5'-CTTGGGACAGTGTCGTAATCCT-3' and 5'-ATCTTGTGGAGAACCT-GTG-3'.

Electrophysiology
Surface ECG was obtained at 8-10 weeks as described (Bruneau et al., 2001). Telemetry devices (Data Sciences International, St Paul, MN, USA) were implanted dorsally with electrodes in lead II configuration. After 60 hours of recovery post-surgery, data were collected and analyzed using Dataquest A.R.T. (DSI) and Chart (ADInstruments).

Data analysis
Statistical comparisons were performed by Student’s t-test with P<0.05 considered significant. Microarrays were normalized for array-specific effects using Affymetrix Robust Multi-Array (RMA) normalization. Normalized array values were reported on a log2 scale. For statistical analyses, we removed array probesets where no experimental groups had an average log2 intensity greater than 3.0. Linear models were fitted for each gene using the Bioconductor limma package in R. Moderated t-statistics, fold-change and the associated P-values were calculated for each gene. To account for testing thousands of genes, we reported false discovery rate (FDR)-adjusted values, calculated using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). FDR values indicate the expected fraction of falsely declared differentially expressed genes among the total set of declared differentially expressed genes. Two-way hierarchical agglomerative clustering was applied to the gene expression data generated for each probe. For each gene, each group was centered to the median. Clusters were visualized using TreeView software. Microarray data are available in GEO with accession number GSE38826.

RESULTS
Expression pattern of Irx3 and Irx5 in the embryonic heart
Irx3 and Irx5 are expressed in complementary domains, at least up until E12.5 (Christoffels et al., 2000). We analyzed the expression pattern of Irx3 and Irx5 at E12.5 and E14.5, with Irx3flloxZ/ and Irx5EGFP alleles and immunostaining for β-gal and EGFP, to define cell types that express one or more of these factors. At E12.5, Irx3-expressing cells were detected in the ventricular trabeculae (Fig. 1Aa, arrowheads) and in the interventricular septum, at the level of the bundle of His (BH) and bundle branches (BBs) (Fig. 1Ad). These cells colocalize with a marker of cardiomyocytes, tropomyosin (Fig. 1Ba), but not with the endocardial marker PECA1 (Pecam1) (Fig. 1Bb). Irx5 was detected in PECA1 + cells lining the ventricular trabeculae and septum (Fig. 1Ab, arrowheads) that did not express Irx3 (Fig. 1Ac). In addition, we identified a set of PECA1 + cells lining the AV cushions that coexpressed Irx3 and Irx5 (Fig. 1Af, arrowheads). Fig. 1C illustrates the expression profile of Irx3 and Irx5 at E12.5. By E14.5, coexpression of Irx3 and Irx5 broadens to include tropomyosin+ cells of the ventricular trabeculae (Fig. 1Da-c, arrowheads) as well as BH and BBs (Fig. 1Dd-f, arrowheads). Fig. 1F summarizes the expression pattern of Irx3 and Irx5 at E14.5.

Cardiac defects in Irx3/Irx5 DKO embryos
Irx3 and Irx5 are located within 600 kb of each other on mouse chromosome 8. To create DKOs, the two genes must be targeted in cis. We used an allele that included a replacement of Irx5 with EGFp and a conditional deletion of Irx3 by flanking exons 2-4 with loxP sites. Crossing the Irx3fllox/Irx5EGFP/Irx3fllox Irx5EGFP to a mouse line that expresses Cre recombinase in the germ line, we obtained heterozygous mice (Irx3±, Irx5±/Irx3KO Irx5EGFP) that were viable and fertile. The mating of these mice did not yield...
homozygous offspring, suggesting that the *Ir*3;*Ir*5 DKO was embryonic lethal. Western blot analysis confirmed the absence of *Ir*3 and *Ir*5 proteins in the DKO embryos at E11.5 (R.S. and C.-C.H., unpublished). To determine the onset of embryonic lethality, we collected and genotyped embryos from *Ir*3+/+ *Ir*5+/+/*Ir*3KO *Ir*5EGFP matings at different stages of development. At E14.5, the ratio of the expected genotypes showed normal Mendelian transmission (Table 1). However, from E15.5 onwards, the *Ir*3KO *Ir*5EGFP genotype was not represented, suggesting lethality of *Ir*3;*Ir*5 DKO embryos after E14.5.

Given that *Ir*3 and *Ir*5 are coexpressed in the heart and regulate cardiac function, we determined whether embryonic lethality of the *Ir*3;*Ir*5 DKO mice was associated with cardiac structural defects. We produced 3D reconstructions of embryonic hearts by optical projection tomography (OPT) and examined serial transverse sections of wild-type and DKO embryos at several developmental stages. Cardiac malformations were first observed at E11.5. OPT analysis showed an improper orientation of the OFT (Fig. 2A,B). By E12.5, transverse sections revealed a misalignment of the OFT, such that the aorta was abnormally oriented toward the right ventricle (RV) (Fig. 2C,D). Although the growth of the muscular ventricular septum was normal at this stage (Fig. 2E,F), a defect was observed at the level of the atrial septum, with an absence of the dorsal mesenchymal protrusion (DMP) and septum primum (Fig. 2G,H, asterisk). At E14.5, DKO hearts demonstrated an abnormal arrangement of the aorta alongside the pulmonary artery, leading to a double-outlet right ventricle (DORV) (Fig. 2I-L). In addition, we observed a membranous ventricular septal defect (VSD) at the level of the AV canal (Fig. 2M,N, arrowhead) at E12.5, a defect in DMP formation, an atrial septal defect (ASD), and defects in the growth of septa primum and secundum (Fig. 2O,P, asterisk).

Table 1. Lethality of *Ir*3;*Ir*5 DKO embryos after E14.5

<table>
<thead>
<tr>
<th>Stage</th>
<th><em>Ir</em>3+/+;<em>Ir</em>5+/+</th>
<th><em>Ir</em>3+/–;<em>Ir</em>5+/–</th>
<th><em>Ir</em>3–/–;<em>Ir</em>5–/–</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>E10.5</td>
<td>25</td>
<td>45</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>E12.5</td>
<td>24</td>
<td>54</td>
<td>22</td>
<td>50</td>
</tr>
<tr>
<td>E14.5</td>
<td>25</td>
<td>53</td>
<td>22</td>
<td>77</td>
</tr>
<tr>
<td>E15.5</td>
<td>30</td>
<td>65</td>
<td>4 (resorbed)</td>
<td>23</td>
</tr>
</tbody>
</table>

Percentage of embryos of each genotype at different embryonic stages. At E15.5, *Ir*3+/-;*Ir*5+/- embryos were either absent or resorbed (n=1) with still enough embryonic material for genotyping. n, number of embryos analyzed.
These findings demonstrate that Irx3 and Irx5 function redundantly in cardiac formation. The location and timing of Irx3 and Irx5 activity required for OFT development are not known. Although it is unclear whether the morphological defects alone result in lethality, altered cardiac function is a possible contributing factor.
Cardiac morphogenesis requires endocardial Irx3 and Irx5 expression

Our data demonstrate that Irx3 and Irx5 are coexpressed in myocardial and endocardial cells at different embryonic stages. In addition, they might also be expressed in cardiac neural crest cells (Houweling et al., 2001). To determine the cellular origin of the DKO heart defects, we deleted Irx3 in each of these cardiac subdomains, in the background of an Irx5 deletion. We employed the cis-targeted alleles of Irx3^flox Irx5^EGFP/Irx3^flox Irx5^EGFP to delete Irx3 conditionally with different Cre-expressing mouse lines, and compared the phenotypes at the level of the OFT and AV canal, with those of Irx3^+/Irx3^+/Irx5^+/Irx5^+ (Fig. 3A,B) and Irx3^flox Irx5^EGFP/Irx3^flox Irx5^EGFP embryos (Fig. 3C,D). Cre-mediated deletion results in complete loss of Irx3 protein 36-48 hours after the onset of Cre activity (R.S. and C.-C.H., unpublished).

Crossing with Wnt1::Cre (Brault et al., 2001), which deletes in the neural crest cells that populate the heart and contribute to the development of the OFT, did not result in any structural abnormalities in the heart (Fig. 3E,F). The OFT but not the AV canal defect was recapitulated using Mef2cAHF::Cre mice, which express Cre in the myocardial and endocardial cells of anterior heart field derivatives (Verzi et al., 2005), which have been linked to OFT defects, such as DORV (Fig. 3G,H) (Park et al., 2006). To further delineate the specific cell type in which Irx3 and Irx5 are required, we used the Nkx2.5::Cre transgene to delete these genes in the ventricular myocardium (McFadden et al., 2004; Takeuchi et al., 2011) and Tie2::Cre (Tie2 is also known as Tek – Mouse Genome Informatics) to delete in the endocardium (Kisanuki et al., 2001). Whereas the myocardial-specific deletion resulted in no morphological defects (Fig. 3I,J), deletion of Irx3 and Irx5 in the endocardium phenocopied the DKO mutant (Fig. 3K,L), with the exception of the DMP defect (Fig. 3L, dashed line). Indeed, the ASD observed using Tie2::Cre (Fig. 3L, asterisk) seemed to be due to a defect in the fusion between the AV endocardial cushions and the DMP. In an attempt to understand the cellular origin of the DMP defect and given that the DMP derives from Isl1-expressing second heart field derivatives (Goddeeris et al., 2008), we used Isl1::Cre mice, which effectively delete within all second heart field derivatives (Yang et al., 2006). Interestingly, this conditional mutant recapitulated the DKO phenotype, including the lack of DMP, with an additional defect in the tissues targeted in this Cre line in the form of a persistent truncus arteriosus (Fig. 3M, arrowhead), suggesting a genetic interaction between Irx3, Irx5 and Isl1 (Fig. 3M,N) or that deletion by Isl1::Cre in neural crest cells (Engleka et al., 2012) results in an additional phenotype.

Our data demonstrate that the deletion of Irx3 and Irx5 in endocardial cells leads to defects in septum formation and OFT alignment, and that there is an additional function for Irx3 and Irx5 in the development of DMP.

BMP signaling is misregulated in Irx3;Irx5 mutant hearts

To identify genes that are misregulated in the Irx3;Irx5 DKO mutant, we used DNA microarrays to analyze mRNA expression in E12.5 embryonic hearts. At this developmental stage, Irx3 and Irx5 are coexpressed in the endocardium lining the AV cushions,
and there are no detectable cardiac abnormalities, such that altered
gene expression is therefore likely to reflect early molecular events
in altered OFT development. EGFP-positive cells were isolated
from dissected hearts using fluorescence-activated cell sorting
(FACS) and analyzed for four groups of mice: Irx3+Irx5EGFP/Irx3+
Irx5− (Irx5het), Irx3KO Irx5EGFP/Irx3KO Irx5+ (Irx3KO;Irx5het),
Irx3+Irx5EGFP/Irx3KO Irx5− (Irx3KO) and Irx3KO Irx5EGFP/Irx3KO
Irx5EGFP (Irx3;Irx5 DKO) (Fig. 4A). Owing to the need for FACS
sorting from the Irx5EGFP allele, all genotypes must be at least
heterozygous for loss of Irx5.

We found 859 genes that were differentially expressed in at least
one group versus the wild-type group, with the largest number of
misregulated genes in the DKO group. Irx3 and Irx5 act mainly as
transcriptional repressors (Costantini et al., 2005; Zhang, S. S. et
al., 2011). Accordingly, almost two-thirds of the genes
misregulated in DKO hearts are upregulated compared with the
Irx5het group (Fig. 4B). Unsupervised two-way hierarchal
clustering used to group samples based on their gene expression
similarities (Fig. 4C) clearly separated DKOs from other samples,
showing that they have a distinct transcriptional expression
signature. Two discriminatory gene clusters were identified. Cluster
A grouped genes downregulated in DKO samples as compared
with the other samples and cluster B grouped upregulated genes.

Within these two clusters, ten genes are known to be implicated in
OFT and vascular defects: Eln, Efnb2, Fli1 (Kdr – Mouse Genome
Informatics), Flt1, Angpt2, Slit2, Ecsdr, Adamts9, Bmp10 and
Vcan. Several genes that have been linked to AV canal defects,
including Bmp2, Bmp5, Bmp10, Tgfrb3 and Runx111, were also
significantly changed (Fig. 4C). Misexpression was confirmed by
qPCR. Bmp10 was the most overexpressed (Fig. 4D). Interestingly,
several genes linked to the BMP signaling pathway, such as Sfrp2,
Tbx20 and Foxb, were also misexpressed, consistent with BMP
upregulation (Fig. 4E).

Irx3 and Irx5 coordinate regulate Bmp10
expression
To define the mechanism responsible for misregulation of Bmp10
in the Irx3;Irx5 DKO heart, we performed in situ hybridization for

Fig. 4. Dysregulation of cardiac
genes in Irx3 and Irx5 mutant
embryos. (A) Genotypes used for
microarray. EGFP-positive cells
were isolated by FACS. (B) Number of genes statistically
(P<0.01) upregulated
(orange) and downregulated (blue) in
each mutant as compared with wild
type. (C) Unsupervised two-way
hierarchal clustering applied to the
wild type, Irx3KO, Irx5KO and
Irx3;Irx5 DKO samples (horizontally) and the
458 statistically differentially expressed
genes (vertically). Input consists of
replicate expression values for each
gene and sample. Gene expression
values were centered to the median.
Therefore, each color patch represents
fold change between expression value
and the median, with a continuum
from bright blue (lowest) to bright
yellow (highest). Two clusters (A and
B) containing genes relevant to the
discrimination between Irx3;Irx5 DKO
and the other genotypes are shown
on the right. (D) qPCR confirmation of
a number of genes identified by
microarray as either downregulated,
upregulated or unchanged in the
Irx3;Irx5 DKO. Values are mean ± s.d.
a, P<0.05; b, P<0.01; c, P<0.001; in
Irx3;Irx5 DKO versus wild type. (E) The
BMP pathway and genes described as
upstream or downstream components
of this pathway. Red arrows illustrate
how these genes are misregulated in
the Irx3;Irx5 DKO embryos according to
the microarray data.
Bmp10 at E12.5 and E14.5. At E12.5, Bmp10 was restricted to the ventricular trabecular myocardium, where Irx3 alone is expressed. Indeed, expression of Bmp10 was increased in ventricular trabecular myocardium of the Irx3KO but not the Irx5KO (Fig. 5Aa). In Irx3;Irx5 DKO hearts, we observed upregulation of Bmp10 in tissues that express either Irx3 or Irx5, including

![Fig. 5. Regulation of Bmp10 by Irx3 and Irx5. (A) Bmp10 expression was examined by in situ hybridization on transverse sections of wild-type, Irx3KO, Irx5KO and Irx3;Irx5 DKO mouse hearts at E12.5 and E14.5 (Aa,b). Summaries of the expression pattern of Irx3 and Irx5 at E12.5 and E14.5 are shown to the right. Immunofluorescence of Smad1/5/8 on wild-type and Irx3;Irx5 DKO E14.5 hearts (Ac,d). Arrows indicate areas of increased Bmp10 expression. Arrowheads indicate Bmp10-expressing endocardium. (B) Change in Bmp10-luciferase reporter construct activity by transfection of Irx3 and/or Irx5 and/or Tbx5 expression constructs into Cos7 cells. Values are mean ± s.d. b, P<0.01; c, P<0.001; versus Luc alone. ***P<0.001, versus Tbx5 (ANOVA). Tbx5 BS, Tbx5 binding site. (C) SYBR Green PCR amplification (red arrows indicate primers) of an intergenic region and of the Iroquois binding site of the Bmp10 promoter, after chromatin immunoprecipitation of Irx3 (left) or Irx5 (right) on E12.5 and E14.5 wild-type hearts. a, P<0.05; c, P<0.001; versus intergenic region (t-test). (D) Serial sections stained with H&E at the level of the OFT (top row), interventricular septum (middle row) and AV canal (bottom row). Sections of a wild-type embryo and of two ca-Bmpr1a embryos crossed with either Isl1::Cre or Tie2::Cre. Arrowhead indicates ventricular septal defect. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; OFT, outflow tract; Ao, aorta; PA, pulmonary artery; IVS, interventricular septum; AV, atrioventricular; DMP, dorsal mesenchymal protrusion.]


myocardium and endocardium (arrowheads) of ventricular trabeculae (Fig. 5Aa). We also observed Bmp10 upregulation in the cardiac domain that coexpresses Irx3 and Irx5, including the endocardium lining the AV cushions, consistent with the observation that the endocardial-specific DKO recapitulates the cardiac phenotype (Fig. 5Aa). At E14.5, Irx3 and Irx5 are coexpressed in the ventricular conduction system and trabeculae, and concordantly Bmp10 expression is increased in these cellular compartments in Irx3;Irx5 DKO hearts (Fig. 5Ab).

We investigated whether the pathway downstream of Bmp10 was affected in Irx3;Irx5 DKO heart. Bmp10 induces Smad1/5/8 phosphorylation (pSmad1/5/8), which leads to Smad translocation to the nucleus to mediate its signaling. By immunofluorescence, we found that the amount and intensity of nuclear pSmad1/5/8 were higher in the DKO ventricular trabeculae, suggesting that Bmp10 activates Smad1/5/8 in the Irx3;Irx5 DKO hearts (Fig. 5Ac,d).

We then investigated the function of Irx3 and Irx5 in Bmp10 transcription. We analyzed a 1.1 kb region of the Bmp10 promoter, located ~1.7 kb upstream of the ATG, which contains a putative consensus Irx binding site (Bilioni et al., 2005). Luciferase analysis showed that Irx3 and Irx5 reduced the baseline activity of this promoter. We also identified two T-box binding sites within the 1.1 kb Bmp10 promoter. Tbx5 activated the promoter and cotransfection of Irx3 and Irx5 expression constructs prevented this activation. These results show that Irx3 and Irx5 individually or cooperatively repress the expression of Bmp10 and that they prevent activation of this promoter by Tbx5 (Fig. 5B).

To explore the possibility that Irx repression of Bmp10 occurs via direct binding, we performed chromatin immunoprecipitation of Irx3 and Irx5 in E12.5 and E14.5 hearts. We tested for enrichment of Irx3 and Irx5 at the putative Irx binding site located in the 1.1 kb promoter region used for the luciferase assay. Irx3 levels at the Bmp10 promoter were double those at an intergenic region at E12.5 and E14.5 and there was 5-fold and 32-fold more Irx5 in E12.5 and E14.5 hearts, respectively (Fig. 5C). These results show that Irx3 and Irx5 directly bind the Bmp10 promoter to regulate its expression.

We then further investigated the role of the ectopic endocardial expression of Bmp10 in the Irx3;Irx5 DKO phenotype. Knowing that Bmp10 signals through the Bmpr1a receptor (Mazerbourg et al., 2005), we used a genetic manipulation that would be a proxy for increased Bmp10 expression: we induced cell-specific constitutive activation of Bmpr1a by crossing ca-Bmpr1a mice (Kamiya et al., 2008) with Isl1::Cre or Tie2::Cre mice. At E14.5, the mutant embryos did not display any gross physical abnormalities (data not shown). However, in both cases, expression of ca-Bmpr1a led to a membranous VSD similar to that observed in the Irx3;Irx5 DKO (Fig. 5Db). Conversely, the orientation of the aorta (Fig. 5Da) and the formation of DMP (Fig. 5Da) appeared normal in these mutants. Together, these data further support the proposal that the endocardial transcriptional repression of Bmp10 by Irx3 and Irx5 is necessary for proper ventricular septation.

Irx3 and Irx5 are coexpressed in the adult heart

The effect of the combined deletion of Irx3 and Irx5 on embryonic heart development clearly indicates overlapping and redundant functions of these two transcription factor genes. As both genes have demonstrated roles in postnatal cardiac physiology, we addressed their roles independent of defective embryonic development. We first analyzed their expression pattern in the adult heart and found that Irx3 and Irx5 are coexpressed in the ventricular conduction system, BH and BBs (Fig. 6A-C), as identified by acetylcholinesterase staining (Fig. 6D). The domain of coexpression of Irx3 and Irx5 in the ventricular conduction system extends to the lower nodal cells (LNCs) that connect the AV node to the BH (Fig. 6E-H) (Aanhaenen et al., 2009). This suggests a shared role for Irx3 and Irx5 in the function of the conduction system. Within the ventricular septum, Irx3 and Irx5 were expressed in a gradient, specifically in myocardial cells, as shown by their coexpression with tropomyosin (Fig. 6I-L). This gradient of expression was found in the myocardium of the right and left ventricular walls, with highest expression in the endocardial region (Fig. 6M-T).

Coexpression of Irx3 and Irx5 is necessary for cardiac function

To address the combinatorial roles of Irx3 and Irx5 in the adult heart, we bypassed the embryonic lethality and cardiac structural defects by inducing the deletion of Irx3 in the postnatal heart with concomitant loss of Irx5 function. We used the Myh6-Cre/Esr1 mouse strain, which expresses the Cre-estrogen receptor fusion protein under control of the cardiac myocyte-specific Myh6 promoter (Sohal et al., 2001), and activated the Cre with five consecutive tamoxifen injections. We confirmed that our protocol reduced the number of Irx3 transcripts (Fig. 7A). Telemetry electrocardiogram analysis of 9-week-old WT (Irx3+ Irx5+/Irx3;Irx5−) mice was restored to normal in the inducible DKO (Fig. 7B, red arrow), suggesting a counterbalance of Irx3 and Irx5 function in regulating the ventricular repolarization. The DKO mice were uniquely characterized by a prolonged PR interval, reflecting a conduction delay through the proximal part of the ventricular conduction system, at the level of the AV node, BH and/or BBs (Fig. 7C). No spontaneous arrhythmias were observed in any of the genotypes throughout the telemetry traces (data not shown). These results clearly show a postnatal genetic interaction between Irx3 and Irx5.

Cardiac electrophysiological activity is a reflection of the combined function of multiple ion channels and gap junctions. Thus, failure in their normal activity leads to changes in the ECG waveforms and ultimately to various types of inherited arrhythmia syndromes. We analyzed the expression pattern of several ion channel and gap junction proteins that sculpt the ECG features affected in the DKO mice. The prolongation of the QRS in the Irx3;Irx5 DKO mice is due to the deletion of Irx3 only. Reduction in the levels of the sodium channel protein Nav1.5 have been implicated in PR prolongation (Papadatos et al., 2002; Smits et al., 2002; Royer et al., 2005; Pfeuffer et al., 2010), and we found that its expression was much reduced in the BH in DKO mice compared with the other genotypes (Fig. 7E). Therefore, Irx3 and Irx5 have individual roles in some aspects of the conduction system, as well as redundant functions in regulating Nav1.5 expression.

Restoration of the repolarization wave in the ECG of the DKO mice indicated a more complicated relationship between Irx3 and Irx5.
This was borne out by molecular analysis of components of the repolarization gradient. As reported, loss of the T wave in the Irx5KO was associated with the loss of gradient in the potassium channel Kv4.2 (Kcnd2 – Mouse Genome Informatics) within the ventricular wall. We found that this gradient was maintained in the Irx3KO and was recovered in Irx3;Irx5DKO mice, in which the T wave is present (Fig. 7F). This indicates that Irx5 normally prevents Irx3 from activating the expression of Kv4.2 and that, in the absence of both Irx factors, the normal pattern of Kv4.2 expression is maintained, suggesting the existence of additional activating or repressing factors that control the repolarization gradient.

**DISCUSSION**

We showed essential redundant roles of Irx3 and Irx5 in the developing mouse heart. Their overlapping expression and functional redundancy in the endocardium ensure completion of atrial and ventricular septation and proper positioning of the great arteries. In addition, our data show that continued expression of Irx3 and Irx5 in adult cardiomyocytes is necessary for proper cardiac electrophysiological activity. Thus, we found two temporalspecific roles for Irx3 and Irx5 in the heart: control of embryonic morphogenesis and regulation of adult heart function.

**Functional redundancy of mammalian Irx transcription factors during embryogenesis**

We demonstrated genetic redundancy of Irx factors, as the combined deletion of Irx3 and Irx5 leads to striking structural heart defects not observed in the Irx3 or Irx5 singly deficient mice. Recapitulation of the abnormal septation phenotype with the endothelial-specific Tie2::Cre strongly suggests functional significance for both Irx3 and Irx5 in the endothelial cell lineage. This indicates a sensitive subset of cardiac cells within which the redundancy between Irx3 and Irx5 is most apparent, and suggests that other Irx genes expressed in myocardium might compensate for their combined loss in the myocardium.

**Fig. 6. Expression of Irx3 and Irx5 in the adult heart.** (A-C) Immunofluorescence colocalization of Irx3LacZ (red) and Irx5EGFP (green) in the ventricular conduction system of Irx3LacZ+/Irx5EGFP+ adult mouse heart. (E-G) Coexpression of Irx3 and Irx5 in the lower nodal cells (LNCs) that connect the AV node to the BH. (D,H) Acetylcholinesterase staining (brown) of serial sections marks the conducting cells and confirms the expression of Irx3 and Irx5 in the ventricular conduction system. (I-L) In the interventricular septum, Irx3 and Irx5 are expressed in a gradient and colocalize with tropomyosin (L). (M-T) Immunofluorescence at the level of the left ventricular wall (M-P) and the right ventricular wall (Q-T) showing a gradient of expression of Irx3 and Irx5, with higher expression in the endocardial region that colocalizes with tropomyosin. BH, bundle of his; RBB, right bundle branch; LBB, left bundle branch; RV, right ventricle; LV, left ventricle; IVS, interventricular septum; MV, mitral valves; TV, tricuspid valves; RVC, right ventricular chamber; LVC, left ventricular chamber.
The Irx3;Irx5 DKO mice also have a DMP defect. DMP derives from Isl1-expressing progenitors but is not endothelial-derived (Mommersteeg et al., 2006), suggesting that the DMP deficiency is not directly due to the Irx3 and Irx5 deficiency in the AV cushion endocardium. As expected, the DMP is present when endocardial Irx3 expression is removed using Tie2::Cre, but it fails to fuse to the AV cushions, leading to an ASD. Conversely, the use of Isl1::Cre recapitulates the absence of the DMP.

Dysregulation of a broad transcriptional program in the DKOs further demonstrates the functional redundancy between Irx3 and Irx5. Among these genes, we identified several that are implicated in OFT and AV canal formation, including members of the BMP family, which are upregulated in the Irx3;Irx5 DKO hearts. These growth factors have crucial roles in the embryonic development of several organs, including the heart: their overexpression, deletion or mutation leads to various cardiac defects (Evans et al., 2010). A link between Iroquois genes and BMPs was reported in Xenopus, in which the ortholog of mouse Irxl inhibits Bmp4 expression during neural differentiation (Gomez-Skarmeta et al., 2001).
Here we show that, in the mammalian heart, Irx3 and/or Irx5 directly inhibits the expression of Bmp10. In the Irx3;Irx5 DKO E12.5 hearts, Bmp10 is misexpressed in the endocardial cells that line the ventricular trabeculae and the endocardial cushions. The ectopic expression of Bmp10 in the endocardium supports our model of redundancy between Irx3 and Irx5 in repressing expression of this gene. By E14.5, however, Bmp10 returns to its myocardial-specific pattern, consistent with the restricted expression of Irx3 and Irx5 to myocardium at this stage. Bmp10 regulates cardiac morphogenesis. Bmp10-deficient mice do not survive past E10.5 due to a range of cardiovascular defects, including abnormal OFT and AV endocardial cushion development (Chen et al., 2004). Myocardial-specific Bmp10 overexpression leads to normal embryonic development but abnormal postnatal cardiomyocyte hypertrophic growth and subaortic narrowing (Chen et al., 2006). As proxy for the overexpression of Bmp10, we tested the effect of activating BMP signaling in the endocardium, and found a defect in cardiac ventricular septation, suggesting that ectopic endocardial expression of Bmp10 in the Irx3;Irx5 DKO contributes to the phenotype. This effect is specific, as we found that activation of BMP signaling within a tissue that is not known to be directly dependent on BMP signaling (in the DMP using Isl1;Cre) does not lead to defects in its formation.

The T-box transcription factor Tbx20 is a direct target of the Bmp10/Smad1 signaling pathway, and is upregulated in the myocardium of αMHC-BMP10 transgenic mice (Mandel et al., 2010; Zhang, W. et al., 2011). During embryonic development, Tbx20 is strongly expressed in the atria and the endocardium lining the AV cushions and exhibits more modest expression in the ventricular myocardium (Zhang, W. et al., 2011). A Tbx20 gain-of-function mutation in human causes AV canal defects including ASD (Posch et al., 2010), and Tbx20 is upregulated in patients with tetralogy of Fallot (Hammer et al., 2008). We observed an increase in nuclear staining of Smad1/5/8 in Irx3;Irx5 DKO hearts and upregulation of Tbx20 in the endocardial sorted cells of the Irx3;Irx5 DKO hearts as compared with control hearts. This suggests that, during cardiac development, Irx3 and Irx5 might participate in a BMP-dependent pathway that represses Tbx20 expression in the endocardium, ensuring proper OFT and AV canal formation.

**Redundant and unique regulation of adult electrophysiological activity by Irx genes**

Ir3 and Irx5 singly deficient mice are characterized by specific defects in adult electrophysiological activity of the heart, suggesting that, unlike their role in embryonic heart morphogenesis, these transcription factors are not fully redundant in the regulation of postnatal electrical activity. The deletion of Irx3 slows and disorganizes the ventricular conduction of the electrical influx through reduction of Cx40 and ectopic expression of Cx43 (Gja1 – Mouse Genome Informatics) in the proximal BBs (Zhang, S. S. et al., 2011). Irx5-deficient mice exhibit abnormal cardiac ventricular repolarization due to a defect in the establishment of the epicardium-to-endocardium gradient of the ion channel Kv4.2 and its current I_{Ks} (Costantini et al., 2005).

Bypassing the embryonic cardiac structural heart defects, we created a postnatal Irx3- and Irx5-deficient mouse and observed several electrophysiological defects. The prolongation of the QRS complex was found in both Irx3;KO and Irx3;Irx5 DKO hearts, and, accordingly, expression of Cx40 was reduced in the BH in these two genotypes. This suggests that the prolongation of the QRS is due specifically to the lack of Irx3 and that Irx5 does not significantly contribute to regulation of Cx40 expression. One characteristic of the DKO ECG that has not been observed in the single KOs is the prolongation of the PR interval, and, together with this result, we found a DKO-specific reduction in expression in the sodium channel Nav1.5 in the BH. This PR prolongation might be due to a delay in conduction through the atria, the AV node and/or the BH. Increase in duration of the PR interval has been described previously in mice heterozygous for a deletion of the Nav1.5 (Scn5a) gene (Papadatos et al., 2002; Royer et al., 2005). Furthermore, Brugada syndrome patients carrying a loss-of-function mutation in SCN5A have a prolonged PR interval associated with a delay in BH to ventricular conduction (Smits et al., 2002). In the adult, Irx3 and Irx5 are coexpressed in the ventricular conduction system, including LNCs and BH, but are not expressed in the AV node, suggesting that the PR prolongation is due to the downregulation of Scn5a in the BH domain. The additional reduction of expression of Cx40 might also contribute to this phenotype.

Surprisingly, the lack of a T wave in the Irx5-deficient mouse was not observed in Irx3;Irx5 DKO mice, suggesting a rescue of the cardiac repolarization gradient. We analyzed the expression pattern of Kv4.2, which underlies the I_{Ks} current essential for the formation of the T wave. Whereas its ventricular gradient of expression was lost in the Irx3;KO mice, it was present in Irx3;KO mice and recovered in the Irx3;Irx5 DKO mice. This suggests antagonistic effects between Irx3 and Irx5 in the regulation of this ion channel. Consistent with this notion, an in vitro study in the rat showed that Iroquois genes have antagonistic effects in the regulation of the Kv4.2 gene, as Irx4 suppresses the effect of Irx5 on its promoter (He et al., 2009). The present expression analysis suggests that, in the subendocardial myocardium, Kv4.2 expression is regulated by several factors, with different levels of control. Concordant with previous reports, in wild-type and Irx3;KO mice, the dominant regulator, Irx5, inhibits Kv4.2 expression to create the gradient (Costantini et al., 2005). A possible explanation for this phenotype is that, in the Irx3;KO mice, Irx3 is the transcriptional regulator that is likely to take over, upregulating this ion channel in the subendocardial myocardium, abolishing its expression gradient.

When both Irx3 and Irx5 are absent, we propose that another, as yet unidentified, factor with a similar endocardium-to-epicardium gradient of activity inhibits the expression of Kv4.2, recreating its gradient. In pathological situations, other transcription factors regulate the expression of Kv4 ion channels, such as Gata4- and Gata6-Fog2 (Zifpm2) and Calcineurin/NFATc3 (Jia and Takimoto, 2003; Rossow et al., 2004). It will be interesting to investigate whether one of these pathways inhibits Kv4.2 expression and recreates its gradient of expression in the absence of Irx5 and Irx3.

**Summary**

We have shown that Irx3 and Irx5 function redundantly in heart development and both redundantly and antagonistically in regulating postnatal cardiac electrophysiology. Recessive IRX5 mutations result in cardiac structural and electrophysiological defects as part of Hamamy syndrome (Bonnard et al., 2012). It is not clear whether the human IRX5 mutations lead to simple loss of IRX5 function or whether they cause a broader dominant effect, but the disparity between mutations in IRX5 and the mouse Irx5 loss of function might also be explained by differences in the function of Irx5 in mouse and human. Nonetheless, the similarities in phenotype between the Irx3;Irx5 DKO mice described here and the cardiac features of Hamamy...
syndrome provide a direct link to human disease and might lead to new insights into the as yet poorly understood etiology of congenital heart defects (Bruneau, 2008).

Acknowledgements

We thank Linda Ta (Gladstone Genomics Core), Alex Williams (Gladstone Bioinformatics Core) and Caroline Miller (Gladstone Histology Core) for technical support and G. Howard for editorial assistance.

Funding

N.G. was funded by American Heart Association fellowships. This work was funded by grants from the Canadian Institutes of Health Research (C.-C.H.), the National Institutes of Health (NIH) National Heart, Lung, and Blood Institute (NHLBI) [R01 HL93414 ARRA to B.G.B.]; and the Lawrence J. and Florence A. DeGeorge Charitable Trust/American Heart Association Established Investigator Award [to B.G.B.]. This work was also supported by an NIH National Center for Research Resources (NCRR) grant (C06 RR018928) to the J. David Gladstone Institutes and by William H. Younger, Jr [B.G.B.]. Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

References


Development 139 (21)


