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Probing Human Cardiovascular Congenital Disease Using Transgenic Mouse Models

Paige Snider and Simon J. Conway

Developmental Biology and Neonatal Medicine Program, HB Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, Indiana, USA

Abstract

Congenital heart defects (CHDs) impact *in utero* embryonic viability, children, and surviving adults. Since the first transfer of genes into mice, transgenic mouse models have enabled researchers to experimentally study and genetically test the roles of genes in development, physiology, and disease progression. Transgenic mice have become a bona fide human CHD pathology model and their use has dramatically increased within the past two decades. Now that the entire mouse and human genomes are known, it is possible to knock out, mutate, misexpress, and/or replace every gene. Not only have transgenic mouse models changed our understanding of normal development, CHD processes, and the complex interactions of genes and pathways required during heart development, but they are also being used to identify new avenues for medical therapy.

I. Introduction

Congenital heart defects (CHDs) are the most common of all human birth defects and are the leading cause of neonatal and infant morbidity and mortality,^{1,2} yet their underlying molecular, biochemical, cellular, and physiological causes as well as etiologies remain poorly understood. It has been estimated that at least 60% of human embryos die *in utero*, the majority due to early postfertilization and/or implantation anomalies but approximately 15% undergo gastrulation and die during pregnancy or prenatally.³ Examination of the myriad of mouse knockouts, gene traps, transgenic alleles, and chemically induced and spontaneous mouse mutants reveals that defects within the heart and vascular system are largely responsible for the observed *in utero* lethality of the embryo and early fetus.⁴ If a transgenically altered mouse embryo survives implantation but fails to be born, it usually indicates that there is some form of lethal cardiovascular defect present. Significantly, the heart is the first organ to develop in the mammalian embryo and remarkably it functions even before it is even fully formed. Most CHDs are the result of anatomical and/or functional malformations that result from errors during embryogenesis, which implies that they are due to alterations in genes involved in cardiac development that underlie or predispose the embryo toward congenital disease. Although an endeavor in itself, merely identifying the genetic mutations resulting in CHDs does not help the patients; however, it may help in risk stratification.⁵ Designing successful therapies often requires a thorough understanding of the underlying pathological mechanisms and potential target pathways within *in vivo* animal models.⁶ This review summarizes the types and use of mouse model systems currently being used to understand the embryology, pathogenesis, classification, and molecular mechanisms involved in cardiac development in order to clarify the genetic basis of CHDs. The hope is that if these complex CHDs can be prevented and/or nullified in genetically defined mouse mutant models, then the knowledge gained will help engineer potential treatments for pediatric patients.

II. Congenital Heart Defects

The term CHDs can describe a number of different problems affecting the heart, but usually refers to problems with the heart's structure which are present at birth and can often lead to inadequate cardiac performance (reviewed by Hoffman and Kaplan¹ and Hoffman⁷). Although just as problematic but less appreciated, congenital functional defects^{8–11} can also result in deleterious changes to the developing heart. CHDs are the most common type of birth defect and are responsible for more deaths in the first year of life than any other birth defects.^{1,12,13} Despite advances in detection and treatment, congenital heart disease accounts for 3% of all infant deaths and 46% of those deaths result from congenital malformations.¹⁴ All infants are at risk for having a congenital defect, regardless of age, race, or socioeconomic status.¹⁵ The Centers for Disease Control reports cyanotic (blue discoloration caused by a relative lack of oxygen) heart defects occurred in 56.9 per 100,000 live births in the United States in 2005, with higher rates noted when maternal age exceeded 40 years.¹⁴ The incidence of CHD in premature infants is 1.25%,¹ excluding isolated patent ductus arteriosus and atrial septal defect (ASD). It has been estimated that 10% of the children born with heart defects will require surgery in infancy or childhood.¹³ CHDs can be severe and complex with life-threatening symptoms and require immediate medical care after birth, or they can be simple defects that have few if any symptoms and can be fixed easily or require no treatment. The occurrence of less severe CHDs, such as small ventricular septal defects (VSDs), within the newborn population should not be trivialized. The impact of stress on the family and the cumulative impact on healthcare expenditures of cardiovascular defects that generally have a favorable outcome can be considerable. Studies on the incidence of congenital heart disease have generally been from collection and reporting of recognized cases rather than careful population screening with currently available diagnostic technologies. Significantly, the incidence of CHDs has remained relatively constant over time and between populations, suggesting a greater contribution from genetic factors and a smaller etiological environmental contribution.¹² A genetic basis for etiology of CHD was founded by a combination of twin studies, demonstration of familial aggregation, and animal homologies.¹⁶

CHDs may occur alone or together as part of a wide ranging spectrum of other congenital defects. The majority of CHDs occur as isolated (or nonsyndromic) CHDs.¹² However, they can also be found in association with various genetic and chromosomal syndromes such as Down syndrome, Trisomy 13, Turner syndrome, Marfan syndrome, Noonan syndrome, and 22q11 DiGeorge syndrome.^{12,17} Microdeletions of chromosomal regions are a significant cause of syndromal CHDs,¹² and extracardiac manifestations occur in approximately 30% of children with CHDs.¹⁷ This is not surprising considering multiple organs and individual cell lineages have common growth and differentiation signals.^{17,18} This often requires multiple surgeries to correct the various defects, resulting in both detrimental postoperative sequelae and compromised quality of life. Moreover, infants with either isolated and/or syndromic CHDs can develop developmental and psychological problems, even without having undergone surgery, indicating a significant secondary effect of CHDs *in utero*.¹⁹ Although much still needs to be learned of genotype–phenotype correlations, it is already clear that CHDs represent a problem that ultimately affects a great number of families. Gratifyingly, the survival of patients with CHDs has significantly improved during the past several decades, particularly in neonates but unfortunately less so in children.²⁰ This achievement is jointly attributable to a growth in basic knowledge, individual therapeutic successes, advances in clinical management, and the commitment of patients and families. However, adult CHDs represent an increasing proportion of heart transplant recipients, and compared with adult recipients, patients with adult CHD experience higher post-heart transplantation mortality and retransplantation.²¹ Moreover, immunosuppression differs among patients with both grown-up patients with congenital heart disease (GUCH) and adult recipients.^{20,22}

Further research directed at the cause and ultimately the prevention of structural birth defects of the heart is an essential component of any agenda to reduce premature death and morbidity due to cardiovascular disease.

III. Genetic Basis of Disease

The genetic basis of disease can be summarized into three major categories: single gene, chromosomal, and multifactorial causes.^{12,16,23} First, single gene mutations conform to standard Mendelian inheritance patterns and can be assessed as autosomal or X-linked dominant mutations, as well as recessive mutations.^{12,16,23} For example, single gene mutations have been demonstrated to result in both isolated CHDs (i.e., *NKX2.5*, *PROSIT240*, *TFAP2B*) and syndromic CHDs (i.e., *JAGGED1*, *NKX2.5*, *TBX5*, *ZIC3*) in patients.²⁴ One of the first single gene mutations demonstrated to give rise to an inherited CHD was in the T-box transcription factor gene family.^{25,26} Holt–Oram syndrome was shown to be caused by mutations in *TBX5*, in both Holt–Oram syndrome families and sporadic Holt–Oram syndrome cases.^{26,27} Like Holt–Oram patients, adult *Tbx5* heterozygous mutant mice were found to have ASD CHDs and conduction system abnormalities including atrioventricular conduction delay.²⁸ CHDs that fail to follow clear Mendelian inheritance are often assumed to result from sporadic mutations and/or multiple genetic insults, and identification of these CHD-causing genetic determinants is extremely difficult.²⁹

The second category of genetic disorders is chromosomal aberrations.^{12,16,23} When chromosomal regions are identified, they tend to be large with multiple genes localized to that region and this has made fine mapping and gene identification challenging.²⁹ Until the recent and dramatic growth in mouse genetics and increased understanding of molecular biology of cardiac development, chromosomal causes of CHDs were only investigated if syndromic anomalies or other organ systems were involved.³⁰ Since the development of sophisticated karyotyping techniques and advancements in localization, sequencing, and detection of loci critical for cardiac development, many associations between CHDs and chromosomal aberrations have started to be delineated.^{13,30} For example, molecular advances in the 22q11 microdeletion syndrome (also known as DiGeorge syndrome) have led to greater understanding of the basic knowledge as well as advances in clinical management of young patients.^{30,31} The 22q11 microdeletion syndrome is the most common human deletion syndrome, and the spectrum of cardiac defects was initially outlined in the mid-1970s.³² Initially, the entire proximal region of mouse chromosome 16 (analogous to human chromosome 22) was deleted, and these heterozygous deletion mice mutants exhibited CHDs similar to interrupted aortic arch (IAA) CHDs.³³ Subsequently, transgenic complementation and gene targeting studies confirmed that the loss of *Tbx1* was the gene responsible for the structural abnormalities observed.^{34–36} Significantly, the *Tbx1* mutant mice have gross anatomical defects that are thought to reflect a severe 22q11 microdeletion syndrome phenotype.³⁷ More recently, a hypomorphic *Tbx1* mouse mutant has been used to demonstrate that *Tbx1* is required to activate expression of two key downstream target genes, namely the *Forkhead transcription factor* (*Foxa2*) and *Fgf8* growth factor.³⁸ In addition to the congenital aspects, the 22q11 microdeletion syndrome is also associated with late-onset features and an association with psychological problems, such as schizophrenia.^{19,39} Initially, it was impossible to determine whether these developmental difficulties were due to genetic causes or postoperative complications, but further understanding of the molecular biology, mutation spectrum, and clinical subclassifications, in conjunction with availability of complementary animal models, is helping to distinguish primary and secondary genotype–phenotype correlations and underlying pathology.

The third category is multifactorial whereby genes interact with environmental influences to result in disease.^{12,16,23} Estimations suggest that approximately 8% of CHDs are due to genetic factors, approximately 2% are due to environmental factors, and the rest are multifactorial.⁴⁰ Although epidemiological data have highlighted environmental influences,⁴¹ these studies have mostly suggested risk rather than pinpointing underlying CHD mechanisms.¹⁸ The joint and/or synergistic effects associated with the interplay between genes and the environment are termed “gene–environment interactions” and do not exhibit any recognizable Mendelian pattern of inheritance and are not associated with any identifiable chromosomal abnormalities.^{15,23} The description “multifactorial” is used as it refers to the additive effects of several genes that can be modified to a greater or lesser extent by environmental factors, and that an individual who is genetically susceptible, may or may not develop a CHD depending on the interaction of risk factors, both genetic and environmental.^{12,15} Examples of multifactorial causes of CHDs have largely come from mice mutant studies, specifically the findings that TBX5 and NKX2.5 proteins interact physically to activate target genes²⁸ and that defective interactions between GATA4/TBX5 and GATA4/NKX2.5 may underlie CHDs caused by *GATA4* mutations.⁴² The recent discovery that many developmental events have to be additionally synchronized via posttranscriptional microRNA (miRNA) regulation, specifically *miR-1*, *miR-138*, and *miR-133*, and that each participates in multiple aspects of cardiac development and homeostasis,^{43,44} suggests that a mutation within a single miRNAs may aberrantly affect multiple signaling pathways. These genetic studies have provided two important insights. First, that developmentally important transcription factors are involved in the etiology of CHD. Second, that the dosage of key cardiac developmental factors must be precisely regulated, as most disease causing mutations result in haploinsufficiency and some are the result of activating mutations.

Routine testing for the many genes that have been shown to be involved in causing CHDs (see Table I) is presently not a viable cost-effective patient option except on a research basis, although there is significant interest in transitioning this testing from the laboratory to clinic.²⁴ However, a recent pilot “personalized genome sequencing” report,⁴⁵ in which the complete genome of an individual was determined by massive parallel DNA sequencing, suggests that identifying the key genome sequences that may be associated with disease is predictive of the response to medication and would enable individual genotype–phenotype correlations may be possible in the near future.

IV. The Mouse as a Genetic Model

The development of animal models to study cardiac embryology has been driven by a need to understand both normal and abnormal heart formation during *in utero* development.^{4,29,46} Transgenic technology is invaluable as it can be used to answer physiological questions that can only be answered within the intact organism.⁴⁷ Significantly, both mice and humans have evolutionary similarity,⁴⁸ share 90% identity in their amino acid sequence of homologous proteins, and their genomes are of similar size (~20,000–25,000 genes; The International Human Genome Sequencing Consortium, 2004; www.genome.gov). Moreover, mouse and human genes are similarly ordered along the chromosomes.⁴⁸ Importantly, mice have been extensively studied, and their genetics, embryology, and physiology are very well understood.^{5,48,49} Additionally, mice are decidedly fertile, easy to feed and house, and are highly amenable to assisted reproductive techniques including cryopreservation which can preserve the sperm from genetically manipulated mouse lines.⁵⁰ Since the sequencing of the entire mouse genome, it is now feasible to remove and/or mutate every gene.^{51,52} Furthermore, embryonic stem (ES) technology now allows for the generation of hypomorphic (partial loss of gene function), spatiotemporal lineage-restricted gene replacement, and/or overexpression in normal or ectopic locations in mice. If a human

sequence is in hand, the homologous mouse gene can be mutated by gene targeting and/or transgenically forced to be expressed, and the consequent physiological outcome determined.⁴⁸ Moreover, known human mutations can be introduced in mice to examine their *in utero* effects and assess genotype–phenotype correlations.^{53,54} Additionally, precise regions of the *in utero* developing cardiovascular system can be genetically ablated.⁵⁵ In addition, mice have the advantage over flies, worms, and fish in that they have the mammalian four-chambered heart, making the data more applicable to human CHDs.⁵ Knowledge of a mutation that predisposes or causes CHDs alone cannot translate directly into treatment, but an understanding of pathological processes will be crucial to the development of eventual treatments.⁵ Animal models are one of the few approaches that we have to help with our understanding of the multifactorial basis for CHDs, including genetic predisposition to disease and environmentally acquired factors such as viral infection.⁵

Human diseases are mostly polygenic with the clinical phenotype being a result of interplay between multiple genes and environmental influences.⁵⁶ Animal models of cardiovascular physiology have mostly been large animals which have limitation due to genetic variance and difficulties of molecular–genetic manipulation.⁵⁶ However, mice are the most genetically manipulative vertebrate model system and are available on approximately 500 strains of inbred (http://www.informatics.jax.org/external/festing/search_form.cgi) and numerous mixed genetic backgrounds. In fact, strain-dependent phenotype variability can be used to examine the multifactorial basis of congenital defects. For example, *TGFβ1*-deficient mice placed on various inbred genetic backgrounds can exhibit phenotypes ranging from preimplantation to weaning-age lethality.^{49,57} This strain-dependent variability facilitates the potential mapping of modifier genes.⁵⁸ Thus, researchers now have tools to modify and control the protein content of the developing mammalian heart, resulting in mouse models that can potentially provide the critical links between mutated or absent protein and CHD pathogenesis at molecular, biochemical, and cellular levels.⁵⁹

V. Transgenic Mice

A transgenic line is one in which artificially introduced genetic material is microinjected *in vitro* into the large male pronucleus fertilized mouse eggs.^{60–62} Following random chromosomal integration of the transgene, injected eggs are subsequently implanted in the oviduct of pseudopregnant surrogate mothers and their progeny is screened for transgene integration. The microinjected DNA tends to integrate as multiple tandemly arranged copies at a random position in the genome, often after one or two cell divisions have occurred. Thus, the resulting mouse is only partially transgenic. If the transgenic cells contribute to the germ line, then some transgenic eggs or sperm will be produced and the next generation of mice will be fully transgenic. These are referred to as “stable transgenic” lines as the transgene has stably integrated in both somatic and germline cells so that the foreign construct is disseminated across future generations.⁶² However, the use of transient generation of transgenic mice has been employed.^{63,64} Here, no breeding is required because females with potential transgenic pups *in utero* are sacrificed during gestation and expression and/or effects of the transgene directly assessed. In order to express a recombinant protein in the transgenic animal, the DNA that is microinjected must contain the region that encodes a coding sequence of interest. This is placed downstream of a selected cell-specific promoter/enhancer (transcriptional regulatory region) which directs gene expression to a specific tissue, cell type, or developmental stage and upstream of a polyadenylation signal sequence.^{47,64} Where possible, it is best to select promoter sequences with proven tissue-specific expression in transgenic mice, as this will increase likelihood of experimental success.

Transgenesis enables several types of experiments to be performed.⁴⁷

Overexpression: wherein a protein in a cell that normally expresses that protein is overexpressed, in order to experimentally address the consequences of producing an abnormally high level of that protein in a particular cell or tissue. Transgenic expression cassettes that use genomic DNA rather than cDNA are generally more efficiently expressed in mice. It is usually a good idea to include (where possible) generic introns when cDNA is used and levels of expression are important.

Ectopic expression: wherein the ramifications of ectopic expression in which a protein is expressed in cell type that does not normally express it are addressed.

Gain-of-function: wherein a mutation can be studied by designing a transgene that encodes a mutated protein that is constitutively active.

Loss-of-function: wherein a dominant negative form of a specific protein is studied.

Reporter expression: a reporter gene does not influence the normal biological activity of the cells in which it is expressed, but rather is used to explore the pattern of the promoter as well as to identify any *cis*-acting elements required for function of the transcriptional regulatory sequence attached to the reporter gene.^{47,56}

The most commonly used reporter genes are *lacZ* and *green fluorescent protein (GFP)*. *LacZ* is an enzyme which catalyzes the hydrolysis of X-gal (5-bromo-4-chloro-indolyl- β -D-galactosidase) and forms a bright blue precipitate within a single cell. In order to stain for *lacZ*, a tissue must be fixed and a chromogenic substrate is added. Although not preset in the heart, mammals have endogenous β -galactosidase activity which can result in background staining. By cloning a nuclear localization signal into the β -galactosidase gene, the nuclear transgene staining can be easily differentiated from the endogenous cytosolic signal.⁵⁶ Another popular reporter is GFP which can be used to analyze living (rather than fixed) cells. GFP is a small protein cloned from jelly fish that functions as a fusion protein and upon long-wave UV stimulation, emits green light.⁵⁶

Like all molecular biological techniques, in addition to their power and flexibility, there are also several caveats. Once the injected DNA has been integrated into the murine genome, it can theoretically exhibit its function. However, it must be pointed out that since the insertion occurs randomly, positional/insertional effects must be considered as both the function of the inserted gene and endogenous genes may be altered.⁶⁴ Variegation effects must also be considered, since the expression of the transgene may be altered by surrounding elements. It has been estimated that 5–10% of transgene integration events cause disruptions, deletions, or translocations that cause a mutant phenotype.⁶⁵ In order to bypass the variegation position effects and study transcriptional regulatory elements, transient transgenic approaches can be employed,^{63,64} as a comparison of multiple founders will ensure that any phenotypes due to positional effects of the transgene are readily apparent.⁶⁴ An additional disadvantage of this technology is the inability to control the number of transgene copies inserted into the host genome.⁵⁹ Therefore, many of the mouse models express the transgene at very high levels and raises questions of nonphysiological consequences due to atypical levels of gene expression.⁵ Most times, the transgene is transmitted in a Mendelian fashion. If you have a mosaic mouse, transmission may be much sparser than Mendelian, and you may have to breed several litters to find your transgene. Once you have a positive offspring, breeding from that one should be Mendelian. At other times (rarely), you may have more than one integration site, in which case your transgene will be transmitted in greater than Mendelian numbers. Very rarely, the transgene is not in the germ line and is not transmitted. Additionally, the endogenous gene and protein product are present and may make interpretation of the data perplexing and inconclusive.⁵⁹

Since the first transgenic animals were mice created by Rudolf Jaenisch⁶⁶ and Gordon *et al.*,⁶⁰ who demonstrated that a microinjected a recombinant plasmid in pronuclei of fertilized mouse oocytes could be passed onto their offspring to generate large numbers of transformed animals, there has been an explosion in the use of transgenic mice models in biomedical research and industry. Recombinant DNA technology of transgenesis has become vital in studying mammalian embryonic development, patterns of gene expression, physiological gene function, as well as the problems of gene regulation and cell differentiation in the complex mammalian system.^{47,60,62} Other uses include the production of human hormones (e.g., insulin) and for testing in biomedical research. Furthermore, transgenic mice are routinely employed to mimic human diseases by cloning human genes that are known to underlie various human disorders, thus making them an invaluable research tool.⁶² Additional transgenic species are also beginning to be generated, including sheep, goats, pigs, some chickens, and most recently a stable line of breeding transgenic marmosets. Marmosets are primates and thus our closest relatives (so far) to be genetically engineered.⁶⁷

VI. Gene Knockout and Gene Knockin Mice

A knockout mouse is a genetically engineered mouse in which one or more genes have been turned off through a targeted mutation. Targeting is achieved via either “homologous recombination” or “gene trapping” within ES cells. ES cells are pluripotent and have the capacity to differentiate into nearly any type of adult cell and generate a mouse.^{68,69} The targeting vector is electroporated into the ES cells *in vitro* and various drug resistance cassettes are used to select for the targeted lines that have undergone targeting.⁵⁹ ES cell colonies are then screened for the presence of the targeting gene, then once confirmed, the genetically modified ES cells are injected into a blastocyst harvested from a black mouse and implanted into a pseudopregnant agouti mouse.^{59,69} The “chimeric” offspring will have patches of both the black and agouti coat color. Male mice with the highest percentages of chimerism are then selected based on coat color, and subsequently out crossed with wild-type black females to confirm germline transmission.^{59,69} If the animals are viable, the mice can be bred to homozygosity and analysis of the heterozygotes and homozygotes can assess the effects of gene dosage. Knockout mice usually follow Mendelian inheritance strictly, and thus it is relatively straightforward to generate double, triple and even quadruple compound heterozygous and/or homozygous mutants to examine genetic interactions and redundancy amongst family members and coexpressed genes.

For homologous recombination (also called gene targeting or knockouts), a targeting vector is generated that shares identical, or homologous, sequence to the gene being targeted.^{48,64,70} This homologous sequence flanks the existing gene’s DNA sequence both upstream and downstream of the gene’s location on the chromosome, and then the cells’ own nuclear machinery automatically recognizes the identical stretches of sequence and swaps out the existing gene or portion of a gene with the artificial piece of DNA. Gene targeting constructs also contain a positive selection cassette conferring antibiotic resistance (typically neomycin) and a core region with the appurtenant genetic modification flanked by homology arms.^{48,64} As homologous recombination is rare compared to random DNA integrations, strategies are used to enrich for recombinant ES cell clones.⁶⁴ These include use of negative selection markers such as *Diphtheria toxin* α -chain coding region or *herpes simplex virus type-I Thymidine* gene located outside of the targeting construct.^{71,72} Thus, ES cells that have correctly undergone homologous recombination will not contain a negative selection marker, as nonhomologous flanking sequences will be removed prior to integration; however, randomly integrated constructs will retain the negative selection marker and be eliminated.⁶⁴ Given the usefulness of knockout mice lines, the National Institutes of Health has established The Knockout Mouse Project (KOMP) that aims to

generate a comprehensive and public resource comprising mice containing a null mutation in every gene in the mouse genome (<http://www.nih.gov/science/models/mouse/knockout/index.html>). For gene trapping, instead of directly targeting a gene of interest via homologous recombination, random insertion in ES cells is used. Gene trap vectors principal element is a gene trapping cassette consisting of a promoterless reporter gene, and/or selectable marker flanked by an upstream 3' splice site (splice acceptor, SA) and a downstream transcriptional termination/polyadenylation sequence.^{73,74} The inserted piece of artificial DNA prevents the cell's RNA "splicing" machinery from working properly, thus preventing the existing gene from producing its designated protein and knocking out its function. As the vector simultaneously inactivates and reports the expression of the trapped gene at the insertion site and provides a gene-trap sequence tag (GTST), it is possible to quickly identify the location of the disrupted gene. Thus, using this approach, it is possible to track the activity of the artificial reporter gene to figure out the existing gene's normal pattern of activity in mouse tissues.⁷⁴ An international public consortium provides a central registry of the all lines available (<http://www.genetrap.org/>).

One advantage gene targeting has over transgenic techniques is that ES cell homologous recombination clearly defines the integration site allowing for precise genetic changes to be introduced.^{64,75,76} "Knockout" mouse models are also able to provide useful information on gene function as well as being useful to study human diseases caused by either total or partial protein loss-of-function mutations.⁶⁹ This is particularly evident, when either reporters (such as *lacZ* or GFP) are inserted into specific sites and used to monitor gene expression under the control of the gene's own *cis* and *trans* transcriptional machinery. Moreover, "knockin" mice, wherein desired sequences are targeted to specific loci, not only allow the study of gain-of-function mutations through targeted mutagenesis but also allow the replacement of one gene by another functional and/or mutated gene, enabling genetic testing of the subtle variations between genes by replacing one gene with another.⁶⁹ Knockin mice can be used to model human CHDs that are a result of gain-of-function mutations. Several of the caveats with knockout models include early embryonic lethality, which prevents studying the role of a gene at later stages, and if the target gene has many functions in multiple cell types obscuring its cell autonomous role.⁴⁸ However, both of these problems can be readily overcome using conditional lineage-specific gene targeting (see section VII below). Additional problems can be encountered if there is duplication of genes during evolution, which can lead to functional redundancy. In this case, a deficient animal may overexpress an alternative gene product in order to compensate for the deficiency, thus severely limiting phenotypic characterizations.⁴⁸ However, this too can be circumvented via generation of double-knockout mice and removal of the compensating, parallel pathway and/or synergistic gene targets. Finally, the presence of a positive selection marker in the targeted gene can affect the gene of interest and result in "hypomorphic phenotype."⁷⁷ However, hypomorphic alleles can be very useful in the study of somatic-based diseases that generate a phenotype as a consequence of gradual gene function deterioration.⁴⁸ Another advantage of generating a hypomorphic allele is the ability to make compound heterozygotes that carry one hypomorphic allele and one null allele following the proper mouse mating and often have more severe phenotypes than hypomorphic alleles alone.⁴⁸ Despite these drawbacks, knockout mice offer one of the most powerful means now available for studying gene function in a living animal. Such studies will accelerate efforts to translate newfound knowledge of the human and mouse genomes into better strategies for diagnosing, treating, and preventing human CHDs.

VII. Conditional Knockout Mice

Conventional gene targeting generates a mutant allele in all cells of the mouse following fertilization. However, difficulties can be encountered, such as embryonic lethality, and

analysis can be complicated due to indirect effects from ablating the gene in all tissues. This can be overcome by using a two-component mouse system employing recombinase to inactivate the gene of interest.⁷⁸ Cre and Flp are members of the α -integrase family of site-specific recombinases.^{78,79} Application of this technology results in deletion of genetic material at a specific time and in specific cells, depending upon the promoter used to drive Cre or Flp recombinase.⁷⁸ Cre and Flp catalyze DNA strand exchange between recombination sites and depending on the number and orientation of the recombination sites, deletions, duplications, inversions, or integrations can be generated.⁷⁸ Cre and Flp remove segments of intervening DNA between two unique 34 base pair recognition sites termed loxP and FRT sites, respectively. They each comprise two 13 base pair palindromes separated by an asymmetric 8 base pair core.^{47,78,80} In order to study CHDs, a mouse is prepared in which the targeted allele is flanked by loxP/FRT sites, and then this mouse is bred to a mouse expressing Cre or Flp which are expressed under the control of heart- or lineage-specific tissue-restricted promoter/enhancers.⁷⁸ The recombinase then excises the DNA segment between the recombination loxP/FRT sites, and the gene is rendered inactive.⁴⁷ Conditional knockouts are advantageous because both the timing at which the cells are affected and its spatial restriction are under the control of the investigator, thereby circumventing the embryonic lethality and/or multiple sites of expression concerns sometimes encountered with conventional knockouts.⁴⁷ In conditional technology, it is vital to make sure the recombinase recognition sites are placed around an essential part of the gene of interest, without compromising its function.⁶⁴ Several methods have been developed to help with this. Often the entire coding region will be flanked by loxP or FRT sites, or the loxP/FRT sites can be positioned within an intronic sequence located around an essential exon. Additionally, the promoter region of the gene can be targeted.⁶⁴ Further development of the targeted Cre-loxP technology now provides researchers with the ability to genetically ablate specific cardiovascular cell lineages at various different stages of heart development. Biological functions can now be analyzed *in vivo* and *in utero* via targeting the toxic gene *Diphtheria toxin A subunit (DTA)* to specific cell lineages and forcing the intended cells to undergo apoptotic cell death, without any bystander effects.^{55,81,82} Given the enormous strides made using cell ablation within the chick embryo model,^{83,84} it is more than likely that the combined power of mouse transgenesis and toxigenics will also provide a boon to the modeling of mouse mutants for studying CHDs. To date, the Cre-loxP system is the best characterized means of achieving conditional gene inactivation in mice and has contributed extensively as a tool for altering the mammalian genome.

Studies on CHD have been immeasurably enhanced with the development of cardiac-specific promoters that can now be used to direct expression of an engineered protein within only the heart at different developmental stages.⁵ Cardiac-specific promoters in transgenic animals have given researchers a powerful tool to eliminate complicated effects of other organs or muscle systems.⁵ Transgenes influencing structural aspects of cardiac function, physiology, metabolic processes, calcium cycling, ion channels, transport, mechanical stimulation, and electrical properties have been expressed with the use of these cardiac-specific promoters paving the way for murine models to mimic human CHD.⁵ Ultimately, the development of the Cre-loxP recombinase system has revolutionized mouse genetics by proving to be an essential tool for conditional genetic alteration in mice.

VIII. Inducible Mice

In a clinical setting, physicians are often interested in the reversibility of cardiac phenotypes, including whether the cardiac pathology is controllable or reversible.⁵ A more precise control of transgene induction is needed to address these questions, and pharmacological methods have proven the answer.⁵ Tetracycline (tet) or its analog doxycycline (dox) has been most widely used to express a transgene at a precise developmental stage, allowing a

phenotype to develop and then shut off expression to see if the heart returns to normalcy.⁵⁹ This tet-inducible system requires two different constructs. One encodes a tet-repressor herpes virus Vp16 termed rTA which is expressed either from a generic promoter that is nontissue-specific or from a developmentally regulated tissue-specific promoter.⁸⁵ The second construct (tetO) consists of a minimum promoter with no basal activity linked to the gene of interest.⁸⁵ The tet-V16 protein has a very high binding coefficient for the tetO.^{85,86} There are two varieties of tet-regulatory sequences, one in which a gene is repressed by tet binding (rTA or “Tet-Off”) and the other where the gene is activated by tet binding (rtTA or “Tet-On”). Repression of a gene with this system occurs because in the presence of tet or its analog, tet binds rTA and the conformation changes so that it can no longer bind to the tetO sequence leading to downregulation of expression. A mutation in the tet activator (rtTA) promoter causes the mutant protein not to bind the tetO sequence in the target gene unless tet is introduced into the system.⁸⁵ Gene induction can be detected within a few hours and maximal expression occurs within a day.⁸⁵ The constructs can be microinjected separately, and animal with each construct can be mated, or the constructs can be coinjected. The tet-Off (rTA) and tet-On (rtTA) systems have been successfully used in mice to control gene expression, both in adults and *in utero* within the developing heart.^{86,87}

IX. Forward Genetic Approaches

A “reverse genetics” strategy occurs when a gene is altered first and then the phenotype resulting from this alteration is subsequently studied, is the principle behind knockin and knockouts. However, it has been recognized that this strategy is somewhat limited by the fact that it examines genes already thought to be involved in the physiological process under study.⁵⁰ Thus, a “forward genetics” strategy which employs an unbiased approach, that more closely mimics the work of clinicians who tend to identify a disease first, and then try to isolate the genes responsible has been developed.^{50,88} This approach uses *N*-ethyl-*N*-nitrosurea (ENU) as a potent means of inducing random point mutations across the genome.^{50,89} ENU is an alkylating agent that causes base substitutions in DNA, creating allelic series of mutations that has a mutation rate of 1 in 1000 gametes in mice.⁹⁰ Mutations generated in mouse ES cells which retain germline competency can be used to study partial loss, complete loss, and/or gain-of-function alleles.^{90,91} Although ENU is an effective random chemical mutagen capable of generating novel phenotype, the ENU-induced single-base-pair changes responsible for a given mutant phenotype are often difficult to detect and require laborious techniques such as positional cloning.⁹² Upon intronic insertion, gene-trap transposons are capable of reducing wild-type transcript levels by mutating disrupted endogenous genes and producing novel phenotypes, making them useful as a random germline insertional mutagen in mice.⁹² Transposons catalyze the insertion of foreign DNA into host chromosomes, which is the most important step in gene transfer applications.⁹³ This forward genetics approach has the added benefit of generating countless new phenotypes, several which exhibit unique CHDs including the single ventricle phenotype that has been underrepresented using reverse genetic approaches (Table I; Ref. 94).

X. Common CHDs

CHDs can be divided into three major subtypes (Table I): cyanotic (blue discoloration caused by a relative lack of oxygen), left-sided obstruction defects, and septation defects.^{1,18,95} Tetralogy of Fallot (TOF), transposition of the great vessels, tricuspid atresia, total anomalous pulmonary venous return, truncus arteriosus, pulmonary atresia, Ebstein’s anomaly, and some forms of total anomalous pulmonary venous return are the most common cyanotic CHDs. Left-sided obstructive lesions include hypoplastic left heart (HLH), mitral stenosis, aortic stenosis, aortic coarctation, and IAA. Septation defects, which constitute the most prevalent CHD subtype, include ASDs, VSDs, and atrioventricular septal

defects. Other relatively common CHDs that lie outside these subtypes include patent ductus arteriosus (PDA) where a neonate's ductus arteriosus fails to close after birth and bicuspid aortic valve where only two valve leaflets instead of the normal three are formed in the aortic valve. Each of these CHDs may occur alone or together and the severity (and morbidity) of these CHDs varies greatly. The following sections highlight one of each of the subtypes' most common defects and some of the insights provided via mouse transgenics.

A. Ventricular Septal Defect

VSDs are the most common congenital cardiac defects in human infants, and there are both muscular and perimembranous VSDs. Clinically, isolated VSDs can lead to the failure of survival, but the effects of isolated VSD disease are quite variable and are dependent upon anatomical location and size. The larger the communication between the left and right ventricles, the greater the hemodynamic alteration and increased load on the heart, the worse the effects. The genetics of ventricular septation are poorly understood, and in human populations, no single gene disorder primarily causing isolated VSD exists.^{87,96} Isolated VSD can occur in patients heterozygous for mutations in *NKX2.5*⁹⁷ or *TBX5*²⁵; however, this phenotype is less common. More frequently, VSDs are seen in combination with other subtypes of CHDs, particularly outflow tract alignment and septation defects.

The morphogenesis of atrioventricular septation is complex,⁹⁸ as multiple primordia contribute to the formation of the interatrial and interventricular septa, including endocardial cushion tissue (derived from both colonizing extracardiac neural crest and intrinsic epithelial-to-mesenchymally derived lineages), endothelium, and myocardium. The muscular portion of the inter-ventricular septum arises from ingrowth and folding of the myocardial wall of the developing ventricle, whereas the membranous portion of the interventricular septum is derived primarily from endocardial cushion tissue. Despite being the foremost CHD found in patients, isolated VSD in mice mutants is usually found in association with altered myocardial growth, OFT/AAA abnormalities, and/or valvular defects.^{4,96} VSD pathogenesis is complex and multifactorial and may include underlying defects within the cardiomyocyte, cardiac neural crest, endothelial, endocardial cushion, epicardial, and/or cardiac fibroblast lineages that can each alter left-right ventricular morphogenesis, chamber formation, and septal positioning. Although a large number of transgenic models exhibit VSDs, very few have been shown to affect the ventricular septum exclusively, but many, such as *Bmp6/7* double knockouts, *Cited2*, *Fog2*, *Nkx2.5*, *Pax3*, *Sema3c*, *Tbx5*, and *Tll1*, have been shown to affect ventricular septation as part of a larger constellation of congenital cardiac anomalies (Table I). Initially, the *Hey2* knockout phenotype was reported as an isolated VSD,⁹⁶ but subsequent analysis has revealed that *Hey2* mutants exhibit a variable penetrance and spectrum of CHDs, including TOF, deformed valves, and cardiomyopathy.^{99,100} These variable phenotypes probably reflect the fact that *Hey2* is expressed in multiple cardiovascular lineages throughout development, including robust expression within the myocardium.¹⁰⁰ Despite the suggestion in several reviews and textbooks that increased apoptosis within the developing interventricular septum may underlie VSD pathogenesis, there is little experimental evidence to support this implication. Using *Hey2* knockout mice as the model, both apoptosis and cell proliferation can readily be examined in knockout and age-matched littermate controls on the identical genetic background. While *Hey2* mutants exhibit both DORV and VSDs that are structurally similar to those observed in patients with CHDs (Fig. 1), apoptosis was unchanged within the developing heart (examined from embryonic days (E) 9 to 14 (only E14 shown in Fig. 1G), during ontogeny of the interventricular septum and septation of the mouse embryo heart. However, examination of cell proliferation revealed a significant reduction in cardiomyocyte proliferation at the base of the interventricular septum (Fig. 1H). These types of analyses illustrate the usefulness of the mouse transgenic analysis approaches and the

type of detailed informative data that can be obtained. While most of the mouse models exhibit perimembranous VSDs, several mutants also exhibit muscular VSDs. Analysis of *Hand1–Hand2* knockin chimeras¹⁰¹ revealed that stillborn high-percentage chimeric neonatal *Hand1–Hand2* knockins exhibited DORV with concomitant VSDs and PDA. Intriguingly, their hearts had a thinned myocardium and the myocardial architecture was extensively disorganized, with both multiple muscular and obligatory large perimembranous VSDs evident.¹⁰¹ As *Hand1* and *Hand2* transcription factors are both expressed in the early cardiomyocytes, this suggests that growth of septal myocytes, elevated cell death, or positioning of the interventricular septum could underlie the *Hand1–Hand2* knockin chimeras CHDs. Evidence that *Hand1* is indeed an important regulator of the interventricular boundary is observed in *Mlc2v–Hand1* ectopic expressing transgenic mice that die at mid-gestation lacking a septum.¹⁰² The multiple causes underlying VSD causation are further illustrated via the recent finding that suppression of the TGF β superfamily intracellular signaling pathway within post-migratory cardiac neural crest cells in the outflow tract endocardial cushions can result in isolated VSD formation.⁸⁷ Using a three-component triple transgenic system, expression of the inhibitory Smad7 was induced via doxycycline within the neural crest lineage at post-migratory stages, resulting in isolated VSD and neonatal lethality. Although there is a paucity of data regarding the function of postmigratory neural crest within the heart itself, it has been shown via Cre-loxP that neural crest-specific N-cadherin, RhoA, and Cx43 expression are each required for normal OFT morphogenesis, cell shape, alignment, and cell–cell communication.⁸⁷ Thus, further studies are required to understand the link between lineage commitment and the many changes in cushion cell shape, cell–matrix adhesion, and cell–cell adhesion that occur within the heart itself during interventricular septal morphogenesis. One important disparity between the mouse models of VSDs and those seen in human infants is the high newborn mortality of mice compared with patients. In most mouse models, the VSDs are localized to the perimembranous region, are generally large, and are predicted to have significant left-to-right shunting.⁹⁶ It is also thought that mice are particularly sensitive to left-to-right shunting, given their high neonatal heart rates (~600+), as mouse models of PDA are also neonatal lethal^{103,104} despite PDAs being well tolerated in neonatal patients.

B. Tetralogy of Fallot

TOF is the most common cyanotic CHD and the most common cause of the “blue baby syndrome.” Approximately, 9–14% of babies with CHDs will have TOF (American Heart Association, 2010; www.heart.org). In TOF, an anterior placement of the conotruncal septum results in unequal division of the conus, which produces four cardiovascular alterations.⁴⁰ The four CHDs associated with TOF are VSD, right ventricular hypertrophy, pulmonary stenosis, and overriding aorta.^{40,105,106} In TOF, the VSD is present because of an anterocephalad malalignment of the developing ventricular septum and a failure of the OFT cushions to muscularize.^{40,106} Right ventricular hypertrophy is a hemodynamic consequence of the deviated outlet septum.¹⁰⁶ Pulmonary stenosis results from abnormal morphology of the septoparietal trabeculations surrounding the subpulmonary outflow tract, causing a narrowing of the right ventricular outflow and obstructing blood flow.^{40,106} Overriding aorta is a result of the displacement of the outlet septum into the right ventricle, which causes the aortic root to be positioned directly over the VSD.¹⁰⁶ Cyanosis is the major hallmark of TOF and can be present in neonates based on the degree of the blood flow obstruction to the lungs.^{105,106} Surgical intervention can result in complete repair, although adults have chronic issues such as pulmonary regurgitation, recurrence of pulmonary stenosis, and ventricular arrhythmias.¹⁰⁶ TOF is thought to be a multifactorial CHD and genetic associations of TOF are chromosomal aberrations such as trisomies 21, 18, 13 as well as microdeletions in chromosome 22.¹⁰⁶ Specific genetic associations via both mouse mutant analysis and patient screening also include *FOG2*, *JAG1*, *NKX2.5*, and *VEGF*. Cooperative

morphological data analysis from mice and humans shows that TOF may be caused because the lack of longitudinal growth of the OFT prohibits the normal counterclockwise rotation of the OFT region.¹⁰⁷

Several mouse mutants have been shown to exhibit TOF. The isoform-specific *Vascular endothelial growth factor (Vegf) Vegf120/120* knockin mice only express the 120 isoform and are highly predisposed to develop TOF.^{108,109} The proposed etiology is that a localized increase in VEGF signaling within the secondary heart field-derived myocardium results in alterations of Notch signaling, causing OFT cushion hypoplasia, and subpulmonary myocardial apoptosis.¹⁰⁹ Significantly, *VEGF* haplotype correlates with increase risk for TOF in patients.^{109,110} Similarly, as knockouts of mouse *Growth differentiation factor-1 (Gdf1)* left-right patterning gene exhibited CHDs, a population study was performed to determine if a spectrum of CHDs can be attributed to human *GDF1*.^{111,112} Indeed, a heterozygous loss-of-function in the human *GDF1* gene contributed to a distinct class of CHD affecting the conotruncus.¹¹² Thus, these studies confirmed that cardiac development in humans can be affected by left-right patterning signals and mutations in the entire pathway of left-right determination may be considered as candidates for CHD manifestations in humans on the basis of identifying a similar mechanism.¹¹² Finally, as mutations in the *FOG-2 (friend of GATA 2)* transcriptional cofactor that can physically interact with GATA-4/5/6, were shown to occur in patients with TOF,^{113,114} a mouse knockout was generated. Significantly, *Fog2* null mice die midgestation and display several cardiac malformations, including thin ventricular myocardium and TOF.¹¹⁵ These *Fog2* knockout mice are currently being used to examine *Fog2*'s ability to act in a dose-dependent manner and decrease *Fog2*-related pathways.¹¹⁴ Similarly, transgenic expression of truncated *Fog2* alleles is being used to examine its *in utero* function.¹¹⁴ Thus, via combining transgenic mice models and clinical and genetic characterization of patients, it is possible to gain a better understanding of the signaling pathways affecting cardiac development and the underlying causes of CHDs.

C. Hypoplastic Left Heart Syndrome

HLH is a relatively rare CHD in which the left side of the heart is severely underdeveloped and the causes are presumed to be genetic, although the exact genetic mechanism is unknown. Approximately, 4–8% of CHDs are HLH and the left ventricle is small and nonfunctional, while the right ventricle maintains both the pulmonary and systemic circulation.^{105,116} HLH is a heterogeneous group of abnormalities and in addition to the small or missing left ventricle, there is atresia of the mitral and aortic valves and hypoplasia of the ascending aorta.^{105,116} In patients with HLH, extensive reconstructive surgery is required for survival.¹¹⁶ Constriction of blood flow to or from the left ventricle can significantly affect left ventricular development and it is supposed that HLH may occur secondarily to embryonic hemodynamic disruptions, rather than a primary defect intrinsic to the left ventricle.^{116,117} It has been observed that the left ventricular growth worsens as fetal growth proceeds which support the theory that obstructed flow is suppressing myocardial growth.¹¹⁷

Although generating a mouse model has been challenging, as HLH is likely to be dependent on both genetic and epigenetic factors,¹¹⁶ a model may have been developed. Recently, screening of Jacobsen syndrome patients who often exhibit HLH as part of their syndrome identified a 1.2-Mb region of overlap that contains six candidate genes.¹¹⁸ One of the genes in that cardiac critical region is the transcription factor *ETS-1*, shown to be important in many biological functions, including regulation of cellular growth and differentiation.¹¹⁸ Critically, *Ets1* knockout mice exhibit various cardiac defects, but a subset has non-apex-forming left ventricle, which is a hallmark of HLH, although the other structures associated

with HLHS were normal.¹¹⁸ Via mouse transgenic analysis, we have established the beginnings of a road map to understand how ventricular cells become specified, differentiate, and expand into a functional cardiac chamber.¹¹⁹ The key is to now take this molecular and genetic information and devise innovative methods to augment or modify the effects of gene mutations on ventricular development.

XI. Conclusion

Significant advances in mouse genetics have led to a vast improvement in our understanding of cardiac morphogenesis. Multiple pathways and genetic networks have been indentified, and clear evidence accumulated to demonstrate which genes disrupt cardiac morphogenesis and can cause CHDs. Now the goal is to develop new concepts and models aimed at improving the diagnosis, treatment, and intervention of CHDs *in utero* and in newborns.

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Fig. 1.

(A) Echocardiography of 2-day-old newborn patient with DORV. Note aorta (Ao) is exiting from the right ventricle (rv). (B) Echocardiography of 5-month-old infant with perimembranous VSD. Note large hole connecting right and left ventricles (indicated via yellow arrow). (C–F) Histological hematoxylin/eosin stained sections of E14 *Hey2* knockout mutant and control littermate hearts. Note large VSD in (E) (indicated via yellow arrow) and DORV in (C) (as both the aorta and pulmonary (pul)) vessels exit the right ventricle. (G) Analysis of apoptosis via TUNEL immunohistochemistry in E14 *Hety2* knockout heart. Note that there is no elevation of cell death associated with VSD, but there are a few apoptotic (brown) cells localized at the base of the DORV. (H and I) Characterization of cell proliferation in E14 *Hey2* cardiovascular system via BrdU immunohistochemistry. *Hey2* knockouts (H) exhibit VSDs, while the septum is intact in wild-type littermates (I). Heat map inserts (in H and I) indicated the relative differences between the mutant (~13% proliferating cells) when compared to normal control hearts (~27% proliferating cells). Note that BrdU staining is reduced by approximately 50% in the stunted mutant interventricular septum. Abbreviation: lv, left ventricle. Bar in (E) and (F) is 100 μ M.

TABLE I

Three Subtypes of the Most Common CHDs Are Listed: Septation, Cyanotic, and Left-Sided Obstruction Defects

Congenital heart defect	Category	Mouse mutants
Atrial septal defect (ASD)	Septation defect	<i>Activin type II6 receptor, Bmp6/7 dKO, Cited2, Eve, Gata4, MyHC, Nkx2.5, Nt3, Tbx5, Zic3</i>
Atrioventricular septal defect (AVSD)	Septation defect	<i>Alk2 cKO, Cryptic, Cx40/45, Fog2, Gata4, Has2, Lefty-1, Nfatc, Nkx2.5, Pitx2c, RxRa, Trisomy 16</i>
Aortic coarctation	Left-sided obstruction	<i>Foxc1/Foxc2 double hets, Vangl2</i>
Aortic stenosis (AS)	Left-sided obstruction	<i>Elastin, Hox1.5</i>
Bicuspid aortic valve (BV)	Other	<i>Foxc1/Foxc2 double hets</i>
Double outlet right ventricle (DORV)	Cyanotic heart defect	<i>Bmp2/4 compound KOs, Cited2, Cx40, Disheveled compound KOs, Gata3, Gdf1, Hand1-Hd2 replacement K1, Hey2, Jumonji, Lefty-1, Meis1, Nf1, Nfatc, Pbx2/3, Pitx2c, PlexinA2, Rb/p107 dKO, Sema3c, SOX-4, Tolloid-like 1, Vangl2</i>
Ebstein's anomaly	Cyanotic heart defect	<i>Alk3 cKO</i>
Hypoplastic left heart (HLH)	Left-sided obstruction	<i>Ets1</i>
Interrupted aortic arch (IAA)	Left-sided obstruction	<i>Bmp receptor2, Chrd, Eta, Foxc2, Foxc1/Foxc2 double hets, Gata3, Ltbp, Pax3, Sema3c, TGFβ receptor type II cKO, Tbx1, Vegf 120/188 Kls, Zic3</i>
Mitral stenosis	Left-sided obstruction	<i>Foxc1/Foxc2 double hets</i>
Patent ductus arteriosus (PDA)	Other	<i>Hand1-Hand2 replacement K1, Tfp28</i>
Pulmonary atresia	Cyanotic heart defect	<i>Jag1, Ptpn11</i>
Persistent truncus arteriosus (PTA)	Cyanotic heart defect	<i>Alk2 cKO, Alk5 cKO, Bmp receptor IA cKO, Chrd, Cited2, Fak cKO, Fox2c/Eta dKOs, Gata6 cKO, Gata 3, Hox1.5, Ltbp1, NCadherin cKO, Neuropilin1, Nt3, Pax3, Pbx1, Pdgf receptors, Pdgf receptor6, Ptpn11 cKO, Raldh2, Rac1 cKO, RXRa, Smad 4 cKO, Smad7 overexpressor, TGFβ receptor type II cKO, Tbx1, Tbx20 hypomorph, Tfp2, RARα1, RAR6, Trisomy 16, Wnt5a</i>
Single ventricle	Cyanotic heart defect	<i>ENU mutant (chr. 7), Tbx20</i>
Tetralogy of Fallot (TOF)	Cyanotic heart defect	<i>Cx40, Fog2, Gdf1, Hey2, Jag1, Pbx2/3, Meis1, Tbx1, Vegf 120/188 Kls</i>
Transposition of the great arteries (TGA)	Cyanotic heart defect	<i>Activin type II6 receptor, Cryptic, Gdf1, Lefty1, Neuropilin1, Perlecan, Pitx2c, Zic3</i>
Tricuspid atresia	Cyanotic heart defect	<i>Bmp6/7 dKO, Hey2, Lefty1, Pax3-Fkhr K1</i>
Ventricular septal defect	Septation defect	<i>Alk2 cKO, Alk5 cKO, Bmp6/7 dKO, Chrd, Cited2, Cx40/45, eNos, Et1, Eta, Ets1, Fak cKO, Fgf receptor 2-IIIb, Foxc1/Foxc2 double hets, Foxc2, Fog2, Gata3, Gata4, Gdf1, Hey2, Jumonji, Ltbp1, Meis1, MyHc, NCadherin cKO, Neuropilin1, Nf1, Nfatc, Nkx2.5, Nt3, Pax3, Pax3-Fkhr K1, Pbx1, Pbx2/3, Pdgf receptors, Pdgf6, Pdgf, Receptor6, Raldh2, RARα1, RAR6, RxRa, Smad 4 cKO, Smad7 overexpressor, Sox4, Tbx5, Tbx20 hypomorph, Tfp2, TGFβ receptor type I cKO, Tolloid-like 1, Vegf 120/188 kls, Vangl2, wnt5a, zic3</i>

The types of CHD that occur within each subtype and a partial listing of the mouse mutants that exhibit them are indicated. Note that the majority of mouse models are conventional systemic knockouts, unless otherwise indicated.