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Engineered chromosome-based genetic mapping establishes a 3.7-Mb critical genomic region for Down syndrome-associated heart defects in mice

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Abstract

Trisomy 21 (Down syndrome, DS) is the most common human genetic anomaly associated with heart defects. Based on evolutionary conservation, DS-associated heart defects have been modeled in mice. By generating and analyzing mouse mutants carrying different genomic rearrangements in human chromosome 21 (Hsa21) syntenic regions, we found the triplication of the *Tiam1-Kcnj6* region on mouse chromosome 16 (Mmu16) resulted in DS-related cardiovascular abnormalities. In this study, we developed two tandem duplications spanning the *Tiam1-Kcnj6* genomic region on Mmu16 using recombinase-mediated genome engineering, *Dp(16)3Yey* and *Dp(16)4Yey*, spanning the 2.1Mb *Tiam1-Il10rb* and 3.7Mb *Ifnar1-Kcnj6* regions, respectively. We found that *Dp(16)4Yey*/+, but not *Dp(16)3Yey*/+, led to heart defects, suggesting the triplication of the *Ifnar1-Kcnj6* region is sufficient to cause DS-associated heart defects. Our transcriptional analysis of *Dp(16)4Yey*/+ embryos showed that the Hsa21 gene orthologs located within the duplicated interval were expressed at the elevated levels, reflecting the consequences of the gene dosage alterations. Therefore, we have identified a 3.7Mb genomic region, the smallest critical genomic region, for DS-associated heart defects, and our results should set the stage for the final step to establish the identities of the causal gene(s), whose elevated expression(s) directly underlie this major DS phenotype.

Keywords

Down syndrome; trisomy 21; heart defects - congenital; chromosome engineering; mouse models for human genetic disease; genetic mapping

Introduction

Trisomy 21 (Down syndrome, DS) is the most frequent live-born aneuploidy in humans (Epstein 1986; Hassold and Hunt 2001; Roizen and Patterson 2003). In the U.S., it occurs in 1 in every 691 live births, affecting approximately 6,000 newborns per year (Parker et al. 2010). A recent review has shown that, after prenatal diagnosis, termination rates stood at 67% and 85% in the U.S. from population-based studies with 2,593 pregnancies and hospital-based studies with 779 pregnancies, respectively. Evidence suggests that termination rates have decreased in recent years, partly due to improved medical care and social support for DS individuals (Natoli et al. 2012). Trisomy 21 is the most common genetic anomaly associated with congenital heart defects. Although individuals with DS have multiple medical problems, the single greatest risk factor for death during infancy is heart defects (Brookes and Alberman 1996). Heart defects are detected in 40-60% of newborns with DS (Abbag 2006; Freeman et al. 2008; Goodship et al. 1998; Roizen and Patterson 2003; Rowe and Uchida 1961; Torfs and Christianson 1998; Vis et al. 2009). The most frequent heart defects associated with DS are atrioventricular septal defect (AVSD) (23-45%), ventricular septal defect (VSD) (33-43%) and atrial septal defect (17-42%). Other

important heart defects include tetralogy of Fallot (TOF), defects associated with valves, aorta, and pulmonary artery (Torfs and Christianson 1998). AVSD is the most frequent cardiac defect in DS in some studies, while VSD is more prevalent in other studies (Abbag 2006; Kava et al. 2004; Paladini et al. 2000). The mechanism underlying DS-associated heart defects is unknown. Although rare genetic variants located outside Hsa21 may play a role in enhancing the frequency, no variants can explain why such a high percentage of newborns with DS have heart defects. Thus, for a majority of DS individuals with heart defects, an extra copy of Hsa21 is necessary and sufficient to cause this phenotype. This is supported by heart defects observed in mouse models. The prevailing hypothesis is that heart defects, like other DS phenotypes, are caused by the dosage increase of a critical gene(s) on Hsa21 (Epstein 1990). It is possible that altered expression level of the critical gene(s) affects one or more key pathways, which in turn results in abnormal heart development as observed in other genetic disorders associated with the cardiovascular system (Jiang et al. 2013; Moskowitz et al. 2011; Terada et al. 2011; Zhang et al. 2006). Although we could seek to identify the disturbance of potentially relevant pathways, it will be strategically most desirable if we could identify the critical gene(s) first. This is because the ensuing analysis of the biological consequences of the triplication of the critical gene(s) would be considerably more focused than the analysis of abnormalities of pathways before the identities of the critical gene(s) is established. Many pathways may be affected by other genes present in three copies that are not causal gene(s) for heart defects. For this reason, human geneticists have, for the last several decades, pursued this critical gene(s) by identifying and analyzing patients carrying segmental trisomy 21. Due principally to the small number of patients with segmental trisomy 21 and to the resulting lack of a complete and informative set of human segmental trisomies, these research efforts have not yet led to identifying the causal gene(s) underlying heart defects in DS (Korbel et al. 2009; Korenberg et al. 1994; Lyle et al. 2009; Sinet et al. 1994).

The genomic regions on Hsa21 are syntenically conserved in three regions in the mouse genome, which are located on mouse chromosome 10 (Mmu10), Mmu16 and Mmu17 (www.ensembl.org) (Fig. 1). Because of this evolutionary conservation, the mouse has become indispensable model organism for DS. To complement the human genetics approach, we are seeking to identify the critical genomic region associated with congenital heart defects in DS based on the syntenic conservation and the shared mutant phenotype of cardiovascular systems between human patients with DS and the mouse models in order to unravel the critical gene(s). In this study, we developed two duplication mouse mutants using recombinase-mediated chromosome engineering and phenotypic characterization of these mutants resulted in the identification of a 3.7-Mb Hsa21 orthologous region underlying congenital heart defects in DS, the smallest critical genomic region associated with this major DS phenotype (Fig. 1).

Materials and methods

Generation of *Dp(16Tiam1-Il10rb)Yey*/+ mice

To generate *Dp(16Tiam1-Il10rb)Yey*, MICER clones MHPN374h24 and MHPP321i13 (Adams et al. 2004) were used as the pTV*Tiam1*- and pTV*Il10rb*-targeting vectors for

inserting *loxP* to the endpoint 1 (EP1) and EP2. AB2.2 embryonic stem (ES) cell line (Bradley et al. 1998), which carries an *Hprt*-null allele, was used for targeting. The pTV*Tiam1* and pTV*Il10rb* vectors were linearized before gene targeting with restriction enzymes *Eco*NI and *Kpn*I, respectively, at the mouse genome homologous regions in the vectors. After targeting, 8 double targeted ES cell clones were isolated. A Cre-expression vector, pOG231 (O'Gorman et al. 1997), was transfected into these double-targeted ES cells to induce recombination between the two targeted *loxP* sites. This led to a duplication [*Dp(16Tiam1-Il10rb)Yey*] and a reciprocal deletion [*Df(16Tiam1-Il10rb)Yey*] in the mouse genome (Fig. 2a). We used Southern blot analysis to confirm the gene targeting as well as the chromosomal rearrangements (Fig. 2b-2c). The presence of *Dp(16)3Yey* and *Df(16)3Yey* was also confirmed by fluorescent *in situ* hybridization (FISH) (see below) (Fig. 2c). The ES cells carrying the desired genomic rearrangements were microinjected into blastocysts that were isolated from C57BL/6J females to generate germ-line transmitting chimeras. The procedural details of ES cell culture, gene-targeting and induction of Cre/*loxP*-mediated recombination, Southern blot analysis and injection of ES cells into blastocysts were described previously (Bradley 1987; Bradley et al. 1998; Ramirez-Solis et al. 1993; Ramirez-Solis et al. 1995).

Generation of *Dp(16Ifnar1-Kcnj6)Yey*/+ mice

To generate *Dp(16Il10rb-Kcnj6)Yey*/+ mice, MICER clones MHPN247l16 and MHPP54c08 were used as the pTV*Ifnar1*- and pTV*Kcnj6*-targeting vectors for inserting *loxP* to EP3 and EP4. The pTV*Ifnar1* and pTV*Kcnj6* vectors were linearized with restriction enzymes *Nhe*I and *Bae*I, respectively, before gene targeting. Afterwards, 8 double targeted clones were isolated. With Cre/*loxP*-mediated recombination between the two targeted *loxP* sites, duplication [*Dp(16Ifnar1-Kcnj6)Yey*] and the reciprocal deletion [*Df(16Ifnar1-Kcnj6)Yey*] between EP3 and EP4 were generated (Fig. 3a). Southern blot analysis and FISH were used to confirm the gene targeting as well as the presence of the chromosome rearrangements (Fig. 3b-3c). The procedures of ES cell culture, gene-targeting and induction of Cre/*loxP*-mediated recombination, Southern blot analysis and injection of ES cells into blastocysts were similar to those used for generating *Dp(16)3Yey*/+ mice (see above).

Fluorescent in situ hybridization

FISH analysis was performed as described previously (Yu et al. 2006). The metaphase chromosome spreads and interphase nuclei of ES cells were prepared as described previously (Robertson 1987). To detect the chromosomal deletion and duplication between *Tiam1* and *Il10rb* or *Ifnar1* and *Kcnj6*, BAC clone RP23-207L13 or RP23-4J4 were labeled with digoxigenin, respectively, and detected with anti-digoxigenin-rhodamine antibody (Figs. 2c and 3c). BAC clone RP23-81D13 was used to identify Mmu16 and labeled with biotin and detected with fluorescin isothiocyanate-avidin (Figs. 2c and 3c). Chromosomes were counter-stained with DAPI (4',6'-diamidino-2-2phenylindole).

Mice

The mutant mice and their wild-type littermates were maintained at a temperature- and humidity-controlled animal facility. The experimental procedures were approved by the Institutional Animal Care and Use Committee.

RNA extraction

RNA was extracted from the pharyngeal arch region and heart of E10.5 embryos with 129Sv background using PureLink RNA Micro kit (Invitrogen Corp., Carlsbad, CA). The boundaries of the pharyngeal arch region were defined as previously described (Prescott et al. 2005). Prior to the RNA extraction, the embryos were genotyped using yolk sac DNA. After the elution step, RNA samples were concentrated by precipitation and resuspended in DEPC-treated nuclease-free water. The quality of the RNA samples was assessed by a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

RNA sequencing and data analysis

For RNA-seq, the pharyngeal arch regions and hearts from 4 E10.5 embryos with the genotype of *Dp(16)4Yey*/+ or +/+ were pooled before RNA extraction. RNA-Seq libraries were prepared using the Illumina TruSeq Stranded Total RNA Sample Prep Kit. Each cDNA library was sequenced for 50 cycles (single end reads), which generated 50 base pair reads, in a single flow-cell lane on the Illumina HiSeq 2000 (Illumina) at the University at Buffalo Next-Generation Sequencing and Expression Analysis Core Facility. Thus, each read consists of a 50-base-pair sequence of a DNA fragment. The total number of reads was obtained from sequencing all the DNA fragments of each individual cDNA library [116 million reads for *Dp(16)4Yey*/+ embryos and 136 million reads for the wild-type control embryos]. The sequence reads were aligned to the annotated mouse genome (NCBI build37.1) using TopHat2 version 2.0.8 (Kim et al. 2013). The resulting alignments were further assembled using Cufflinks v2.1.1 (Trapnell et al. 2010). Cuffdiff 2 (Trapnell et al. 2013) was used to calculate the expression at the gene level and detect differentially expressed gene transcripts between *Dp(16)4Yey*/+ embryos and the wild-type control embryos. Briefly, library sizes were normalized using the geometric mean method. Transcript abundances were estimated from the counts of individual isoforms transcribed from genes and reported as the expected number of fragments per kilobase per million fragments mapped. Fold changes of expression at the gene-level were calculated.

Real-time quantitative reverse transcriptase PCR

Real-time quantitative PCR was used to analyze RNA levels of the selected genes. *Gapdh* is located on Mmu6 and served as a reference gene of the disomic state for all the mice examined. Total RNAs were isolated from the pharyngeal arch regions and hearts of E10.5 embryos, as described above. 1 μg of RNA from each embryo was used to generate cDNA by using Superscript version III reverse transcriptase (Invitrogen Corp., Carlsbad, CA). The specific primers and probes for the genes were obtained from the TagMan® Gene Expression Assays System of Applied Biosystems, Inc. A 0.5 μg of cDNA from each embryo was analyzed by ABI 7900HT Real-Time Thermocycler (Applied Biosystems, Foster City, CA) with the following amplification conditions: an initial activation and

denaturation at 95° C for 10 min, followed by 40 cycles of denaturation at 95° C for 15 sec and primer annealing and extension at 60° C for 1 min.

Results

Development of a mouse model carrying *Dp(16Tiam1-Il10rb)Yey* using chromosome engineering

We previously established that the triplication of the 5.43-Mb *Tiam1-Kcnj6* region on Mmu16, which contains 52 Hsa21 gene orthologs, is necessary and sufficient to cause heart defects (Fig. 1) (Liu et al. 2011b). To dissect the *Tiam1-Kcnj6* region, we, therefore in this study, first generated a new mouse model carrying a 2.11-Mb duplication between *Tiam1* and *Il10rb* within the *Tiam1-Kcnj6* region, which contain 17 Hsa21 gene orthologs. We developed this model using Cre/*loxP*-mediated chromosome engineering (Yu and Bradley 2001). MICER vectors (Adams et al. 2004) were used as pTV*Tiam1* and pTV*Kcnj6* for targeting *loxP* to the regions 403.6-Kb proximal and 75.8-Kb distal to the coding regions of *Tiam1* and *Il10rb*, respectively, in AB2.2 ES cells (Fig. 2a) (Bradley et al. 1998). A duplication and the reciprocal deletion were induced in ES cells by the transfection with a Cre expression vector as described (Liu et al. 1998; Ramirez-Solis et al. 1995) and were confirmed by Southern blot analysis and FISH (Fig. 2b-2c). We used these ES cell clones to generate chimeras. The germ-line transmission of the duplication after crossing C57BL/6J and 129Sv females with chimeric males was confirmed by Southern blot analysis. The duplication was designated as *Dup(16Tiam1-Il10rb)Yey*, abbreviated as *Dp(16)3Yey* or Ts5Yey. The deletion was designated as *Del(16Tiam1-Il10rb)Yey*, abbreviated as *Df(16)3/Yey* or Ms4Yey.

Development of a mouse model carrying *Dp(16Ifnar1-Kcnj6)Yey* using chromosome engineering

The genomic region surrounding a gene contains *cis*-regulatory elements, and the size of such a region affecting gene transcription depends on the individual gene. In order to maximize the probability of including all the *cis*-regulatory elements for the genes near endpoints of the duplications, the proximal endpoint of *Dp(16Ifnar1-Kcnj6)Yey* is about 87 Kb proximal to the distal endpoint of *Dp(16)3Yey* (Figs. 2-3; Supplementary Fig. 1). Therefore, *Dp(16)3Yey* and *Dp(16Ifnar1-Kcnj6)Yey* share a ~87-Kb overlapping region. We generated *Dp(16Ifnar1-Kcnj6)Yey*/+ mice using a similar strategy for generating *Dp(16)3Yey*/+ mice as described above. MICER vectors (Adams et al. 2004) were used as pTV*Ifnar1* and pTV*Kcnj6* for targeting *loxP* to the regions 70.8-Kb proximal and 152.3-Kb distal to the coding regions of *Ifnar1* and *Kcnj6*, respectively, in ES cells (Fig. 3a) (Bradley et al. 1998). A duplication and the reciprocal deletion were induced in ES cells by the transfection with a Cre expression vector and were confirmed by Southern blot analysis and FISH (Fig. 3b-3c). After generating chimeras using these ES cell clones, the germ-line transmission of the duplication after crossing C57BL/6J and 129Sv females with chimeric males was confirmed by Southern blot analysis. However, we could not obtain any *Df(16)4Yey*/+ mice from these crosses. One possibility is that the genotype of *Df(16)4Yey*/+ may have led to embryonic lethality. To test this possibility, we crossed the chimeric males with the *Dp(16)1Yey*/+ females (Li et al. 2007). As predicted, we obtained *Dp(16)1Yey/*

Df(16)4Yey progeny from this cross, providing evidence that the *Ifnar1-Kcnj6* region contains a gene(s) associated with haploinsufficient lethality. This gene(s) may underlie the embryonic lethality associated with human monosomy 21 (Chang et al. 2001; Joosten et al. 1997). The duplication was designated as *Dup(16Ifnar1-Kcnj6)Yey*, abbreviated as *Dp(16)4Yey* or Ts6Yey. The deletion was designated as *Del(16Ifnar1-Kcnj6)Yey*, abbreviated as *Df(16)4/Yey* or Ms5Yey.

Identification of a 3.7-Mb minimal critical genomic region for DS-associated heart defects by genetic mapping in mice

In the process of genetic analysis of heart defects in DS, we have recently identified 5.4-Mb *Tiam1-Kcnj6* region on Mmu16 as a critical genomic region for this syndromic phenotype (Liu et al. 2011b). In the current study, we engineered *Dp(16)3Yey*/+ mice which carries a 2.11-Mb duplication between *Tiam1* and *Il10rb* within the *Tiam1-Kcnj6* region (Fig. 2). We examined the cardiovascular phenotypes of embryos carrying *Dp(16)3Yey*/+ (n=25) at E18.5 and found no heart defects. This result indicates that the presence of three copies of the *Tiam1-Il10rb* region is not sufficient to cause DS-associated heart defects. On the other hand, examination of *Dp(16)4Yey*/+ embryos at E18.5 showed that these embryos exhibit heart defects similar to *Dp(16)1Yey*/+ and *Dp(16)2Yey*/+ embryos with a similar frequency in either the 129Sv background or after crossing to C57BL/6J mice (Fig. 4; Table 1) (Li et al. 2007; Liu et al. 2011b). Therefore, the causal gene(s) for this phenotype is located in the *Ifnar1-Kcnj6* region (Fig. 1).

To examine if the genes located in the duplicated region in *Dp(16)4Yey*/+ mice are expressed, we performed RNA-seq using RNA isolated from the pharyngeal arch region and heart of the E10.5 *Dp(16)4Yey*/+ and wild-type embryos. The RNA-seq result is consistent with the elevated expression levels for the duplicated genes (Table 2). In addition, we used TaqMan real-time quantitative PCR to compare the mRNA levels for 4 genes located within the duplicated interval in *Dp(16)4Yey*/+ mice. *Gapdh* is located on mouse chromosome 6 and served as a reference gene of the disomic state in the *Dp(16)4Yey*/+ and +/+ mice. This analysis showed that the duplication altered the transcript levels of the genes in the pharyngeal arch region and heart of the *Dp(16)4Yey*/+ model at E10.5 (Table 3), reflecting the dosage imbalance for the duplicated region. This result further supports the conclusion that the duplicated genes within the *Ifnar1-Kcnj6* region are expressed with the exception for transcriptionally inactive genes and suggests that the cardiovascular abnormalities are a consequence of elevated expression of the duplicated gene(s).

Discussion

The combined Hsa21 orthologous regions on Mmu10, Mmu16 and Mmu17 are about 26.3Mb and contain about 175 Hsa21 gene orthologs (Fig. 1) (www.ensembl.org). We developed *Dp(10)1Yey*/+, *Dp(16)1Yey*/+, and *Dp(17)1Yey*/+ mouse mutants carrying the duplications spanning the entire Hsa21 orthologous regions on three mouse chromosomes, and the model carrying all three duplications simultaneously is considered as a complete genetic model for DS (Lana-Elola et al. 2011; Roubertoux and Carlier 2010; Yu et al. 2010). To genetically dissect DS-associated heart defects, we first examined the cardiovascular

system of the embryos at E18.5 with the following genotypes: *Dp(10)1Yey*/+, *Dp(16)1Yey*/+, *Dp(17)1Yey*/+ or *Dp(10)1Yey*/+;*Dp(16)1Yey*/+;*Dp(17)1Yey*/+ carrying the triple duplications. Heart defects were observed only in *Dp(16)1Yey*/+ and *Dp(10)1Yey*/+;*Dp(16)1Yey*/+;*Dp(17)1Yey*/+ models. We and others (Moore 2006; Williams et al. 2008) detected heart defects in Ts65Dn model (Fig. 1), so we next engineered a pair of duplication and deletion associated with the *Tiam1-Kcnj6* region, which spans 5.4Mb and contains 52 Hsa21 gene orthologs, because of the following reasons: (a) the region is located within the triplicated region in Ts65Dn, (b) TIAM1 affects the functions of endothelial cells (Birukova et al. 2007a; Birukova et al. 2007b; Singleton et al. 2005), and (c) KCNJ6 has been implicated in affecting heart rate (Lignon et al. 2008). The result from this effort was surprisingly revealing: *Dp(16Tiam1-Kcnj6)Yey*/+ (i.e., *Dp(16)2Yey*/+) embryos exhibited heart defects similar to those observed in *Dp(16)1Yey*/+ embryos with similar frequency (Liu et al. 2011b). On the other hand, we did not detect any heart defects in the mutants carrying *Dp(16)3Yey*/+ nor Ts1Rhr which carries a Dp in the *Cbr1-Fam3b* region (Fig. 1). Our genetic mapping culminated in the discovery that *Dp(16)4Yey* is sufficient to cause heart defects (Figs. 1-3), so we have reduced the critical regions for DS-associated heart defects from 26.3Mb to 3.7Mb and the number of the candidate genes from 175 to 35 (Figs. 1-3) (Liu et al. 2011a; Liu et al. 2011b; Yu et al. 2010; Zhang et al. 2012).

The data from our previous and current studies have clearly shown that there are different critical Hsa21 orthologous regions for different DS-related phenotypes (Zhang et al., 2013). The *Ifnar1-Kcnj6* region on Mmu16 is associated with heart defects, while the *Cbr1-Fam3b* region on Mmu16 as well as the interaction of the Hsa21 orthologous region on Mmu17 and the Hsa21 orthologous region proximal to *Cbr1* on Mmu16 are associated with developmental cognitive deficits (Fig. 1) (Zhang et al., 2013).

Our analysis of gene expression of the *Dp(16)4Yey*/+ model *at* E10.5 showed that 20 genes of the 35 genes in the region are expressed at elevated levels in the heart and the pharyngeal arch which contributes to arteries during subsequent developmental processes (Tables 2-3). It is possible that other genes in this region are expressed at an earlier developmental stage or in specific cell types of the cardiovascular system, which may not be detected at a high level in our analysis. The contributions of many genes in the *Ifnar1-Kcnj6* region to cardiovascular development are unknown, and our results indicate that one or more of the genes in this region underlies the mutant phenotype. Our hypothesis is supported by the studies showing a mutation or a copy number variation of a single gene, such as *Cx40*, *Cited2*, or *Nt3*, can cause a spectrum of heart defects, including heart septal defects, abnormalities associated with aortic/pulmonary arteries and TOF in mouse mutants (Donovan et al. 1996; Gu et al. 2003; Yin et al. 2002). However, no orthologs of these genes are located on Hsa21.

To identify the causal gene(s), we could generate single-copy transgenic mice for every Hsa21 gene orthologs located in the *Ifnar1-Kcnj6* region one by one. However, this approach has some drawbacks. First, to accurately mimic the trisomic state of a gene in human trisomy 21, a transgene construct will need to include all the endogenous regulatory elements. However, we do not know every endogenous regulatory element for most of the Hsa 21 gene orthologs triplicated in *Dp(16)4Yey*, and it will need a very large amount of

effort to identify them. Second, for several genes, transgenic approach is not feasible because the sizes of the structural genes plus the regulatory elements are larger than a BAC clone (www.ensembl.org). Third, if a mutant phenotype is a consequence of a joint effect of two or more triplicated genes, an individual gene transgenic approach may fail to identify the causal genes. Therefore a far more desirable approach is to further reduce the number of the candidate genes by continuing to narrow down the critical genomic region(s) using additional chromosomal duplications and deletions without premature or bias assumptions before initiating the efforts to screen for individual causal gene(s).

The *Ifnar1-Kcnj6* region can be divided into the *Ifnar1-Cbr1* and *Cbr1-Kcnj6* regions. We and the others have shown that the triplication of the *Cbr1-Fam3b* does not cause heart defects in the Ts1Rhr embryos (Fig. 1) (Dunlevy et al. 2010; Liu et al. 2011b). Because of the presence of a 1.6-Mb overlapping duplicated region between *Dp(16)4Yey* and Ts1Rhr, which carries 14 Hsa21 gene orthologs (Fig. 1; Supplementary Table 1), there are two possibilities for the genomic locations of the causal gene(s) for heart defects. First, the causal gene(s) may be located in the *Ifnar1-Cbr1* region (Fig. 1). Second, a causal gene(s) may be located in the *Ifnar1-Cbr1* region and another causal gene(s) may be located in the *Cbr1-Kcnj6* region (Fig. 1). Under the latter circumstance, the mutant phenotype is caused by the combined effect of the triplications of the causal genes in both regions, and triplications of the causal gene(s) in either region alone are not sufficient to cause the phenotype. The aforementioned scenarios could be dissected by engineering and characterization of mutants carrying a duplication and a deletion between *Ifnar1* and *Cbr1*. If the causal gene(s) is located in the *Ifnar1-Cbr1* region, *Dp(16Ifnar1-Cbr1)*/+, but not *Dp(16)4Yey/Df(16Ifnar1-Cbr1)*, should lead to heart defects. If the causal genes are located in both the *Ifnar1-Cbr1* and *Cbr1-Kcnj6* regions and the simultaneous triplications of these genes are required to cause heart defects, neither *Dp(16Ifnar1-Cbr1)*/+ nor *Dp(16)4Yey/Df(16Ifnar1-Cbr1)* should lead to heart defects. If we can confirm that the causal gene(s) are located in the *Ifnar1-Cbr1* region, this genomic region can be further dissected independently, which contains 21 protein- and 1 miRNA-coding Hsa21 gene orthologs. Because the *Slc5a3* gene is located in the middle of the region, the *Ifnar1-Slc5a3* and *Slc5a3-Cbr1* segments will divide the genomic region into two with a similar number of the Hsa21 gene orthologs in each region. Generation and characterization of *Dp(16)4Yey/Df(16Ifnar1-Slc5a3)* mice can inform us if the *Ifnar1-Slc5a3* region contains a causal gene(s) whose triplication is necessary to cause heart defects. On the other hand, generation and characterization of *Dp(16Ifnar1-Slc5a3)*/+ mice can inform us if the same genomic region contains the causal gene(s) whose triplication is sufficient to cause the mutant phenotype. This strategy using a duplication alone and in combination with a smaller deletion could also be employed to dissect the *Slc5a3-Cbr1* region as well as the sub-regions in order to narrow down the critical genomic regions. After a minimal critical genomic region(s) is established through genetic dissection, the identity of a causal gene can be revealed by generation and characterization of a compound mouse mutant which harbors a duplication of the minimal critical genomic region and a knockout allele of a candidate gene located within the region. This genetic analysis approach will be significantly facilitated by the availability of knockout mice generated by the International Knockout Mouse Consortium (www.knockoutmouse.org).

Through our current effort, we have mapped heart defects-associated critical Hsa21 gene ortholog(s) to the 3.7-Mb *Ifnar*1-*Kcnj6* region, the smallest critical genomic region that has been identified. This success lays the groundwork for the final effort to identify the dosage-sensitive gene(s) underlying heart defects in DS. Because human trisomy 21 is the most common genetic anomaly associated with heart defects, the gene(s) identified will be an entry point to better understand normal and abnormal cardiovascular development. Since human trisomy 21 can be detected by sequencing fetal DNA in maternal blood (Benn et al. 2012; Ehrich et al. 2011; Palomaki et al. 2011) and fetal DNA in maternal blood can be detected at 4-5 weeks of gestation (Norbury and Norbury 2008; Rijnders et al. 2003; Scheffer et al. 2010; Sikora et al. 2010), there is a genuine possibility that the causal gene(s) and/or the corresponding protein(s) could serve as the targets for preventing heart malformations in embryos carrying human trisomy 21.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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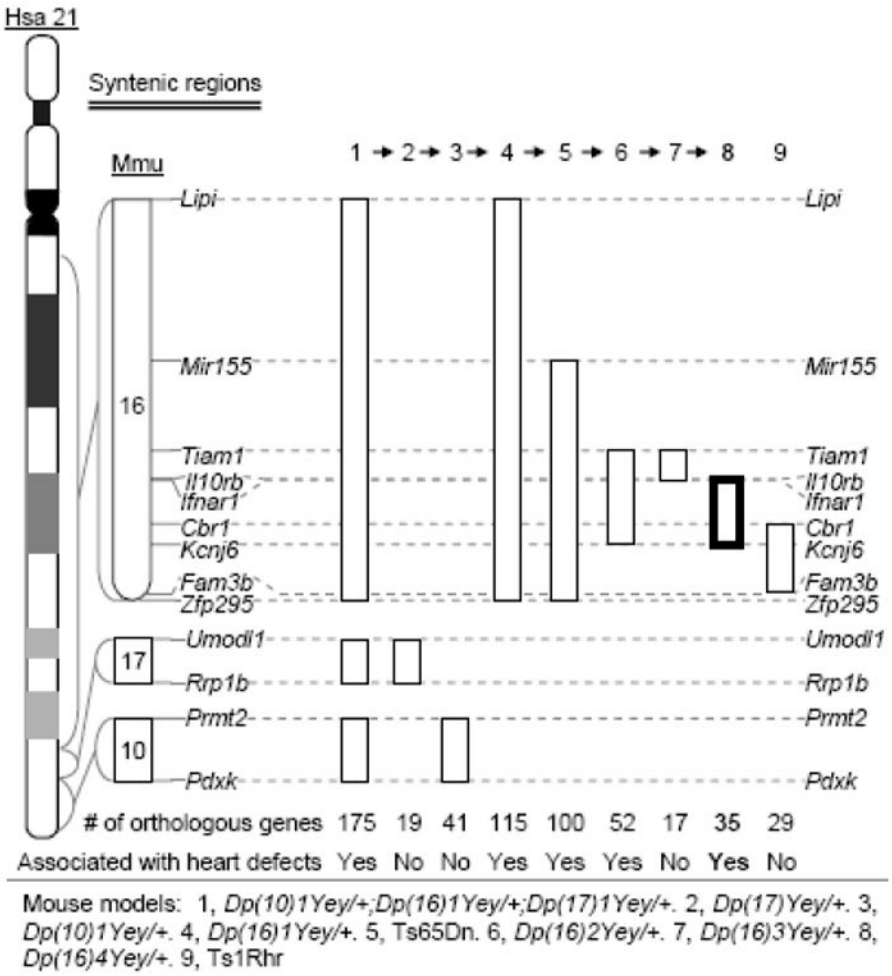

Fig. 1.

Genetic mapping of DS-associated heart defects in mice.

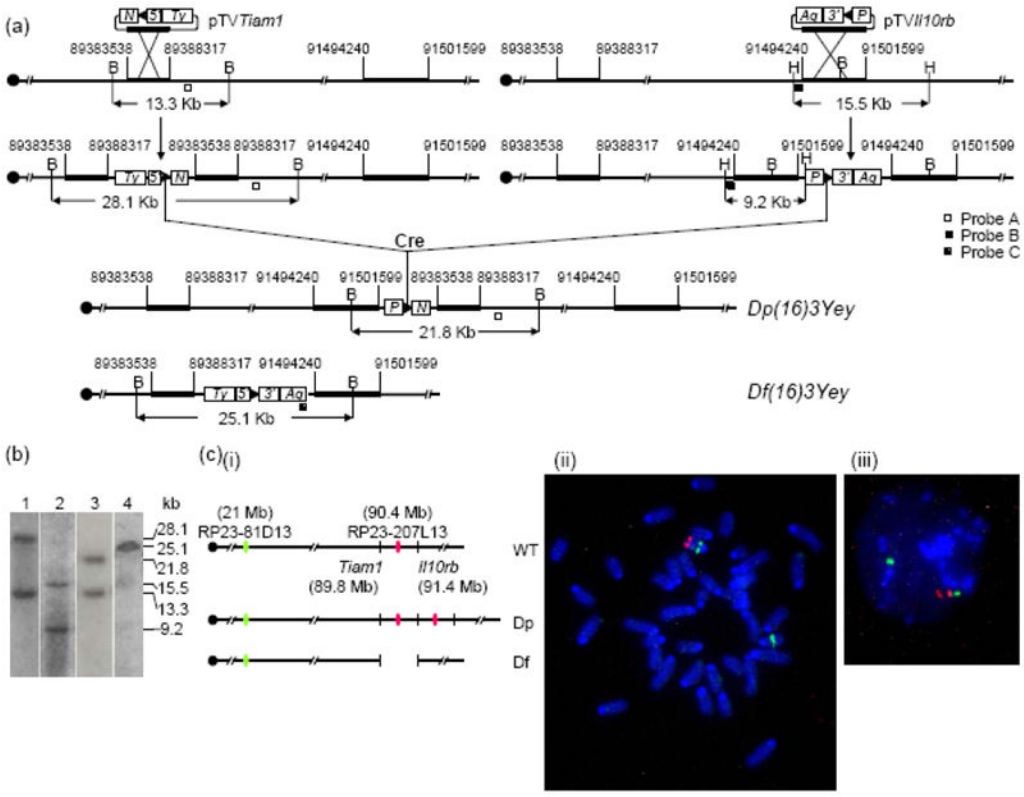

Fig. 2.

Development of *Dp(16)3Yey*/+ mice based on recombinase-mediated chromosome engineering. (**a**) Schematic representation of the strategy used to generate *Dp(16)3Yey* and *Df(16)3Yey*. B, *Bam*HI; H, *Hind*III; 5', *5'HPRT* fragment; 3', *3'HPRT* fragment; *N*, Neomycin-resistance gene; *P*, puromycin-resistance gene; *Ty*, Tyrosinase transgene; *Ag*, Agouti transgene; arrowhead, *loxP* site. (**b**) Southern blot anaylsis of ES cell DNA digested with *Bam*HI (lanes 1, 3 and 4) or *Hind*III (lane 2) using Probe A (lanes 1 and 3), Probe B (lane 2) or Probe C (lane 4), respectively. (**c**) FISH analysis of chromosomes of the engineered ES cells. (**i**) Schematic representation of the genomic locations of BAC probes for FISH analysis. (**ii**) FISH analysis of metaphase nuclei prepared from the ES cells carrying *Dp(16)3Yey/Df(16)3Yey*. (**iii**) FISH analysis of interphase chromosomes prepared from the ES cells carrying *Dp(16)3Yey/Df(16)3Yey*.

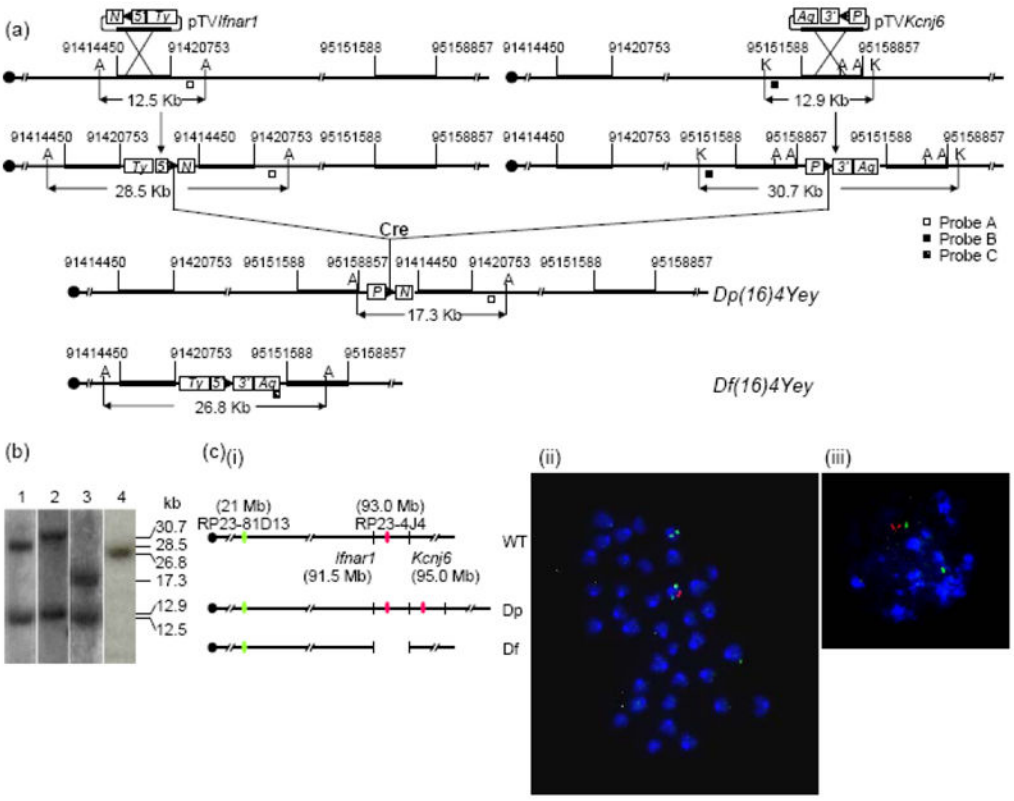

Fig. 3.

Development of *Dp(16)4Yey*/+ mice based on recombinase-mediated chromosome engineering. (**a**) Schematic representation of the strategy to generate *Dp(16)4Yey* and *Df(16)4Yey*. A, *Afl*II; K, *Kpn*I. (**b**) Southern blot anaylsis of ES cell DNA digested with *Afl*II (lanes 1, 3 and 4) or *Kpn*I (lane 2) using Probe A (lanes 1 and 3), Probe B (lane 2) or Probe C (lane 4), respectively. (**c**) FISH analysis of chromosomes of the engineered ES cells. (**i**) Schematic representation of the genomic locations of BAC probes for FISH analysis. (**ii**) FISH analysis of metaphase nuclei prepared from the ES cells carrying *Dp(16)4Yey/Df(16)4Yey*. (**iii**) FISH analysis of interphase chromosomes prepared from the ES cells carrying *Dp(16)4Yey/Df(16)4Yey*.

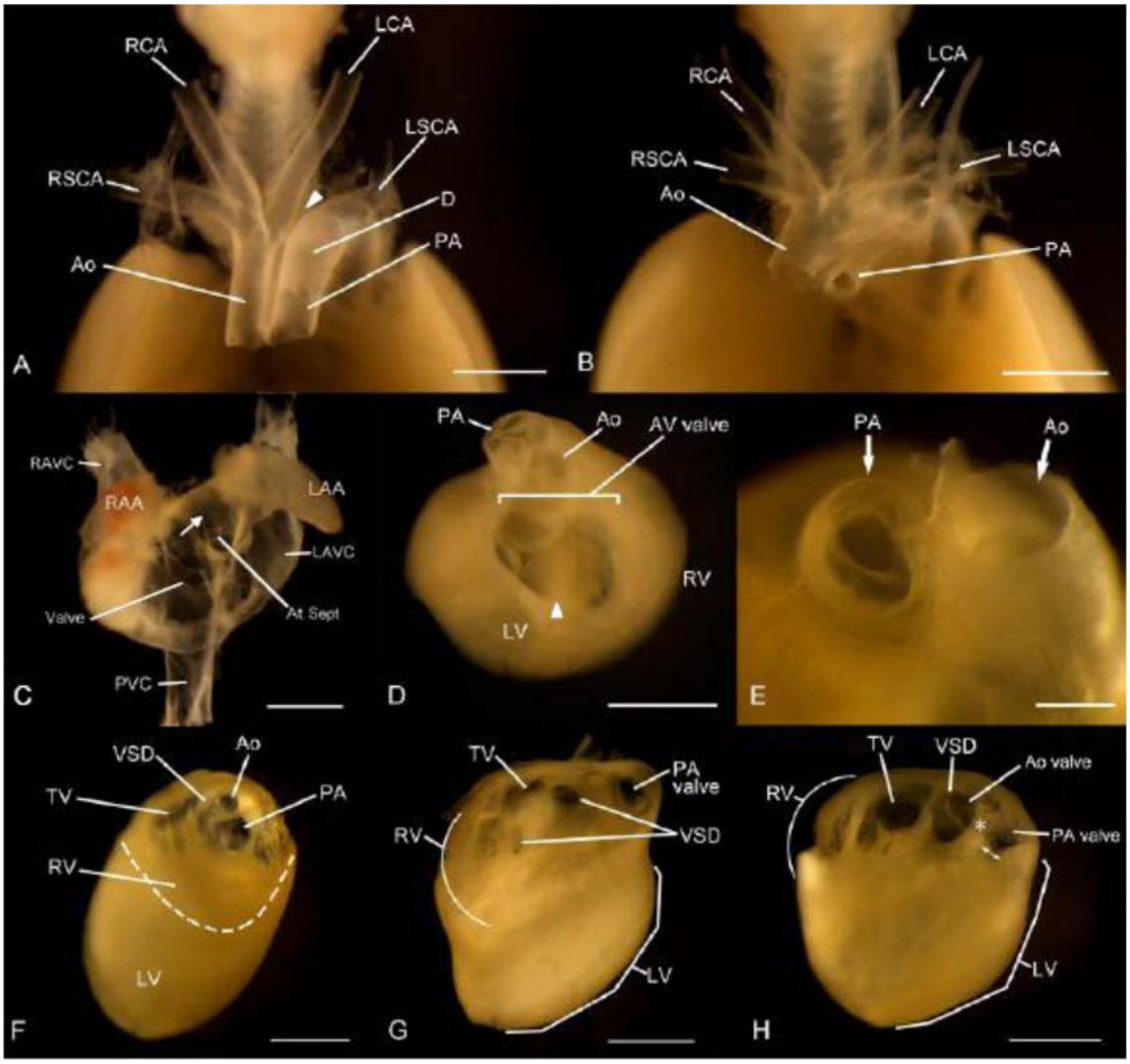

Fig. 4.

Cardiovascular malformations in *Dp(16)4Yey/+* embryos at E18.5. (**a-b**) Ventral views of the great arteries after the hearts were removed. Interrupted aortic arch is indicated by an arrowhead in (**a**) and a stenosis of the pulmonary artery (PA) is shown in (**b**). (**c**) A ventral view of the atria after the ventricles were removed. The arrow indicates atrial septal defect (ASD). (**d-e**) Superior views of the hearts after removal of the atria. Atrioventricular (AV) septal defect is shown in (**d**) with "common AV valve". The arrowhead points to the location of the ventricular septum. A unicommissural unicuspid PA valve is shown in (**e**). (**f-h**) Intracardic views of the right ventricle (RV) after the free wall was removed. A double outlet right ventricle (DORV) and subaortic stenosis is shown in (**f**), and both an inlet and a perimembranous ventricular septal defects (VSD) are shown in (**g**). A tetralogy of Fallot (TOF) is shown in (**h**), which includes a narrowed outflow tract (double arrows) of the right ventricle due to obstruction by the deviation of the parietal band (asterisk), a VSD, and the overriding of the aorta, where the aortic valve is visible through the VSD. Ao, aorta; Ao valve, aortic valve; At Sept, Atrial septum; AV valve, atrioventricular valv; D, ductus

arteriosus; LAA, left atrial appendage; LAVC, left anterior vena cava; LCA, left carotid artery; LSCA, left subclavian artery; LV, left ventricle; PA, pulmonary artery; PVC, posterior vena cava; RAA, right atrial appendage; RAVC, right anterior vena cava; RCA, right carotid artery; RSCA, right subclavian artery; RV, right ventricle; TV, tricuspid valve; VSD, ventricular septal defect. Scale bars, 1 mm.

Table 1

Cardiovascular malformations of *Dp(16)4Yey*/+ embryos at E18.5

Genotype	n2/n1	Type of heart defects	Number of embryos
Dp(16)4Yey/+	7/29	ASD	3
		VSD	3
		AVSD	1
		TOF	1
		Valve defect	1
		DORV	1
		Interrupted aortic arch	1
		Pulmonary stenosis	1

n1, number of embryos examined; n2, number of embryos with heart defects. ASD, atrial septal defect; VSD, ventricular septal defect; AVSD, atrioventricular septal defects; TOF, Tetralogy of Fallot; DORV, double outlet right ventricle.

Table 2

RNA-seq-based relative values of expression*

Gene name	*Dp(16)4Yey*/+ over +/+	Gene name	*Dp(16)4Yey*/+ over +/+	Gene name	*Dp(16)4Yey*/+ over +/+	Gene name	*Dp(16)4Yey*/+ over +/+
Ifnar1	1.77	*Donson*	1.92	*Smim11*	1.28	*Chaf1b*	2.26
Ifngr2	1.58	*Atp5o*	1.38	*Rcan1*	1.33	*Pigp*	1.38
Tmem50b	1.47	*Cryzl1*	1.68	*Cbr1*	1.46	*Ttc3*	1.55
Gart	1.80	*Itsn1*	1.49	*Cbr3*	1.40	*Dscr3*	1.39
Son	1.36	*Mrps6*	1.72	*Morc3*	1.58	*Dyrk1a*	1.41

* RNA was isolated from the pharyngeal arch region and the heart of E10.5 *Dp(16)4Yey*/+ or +/+ embryos. The samples from 4 mutant embryos or the wild-type control embryos were pooled before RNA extraction.

Table 3

Real-time PCR-based relative values (RQ) of expression*

Gene name	*Dp(16)4Yey*/+ over +/+ (RQ ± S.E.M.)
Gart	1.51 ± 0.10
Mrps6	1.69 ± 0.10
Pigp	1.51 ± 0.13
Dyrk1a	1.52 ± 0.07

* The values are calculated based on the means of the samples with different genotypes. *Gapdh* was used as an internal control and is disomic in all strains. RNA was isolated from the pharyngeal arch region and the heart of each E10.5 *Dp(16)4Yey*/+ or +/+ embryo. Three mutant embryos or the wild-type control embryos were used.