Title: PROCOLLAGEN IIA DEFICIENT MICE

Abstract: This invention relates to the production of dual models of Congenital Heart Defects and Hypertrophic Cardiomyopathy with the use of mice which are genetically modified by transgenic (gene-knockout) techniques. The present invention produces knockout mice that show multiple cardiovascular malformations which will serve as a model of cardiovascular diseases for the screening of potential drugs against ventricular remodeling, malignant arrhythmias, primary pulmonary hypertension, and degenerative valvular diseases, and congenital heart disease.
PROCOLLAGEN IIA DEFICIENT MICE

This application claims priority of U.S. Provisional Application No. 60/591,935, filed July 26, 2004, the contents of which are hereby incorporated by reference into this application.

Throughout this application, various publications are referenced. Full citations for these publications may be found immediately preceding the claims. The disclosures of these publications are hereby incorporated by reference into this application in order to more fully describe the state of the art as of the date of the invention described and claimed herein.

Background of the Invention

Sudden cardiac death (SCD) is a major public health problem responsible for up to 63% of cardiac deaths among persons older than 35 in the United States. While acute ischemic heart disease is the major cause of SCD in this population, one of the most common causes of sudden death in adolescents and young adults less than 40 years old is hypertrophic cardiomyopathy (HCM). Other less frequent causes include anomalous coronary arteries, Marfan's syndrome, arrhythmogenic right ventricular dysplasia, myocarditis, aortic valvular stenosis and coronary atherosclerotic disease. HCM is an inherited disease of cardiac muscles where there is asymmetric or symmetric left ventricular hypertrophy with a non-dilated, hyperdynamic chamber in the absence of secondary causes. It affects men and women equally and is characterized by highly variable clinical courses, heterogeneous pathology and complex genetic expression. Depending on underlying mutations, HCM can be compatible with normal longevity at one end of disease spectrum, but at the other end, it is the most common cause of SCD in young athletes. The causes of SCD are assumed to be complex ventricular tachycardia or fibrillation originating on an electrically unstable myocardial substrate, but bradycardias and severe obstruction are also suggested. Prevention of SCD in HCM continues to be a major challenge for clinicians. The use of implantable
cardioverter defibrillator (ICD) in tertiary centers has been reported to be useful, but the patients may still suffer from end-stage heart failure and atrial fibrillation with attendant risk of thromboembolic strokes.\textsuperscript{3,4} A pathological model of HCM will be critical to understand the development of HCM and causes of SCD, and that will be important for prevention and treatment. Currently, the known disease genes account for about 50-70\% of all cases of familial hypertrophic cardiomyopathy.\textsuperscript{5} Most of the primary cardiomyopathies in humans, hypertrophic or dilated, are due to mutations in contractile proteins, cytoskeletal proteins, or gap junction proteins, and are in most cases the direct consequences of structural defects.

The cardiac extracellular matrix plays multiple important functional roles as the substrate for cell adhesions and scaffolding, as the signals for cell survival, as a reservoir for growth factors, and as the major determinant of tissue mechanics. Collagen is a major component of extracellular matrix in the mammalian hearts and exhibits a dynamic expression pattern and isoform diversity during development and in pathological conditions.\textsuperscript{5} In the adult myocardium, type I collagen comprises 80\% of total collagen and together with type III collagen forms a network of fibrillar collagen that supports individual cardiomyocytes and smooth muscle cells, and organizes muscle bundles, conduction system, and coronary vasculature into cohesive contractile units. In contrast, type III collagen is the major component in neonatal rat hearts, comprising about one-third of the total collagen content, and thus the visco-elasticity is different from that of adult myocardium. In rodent models of right ventricular hypertrophy, the ratio of types I and III collagen is clearly altered.\textsuperscript{6} Types II and VIII collagen are transiently expressed during cardiac development and have been implicated in cardiac morphogenesis. Although type II collagen was classically thought to be cartilage specific, a much wider tissue distribution has been shown during mouse embryogenesis. The mouse α1(II) collagen gene (Col2a-1) is transiently expressed in many non-cartilaginous tissues such as the notochord, neural retina, tail tendon, heart, surface ectoderm, calvarial mesenchyme, fetal brain, the sensory epithelium of the inner ear and the apical ectodermal ridge of the forming limb buds (AER). Alternative splicing of exon 2 produces type II procollagen mRNAs that either include (IIA mRNA) or exclude (IIB mRNA) this exon. Type IIA mRNA is abundant in cells of the epimyocardium in both the atria and ventricles of 9.5-day embryos but rapidly diminishes by 10.5 days. After 12.5 days, IIB mRNA
levels increase rapidly and finally exceed IIA mRNAs. By 16.5 days, IIB mRNAs are the major Col2a-1 transcripts, predominantly expressed in maturing chondrocytes.\textsuperscript{13} Postmortem analyses from one hospital revealed that defects in cardiac septation were present in 2 infants with hypochondrogenesis, a disorder of type II collagen. How type IIA collagen contributes to cardiac morphogenesis is not yet clearly defined, but recent studies suggest that it may modulate growth factors. Of note, exon2 of the type II collagen gene (Col2a-1), which is included in IIA mRNA, encodes a cysteine-rich globular domain in the amino-propeptide of pro-\alpha1(II) collagen chains. This cysteine-rich domain has been shown to bind bone morphogenic proteins (eg. BMP2) and Transforming Growth Factor (TGF)-\beta in \textit{in vitro} assays.

TGF-\beta1 and BMP-2 belong to the transforming growth factor-\beta superfamily that regulates growth and differentiation. Like other members of the TGF-\beta superfamily (TGF-\beta/Activin/Nodal and BMP/GDF/MIS subfamilies), their diverse biological effects are mediated by the formation of heteromeric complexes of type I and type II serine/threonine kinase receptors, phosphorylation of the type I receptor by type II receptor and subsequent activation of Smad proteins. Receptor-activated Smads (R-Smads, Smads 1-3, 5, 8) are phosphorylated by type I receptor kinases, form heteromeric complexes with the common mediator Smads (co-Smads, Smad4) and translocate into the nucleus where they interact with specific transcription factors and co-regulators to modulate gene expression. R-Smads2 and 3 respond to the TGF-\beta subfamily, while R-Smads 1, 5, and 8 primarily to the BMP subfamily. Two inhibitory Smads (I-Smad), Smad6 and Smad7, negatively regulate TGF-\beta/BMP signaling by competing with Smad4 for the binding of activated R-Smads, inhibiting phosphorylation of R-Smads, or targeting the receptors for degradation.\textsuperscript{16, 17} Both TGF-\beta1 and BMP-2 play crucial roles in various aspects of heart development, especially in cardiomyocyte differentiation, valvulogenesis, and outflow tract and septal development.\textsuperscript{18-21} TGF-\beta1 exerts diverse biological activities in postnatal hearts. It controls the expression of major histocompatibility complex, induces production of the extracellular matrix proteins by cardiac fibroblasts, regulates vascular smooth muscle cell phenotype, induces cardiac hypertrophy, and enhances \beta-adrenergic signaling. There is limited information about the role of BMP-2 in postnatal mammalian hearts. In rat neonatal cardiomyocyte culture, BMP-2 exerts
anti-apoptotic effect by activation of the Smad1 pathway and enhances contractility by activation of phosphatidylinositol 3 kinase pathway. \(^{22, 23}\) Clinically, primary pulmonary hypertension, a disorder of increased proliferation of endothelial and smooth muscle cells in pulmonary arteries, has been linked to mutations in type II receptors for BMP (BMPR-II).\(^{24, 25}\)

Recent studies in the murine model of Marfan syndrome also highlight the active role of extracellular matrix, in this instance, fibrillin, in modulating TGF-β signaling.\(^{26, 27}\)

In human dilated cardiomyopathy, increased perimysial fibrosis is associated with increase in types I, III, and VI collagen content, and type V collagen is increased in intracellular matrix of myocardium. Type II collagen is not expressed in normal hearts or dilated cardiomyopathy.\(^{28}\) Whether or not type II collagen is ectopically expressed in hypertrophic cardiomyopathy in humans remains to be investigated. A variety of mouse models of cardiomyopathies and altered contractility focus on targeted mutations of aforementioned proteins, cell cycle regulatory proteins, regulator proteins involved in calcium metabolisms, and subunits of the adrenergic receptor signal transduction cascade. The concept of extracellular matrix modulating tissue patterning and cardiomyocyte differentiation is not novel although matrix defects have not generally been considered as possible source of cardiomyopathies. Even in the mouse with targeted deletion of type III collagen, there was no cardiac abnormality and sudden death was due to rupture of the aorta.\(^{29}\) The adult 191-IIA mutant mice described in this application are therefore a very important model for cardiomyopathy-induced death which may occur suddenly.

Taussig-Bing Anomaly (TBA) is a rare but complex congenital heart disease and comprises of a double outlet right ventricle (DORV) with subpulmonary ventricular septal defect (VSD). Aortic and pulmonary trunks are transposed. Next to Tetralogy of Fallot, TBA is the most common variant of DORV. Infants with TBA often present with congestive heart failure and pulmonary hypertension. Its management is complicated by associated vascular defects such as coronary anomalies, subaortic stenosis, aortic arch coarctation or interruption. Currently recommended treatment is complete correction in a single operation, typically involving arterial switch operation,
VSD repair, corrective repair of aortic obstruction, and translocation of anomalous coronary arteries. Therefore, detailed mapping of these vascular defects is crucial to the success of corrective surgery. TOGA is the most frequently diagnosed complex cyanotic heart defects in newborns (ref) and involves ventriculoarterial discordance.


Here we describe that mice carrying the IIA mutation on a C57/BL6 genetic background is a novel mouse model of TBA and TOGA. Mutations in the human COL2A1 gene are dominant, resulting in chondrodysplasias with a spectrum of severity from prenatal lethality to mild deformity. Dominant mutations in exon2 have been found in the Stickler Syndrome and Wagner’s Syndromes which are also associated with eye defects{ Richards, A.J. et al. COL2A1 exon 2 mutations: relevance to the Stickler and Wagner syndromes. Br J Ophthalmol 84, 364-71 (2000).}{ Van Der Hout, A.H. et al. Occurrence of deletion of a COL2A1 allele as the mutation in Stickler syndrome shows that a collagen type II dosage effect underlies
Summary of the Invention

The invention provides two lines of genetically engineered mice where the exon 2 of procollagen IIA gene is flanked by loxP sequences and this exon 2 is deleted by mating with mice expressing Cre-recombinase. Deletion of exon 2 results in deletion of IIA transcript. This in turn results in dual manifestation of cardiovascular defects where one lineage being predominantly C57/BL6 genetic background, dies of severe congenital heart disease (IIA-/-) and the other lineage on an outbred genetic background (C57BL6/ICR/129) develops hypertrophic cardiomyopathy characterized by cardiomyocyte hypertrophy, disarray and valvular fibrosis (191-IIA). This invention provides a method of screening for an agent that diminishes hypertrophy, disarray, and fibrosis, as well as cardiovascular patterning defects by administration of the agent to either 191-IIA adult mice or IIA-/- mutant embryos. Such agent may be synthetic or naturally occurring hormones, growth factors, peptides, or genes carried by suitable vectors for gene transfer. This invention also provides primary cell cultures from hearts or aorta and permanent cell lines derived from the knockout mice. Cells from permanent or primary cell cultures from 191-IIA mice are used to screen for agents that ameliorate or abrogate the processes of hypertrophy, hyperplasia, or fibrosis.
Brief Description of the Figures

Figure 1  Pedigree of 191-11 A mice
The upper panel summarizes the natural history of sudden death in 191-IIA mice. The lower panel shows four examples of cardiomegaly found in these mice at different ages.

Figure 2  H&E stainings of 191.1 heart
A C E - from a wild type mouse. B D F - from 191.1 male, a homozygous null mutant for CollIA that died at 2 months. A B - Two-chamber short-axis view of the hearts from the wild type (A) and the mutant (B). The left ventricle wall of the mutant is less compacted and is hence thicker than that of the wild type. C D - Right ventricle (RV), 100x total magnification. The RV wall thickness is increased in the mutant. E F - Lateral LV wall, 400x total magnifications. While the cardiomyocytes in the wild type are uniform in size and display parallel orientation in the myocardium (E), the mutant myocardium is characterized by cardiomyocyte hypertrophy and disarray (F).

Figure 3  H&E stainings of 191.3 heart
A C - Aortic valve (AV) and mitral valve (MV) of a wild type mouse, respectively. B D - AV and MV of 191.3 male that died at 3 months. In the mutant, both valves appear fibrotic. All panels are at 400x total magnification. Scale.bar- 20 μm.

Figure 4  H&E stainings of older 191-IIA mice
A B - Lateral wall of LV from 254.66 male that died at 8 months. Note the hypercellularity. C - RV of 254.66. Note the hypercellularity and cardiomyocyte hypertrophy. D - Lateral wall of LV from 191.29 male that died at 15 months. As compared with Fig. 2F, 4A, and 4B, cardiomyocytes in this mouse are less disorganized. All panels are at 400x total magnification. Scale bar - 20 μm.

Figure 5  Normal expression pattern of IIA mRNA and generation of IIA-/- mice
A - Asymmetric distribution of IIA mRNA in normal developing hearts. Whole
mount in situ hybridization (WISH) of wild type embryos with IIA-specific probe. At 2-somite pair stage (E8.0), IIA mRNA expression was very strong in the cardiac crescent. At 10-somite pair stage (E8.5), the IIA mRNA expression continues to be very strong in the looping primitive heart tube. At the 27-somite pair stage (E9.5), the IIA mRNA expression is the highest in the bulbous cordis and outflow tract, followed by the left ventricle and the lowest level was found in inflow tracts (atria).

**B** - Schematic diagram of the Col2a1 locus and targeting vector where exon 2 was disrupted by insertion of the Neo gene flanked by loxP sites. The Neo gene was subsequently excised by Cre-mediated recombination. **C, D, E** - Targeted deletion was confirmed by southern blotting, RT-PCR, and WISH, respectively.

**Figure 6. Cardiovascular Defects in Newborn IIA-/- Mutants**

From left to right, transverse sections are displayed from cranial to caudal ends of the thorax. Panels (A-C) are from WT newborn mice. Panels (D-L) are from IIA-/- mutants. **A, B** - In WT, the pulmonary artery (p) arises from the right ventricle (rv) and is situated anterior and cranial to the aorta (a). The aorta curves to the left of the trachea (t) and gives rise to right subclavian artery (rs) and right carotid artery (rc), left carotid artery (c), and left subclavian artery (ls). **C** - An interatrial septum separates the right and left atria (arrowhead) while interventricular septum (ivs) separates the left ventricle (lv) from the right ventricle (rv). Note that right ventricular wall is thinner than that of left. **D-F** - An example of incomplete TOGA with DORV, and subpulmonic VSD. **D** - In IIA-/- mutants, the aorta (a), which gives rise to head vessels, is anterior to the pulmonary artery (p). An anomalous coronary artery (asterick) arises from the ascending aorta. **E** - The aorta (a), where coronary arteries take off (arrows), arises entirely from the RV. A large patent ductus arteriosus (pda) connects to a left descending aorta (da). **F** - The pulmonary artery (p) is caudal to the aorta and arises both from LV and RV. The pulmonary valve (pv) is overriding a subvalvular ventricular septal defect (arrowhead). **G** - An example of complete TOGA, where the aorta (a) arises from RV. The pulmonary artery (p) is transposed caudally and posteriorly and arises entirely from LV. **H** - A large ECD (double arrowheads) I - Persistent truncus arteriosus (PTA), a complete failure of septation of the primitive outflow tract with resultant main pulmonary arteries (p) arising from the posterior wall of the common arterial trunk and sharing
of common abnormal truncal valves (tv).  J - A right lateral view of a IIα^−/− mutants with right aortic arch and descending aorta (rda) curves to the right of the trachea (t), giving rise to separate right carotid artery (rc), right subclavian artery (rs), and a left carotid artery (ic). A large PDA connects the pulmonary artery and descending aorta (rda). K - Interrupted aortic arch in a IIα^−/− mutant. Note that the aortic arch between the left carotid artery (ic) and left subclavian artery (ls) is absent (outlined by broken lines). L - A retro-esophageal left subclavian artery (res) arises from the right descendint aorta (rda) and courses behind the esophagus (e) and trachea.

Figure 7. Defective BMP signaling in IIα^−/− embryos
A - WISH with BMP2 riboprobe in E9.5 IIα^−/− embryos (right panel) shows BMP2 expression is diminished in the heart, including the atroioventricular cushion, and proepicardial organ (PEO). B - WISH with BMP4 riboprobe also shows diminished BMP4 expression in the ventricles, atria, and PEO of the IIα^−/− embryo (right panel).

C - WISH with TGFβ-2 riboprobe shows diminished expression in AV cushion and PEO (right panel). D - Immunohistochemistry against BMP2 protein (upper panels) and BMP4 (lower panels) in E13.5 hearts shows that there is no significant change in protein expression in IIα^−/− mutants (right upper and lower panels).

E Immunohistochemistry against phosphorylated Smad1 (p-Smad1) in serial sections from E13.5 embryos. In IIα^−/− mutants (third and fourth panels), the number of nuclear staining with p-Smad1 is significantly reduced across the entire heart, especially in the right ventricle (RV) and interventricular septum (IVS). F - Co-localization of IIα, BMP2, and BMP4 proteins in wild-type hearts. In the E8.5 (first row), IIα protein and BMP proteins co-localized in the ECM of both endocardium and myocardium. In the OFT of E9.5 looping heart (second row), BMPs and IIα protein remain colocalized in the ECM of myocardium but BMP expression is lost in endocardial lining. In the primitive left ventricle (third row) of the same heart, BMPs
and IIA also colocalize in the ECM of myocardium.

**Figure 8. Defective Nkx2.5 expression in IIA/- embryos**

A - WISH with Nkx2.5 riboprobe. At E9.5, there was significant reduction of Nkx2.5 expression in IIA+/ heart (second column). Transverse sections of E9.5 hearts show both reduction in the size of mutant hearts and reduced Nkx2.5 expression in individual cells (fourth column). B - WISH of E8.5 embryos with Col2a1 riboprobe that detects transcription of the gene and recognizes both IIA and IIB mRNAs. In IIA-/- mutant (right panel), reduced signal was detected in the heart despite the fact that targeted deletion of exon 2 of Col2a1 should not affect transcription of the gene.. C. Comparative genomics at the Col2a1 locus identifies three conserved non-coding regions upstream of the gene using VISTA program. D. One region (region C) contains conservec Nkx2-5, GATA4 and SRF sites. E. Top- diagram of Col2a1 locus showing the positions of three (regions A,B,C) conserved non-coding sequences upstream of the transcriptional start pf the gene, bSY1 is a modification of the bac clone (61F08) containing the whole Col2a1 gene with the LacZ gene inserted into exon 1 of the gene. Middle row, X-gal staining of representative bSY1 transgenic embryo showing strong expression in the heart and lower row, bSY2\(^{nko-neo}\) 8.5dpc embryos. In the bSY1' embryo, intense blue staining in the heart recapitulates endogenous Col2a1 expression pattern. Bottom row, in the bSY2\(^{nko-neo}\) embryo, deletion of Nkx2.5 binding site in the 5' regulatory region of Col2a1 by inserting the neo gene into the Nkx2.5 site results in abolishment of lacZ expression in the heart.

**Figure 9. Summary of roles of IIA procollagen in Nkx2.5 regulatory loop during cardiovascular morphogenesis**

Once a contractile, linear heart tube is established, looping and lengthening of OFT involve further addition of cardiac progenitor cells from the cardiac crescent and anterior heart field and finally participation by cardiac neural crest cells. At this stage, a regulatory loop is initiated where Nkx2.5 enhances or maintains IIA procollagen production by regulating the Col2a1 gene. IIA procollagen in turn acts as facilitator, sequesters BMP2 ligands, presents the morphogen to BMP receptors, and thus augments BMP signaling necessary for deployment of these progenitor
cells. Note that it is not known if IIA procollagen and BMP complexes are processed by proteases (such as furin-like, tolloid) to release the BMP for interaction with the receptor. Nuclear translocation and binding of BMP-responsive phosphorylated smads – Co-smad 4 complexes to the smad responsive element on the 5' regulator region of \textit{Nkx2.5} promotes transcription and translation of \textit{Nkx2.5}. \textit{Nkx2.5} in turn activates \textit{Col2a1} and thus a regulatory loop is completed. This regulatory loop potentially also involves combinatorial interactions of GATA and SRF transcription factors. It is known that GATA4 and Smad4 cooperate to transactivate \textit{Nkx2-5} and GATA6 has been shown to regulate \textit{Nkx2-5} (reviewed in Patient RK, McGhee, JD, Curr Op Gen Dev 12:416-422 (2002); Peterkin T et al Sem Cell Dev Biol 16: 83-94 (2005). \textit{Nkx2-5} has also been shown to regulate GATA6 in a reinforcing regulatory network \{Molkentin, J.D. et al. Direct activation of a GATA6 cardiac enhancer by \textit{Nkx2.5}: evidence for a reinforcing regulatory network of \textit{Nkx2.5} and GATA transcription factors in the developing heart. \textit{Dev Biol} 217, 301-9 (2000).\}. SRF can interact with GATA4 or \textit{Nkx2-5} to activate genes in cardiomyocytes. Note the presence SRF, GATA and \textit{Nkx2-5} binding sites in the conserved \textit{Col2a1} enhancer element (Fig.8.C-D).

\textbf{Detailed Description of the Invention}

During mouse embryogenesis, the mouse α1(II) collagen gene (\textit{Col2a-1}) is transiently expressed in many non-cartilaginous tissues such as the notochord, neural retina, tail tendon, heart, surface ectoderm, calvarial mesenchyme, fetal brain, the sensory epithelium of the inner ear and the apical ectodermal ridge of the forming limb buds. Alternative splicing of exon 2 produces type II procollagen mRNAs that either include (IIA mRNA) or exclude (IIB mRNA) this exon. Type IIA mRNA is abundant in cells of the epicardium in both the atria and ventricles of 9.5-day embryos but rapidly diminishes by 10.5 days. After 12.5 days, IIB mRNA levels increase rapidly and finally- exceed IIA mRNAs. By 16.5 days, IIB mRNAs are the major \textit{Col2a-1} transcripts predominantly expressed in maturing chondrocytes.

To understand the function of IIA, gene targeting was used in embryonic stem cells
to produce mutant mice that can only produce Col2a-1 mRNA without exon 2. Compelling evidence showed that IIA plays an essential role in cardiac morphogenesis by modulating BMP and TGFβ signaling. Mice lacking IIA display multiple cardiovascular malformations with many features of some human congenital heart diseases. Because of the variable penetrance of the severe phenotype, the IIA mutants were backcrossed to the C57B16 background (6 generations). The genetic background of these IIA-/- mutants was predominantly C57/BL6 background (approximately 90-95%) with a small contribution of 129Sv and ICR background. Mice homozygous for the IIA null mutation (IIA-/-) display defects in patterning and morphogenesis of the heart similar to those associated with loss of function of the BMP, TGF-β and nodal signaling pathways and die perinatally from cardiovascular insufficiency. The spectrum of heart defects include ventricular septal defect (VSD), patent ductus arteriosus (PDA), persistent truncus arteriosus (PTA), right ventricular hypertrophy, right aortic arch with or without anomalous origins of subclavian arteries, and the Double Outlet Right Ventricle (DORV) where aorta and pulmonary arteries arise wholly or in great part from the right ventricle, with associated VSD, transposition of great arteries (TOGA), and endocardial cusion defects with primum atrial septal defects. Both BMP/TGFβ signaling activity and expression of Nkx2.5 were reduced in the IIA-/- mutant hearts.

We show that Nkx2.5 regulates the Col2a1 gene, which was reciprocally downregulated in Nkx2.5-null mutants. Our findings highlight a novel role for extracellular matrix proteins such as procollagen IIA which regulates cardiovascular morphogenesis by modulating cardiac transcription factors activity through a BMP-mediated signaling via a regulatory loop. Procollagen IIA deficiency may underpin some common congenital heart diseases. The majority of mutant embryos have a triad of DORV, incomplete TOGA, and subpulmonic VSD, the findings consistent with Taussig-Bing anomaly in humans. Taussig-Bing anomaly is a rare but severe complex cyanotic congenital heart disease, first described by Pernkopf in 1920 and subsequently by Helen Taussig and Richard J. Bing in 1949.30 Its mode of genetic transmission and molecular defects have not been systematically studied. This line of IIA mutant therefore may be useful as a novel animal model of Taussig-Bing anomaly.

In generating these IIA null mutants, a viable line of IIA7-mice (hereafter referred to as 191-IIA) with a mixed genetic background comprising C57/BL6; 129Sv; ICR was
also generated. It is at the 5th generation of homozygous matings. In the second generation, 12 out of 19 animals died shortly after birth. Out of 28 animals in the third generation, 6 died prematurely between 2 to 8 months. Interestingly, there was a preponderance of males among those showing premature death (Figure 1). The following observations were made from limited necropsy:

- Cardiomegaly with atrial enlargement and increased wall thickness in right and/or left ventricles (Fig.1, 2C, 2D)
- Hypercellularity with atypical nuclei, mostly in interstitium (Fig.2F, 4A-C)
- Cardiomyocyte disarray and heterogeneity in size, with prominent cellular hypertrophy (Fig. 2F, 4A-D)
- Valvular fibrosis (Fig. 3B,D)
- Absence of extensive myocardial necrosis or fibrosis
- Absence of outflow tract or aortic arch anomalies
- The severity of cardiomyocyte hypertrophy and disarray seems to correlate with shortened survival (compare Fig.2F with Fig.4A, B, and D).

These adult 191Co/IIA/- mice develop cardiomyopathy because of dysregulation in cardiomyocyte proliferation and growth during fetal and/or postnatal development. One possible mechanism is that the absence of IIA procollagen at a critical period of heart morphogenesis perturbs the delicate balance among different collagen isoforms and alters the extracellular matrix composition in fetal/neonatal/adult hearts. Of note, exon 2 of the type II collagen gene (Col2a-1), which is included in IIA mRNA, encodes a cysteine-rich globular domain that has been shown to bind bone morphogenic proteins (eg. BMP2) and Transforming Growth Factor (TGF)-β1 in in vitro assays. This alteration in the cardiac matrix in turns may perturb the proper local gradients of BMP2 and TGF-β and may alter cardiomyocyte contractility by affecting sarcomeric assembly and/or signal transduction.

Clinical studies have demonstrated that cardiac hypertrophy is a major risk factor for major cardiac events, such as myocardial infarction, heart failure, sudden cardiac death, and stroke. This line of mutant mice can be used to screen potential drugs for the treatment of cardiac hypertrophy, a major risk for sudden death and heart failure. For example, one may administer drug X or a placebo to adult 191-IIA
mice. After 4 weeks of treatment, both groups of 191-IIA mice are sacrificed and examined for the extent of change in cardiac hypertrophy by histological techniques. The myocardial infarction induced in this line of mutant mice will be useful as a platform to screen for potential drugs against ventricular remodeling and malignant arrhythmias. In this case, coronary arteries of 191-IIA mice are ligated to induce myocardial infarction. One group receives drug X and the other a placebo. Both groups have implantable telemetry to evaluate for the occurrence of malignant arrhythmias. Both groups have serial echocardiography to evaluate for any changes in cardiac function. At the end of the drug treatment, both groups are sacrificed and their hearts are processed by paraffin embedding, serial sectioning, Hematoxylin and Eosin stainings, and trichrome Mason's stainings. These cardiac sections are examined for the geometric changes associated with ventricular remodeling as well as cellular disarray and hypertrophy.

Roles of TGF-beta and BMP signalings in development of cardiomyopathy:
Of note, exon 2 of the type II collagen gene (Col2a-1), which is included in IIA mRNA, encodes a cysteine-rich globular domain in the amino-propeptide of pro-c1(II) collagen chains. This cysteine-rich domain has been shown to bind Bone Morphogenetic Proteins (e.g. BMP2, BMP4, BMP7) and Transforming Growth Factor (TGF)-β1 in in vitro assays. Members of the TGFβ superfamily and downstream effectors have been shown to play crucial roles in various aspects of heart development (examples in Table 1), especially in cardiomyocyte differentiation, valvulogenesis, and outflow tract and septal development. TGF-β1 exerts diverse biological activities in postnatal hearts. It controls the expression of major histocompatibility complex, induces production of the extracellular matrix proteins by cardiac fibroblasts, regulates vascular smooth muscle cell phenotype, induces cardiac hypertrophy, and enhances β-adrenergic signaling. There is limited information about the role of BMPs in postnatal mammalian hearts. In rat neonatal cardiomyocyte culture, BMP-2 exerts antiapoptotic effect by activation of the Smad1 pathway and enhances contractility by activation of phosphatidylinositol 3 kinase pathway. The adult 191-IIA mutant mice are therefore a very important model for cardiomyopathy secondary to the dysregulation in BMP/TGF signaling. The 191-IIA mutants are useful models to screen potential drugs, whether it be pharmaceutical or gene therapy, targeted at the BMP/TGF induced Smad signaling pathways on
cellular hypertrophy and proliferation. Such drug candidates can be ligand traps, BMP or TGF-β agonists, receptor antagonists, or Smads proteins. The end-points of such drug testing will be (1) amelioration or abrogation of cardiac hypertrophy and (2) improved survival.

**Roles of TGF-beta and BMP signaling in primary pulmonary hypertension:**

Primary pulmonary hypertension (PPH) is a progressive and fatal disease where mean pulmonary arterial pressure at rest is greater than 25 mmHg or 30 mmHg with exertion in the absence of left-sided heart diseases, chronic thromboembolic disease, underlying pulmonary diseases, or other secondary causes. It is a rare disorder that commonly affects young people, especially women in the third decade of life, leading to progressive right heart failure and death with the median survival of 2.8 years. PPH is characterized by obstruction of small pre-capillary pulmonary arteries with associated plexiform lesions, medial hypertrophy, intimal fibrosis, fibroid degeneration, and thrombotic lesions. Symptoms associated with primary pulmonary hypertension are often non-specific, including dyspnea, palpitations, fatigue, chest pain, near-syncope, syncope, peripheral edema, and as a result, the correct diagnosis is often delayed. Standard therapies include anticoagulation, calcium channel blockers, prostacyclin infusion, and, in refractory cases, lung transplantation and atrial septostomy. Other pharmacological agents in trial include inhaled or oral prostacyclin analogues, phosphodiesterase inhibitors, endothelin-I receptor antagonists, and L-arginine. Central pathogenesis is thought to involve dysregulation in smooth muscle and endothelial cell proliferation rather than abnormal vasoconstriction. Genetic analysis reveals that deleterious heterozygous mutations in type II receptors for BMP (BMPR2) are present within the majority of familial and up to 26% of sporadic cases of primary pulmonary hypertension. In addition, in 9% of patients who developed pulmonary hypertension after exposure to appetite suppressant fenfluramine and dexfenfluramine are found to have mutations in BMPR2. More recently, mutations in ALK-1, a component of TGF-β receptors, are found most commonly in patients with hereditary hemorrhagic telangiectasia and pulmonary hypertension.

Many experimental data have demonstrated the pivotal roles that defective TGF-β and BMP signaling play in the pathogenesis of pulmonary hypertension. For
examples, transgenic mice expressing dominant-negative BMPR2 in smooth muscles develop pulmonary hypertension. While BMP-2 enhances apoptosis of normal human pulmonary vascular smooth muscle cells, primary arterial smooth muscles from PPH patients exhibit abnormal growth responses to BMP and TGF-

p. In one embodiment, the 191-IIA mice will serve as a stand model of PPH to screen for potential drugs, whether it be pharmaceutical or gene therapy, targeted at the BMP/TGF induced Smad signaling pathways in pulmonary vascular smooth muscle cells and endothelial cells. Such drug candidates can be ligand traps, BMP or TGF-P agonists, receptor antagonists, or Smads proteins. The end-points of such drug testing will be (1) amelioration or abrogation of pulmonary pathology found in PPH (i.e., plexogenic or thrombotic arteriopathy), (2) amelioration or abrogation of right ventricular hypertrophy, and (3) improved survival.

Roles of TGF-beta and BMP signaling in valvular fibrosis:

The transient absence of IIA collagen during the critical period of embryonic development results in fibrosis of semilunar and atioventricular valves. Studies in recent years have clearly established the critical roles of TGF-β and BMPs in valvulogenesis during development. Currently, surgical repair, valve replacement, and balloon valvulotomy remain the only treatment options for aortic stenosis and mitral stenosis, both which present with fibrotic, often calcified valves with decreased leaflet mobility. The 191-IIA mice will serve as stand model to develop novel non-surgical therapeutics for treatment of degenerative valvular diseases. Drugs targeted at TGF-β and BMP signaling pathways will be administered to the 191-IIA mice and screened for their anti-fibrotic effects.

Characterization of the hemodynamic alteration and determine the mechanisms of sudden death in adult 191Co/IIA-/- mice:

1) Postmortem Analysis of adult 191Co/IIA-/- mice that died suddenly

a) Light microscopy

Paraffin blocks of the heart, lungs, and aorta are sectioned along specific axes of the heart, processed for H& E stainings and Mason's trichrome stainings and analyzed for inflammatory infiltration, pathological hypertrophy, and interstitial fibrosis.
b) **Electron microscopy**

The cardiac sections are examined by transmission electron microscopy to delineate any ultrastructural defects in the myofibrils and extracellular matrix.

2) **Cardiovascular functional assessment**

Cardiomegaly found in 191 Coll/IIA/- mice could be caused by myocyte hypertrophy, hyperplasia, myocardial fibrosis with chamber dilatation, or infiltrative disease. In order to define the clinical course of cardiomegaly and elucidate the mechanisms of sudden death, we plan to study 191 Coll/IIA/- mice and wild-type littermates in parallel. Heterozygous 191 Coll-IIC+/+ mice are generated by one round of backcrossing 191 191 Coll/IIA/- mice with 1CR mice. Wild type littermates are derived by heterozygous matings. Since the mode of sudden death may be different for different age groups, the following studies will be conducted at 2 months and 8 months of age.

a) **Determination of chamber sizes and function by echocardiography**

Serial transthoracic ECHO will be performed to determine the left and right ventricular chamber size, wall thickness, systolic function, and presence of significant valvular diseases.

b) **Evaluation for systemic hypertension**

Hypertrophy, fibrosis, or chamber dilatation may be manifestations of hypertensive heart disease or valvular diseases. Serial blood pressure measurement are obtained using the cuff-tail method. This non-invasive method allows one to follow these mice longitudinally for development of hypertension.

c) **Evaluation for cardiac arrhythmias**

By mechanisms that are yet to be defined, hypertrophy, fibrosis, and poor left ventricular function often lead to malignant arrhythmias that are major causes of sudden cardiac death. To rule out malignant arrhythmias as potential causes of sudden death, the heart rhythm of IIA/- mice and wild type littermates is monitored with a miniature telemetric device from Data Sciences International.

d) **Determination of left ventricular contractility and compliance**

Any change in the extracellular matrix composition and the architecture
of cardiomyocytes will invariably affect global contractile performance of the heart. To determine any alteration in systolic contractility and diastolic compliance, left ventricular pressure-volume loops will be generated using the specialized conductance catheter from Millar in non-recovery surgery.

e) **Evaluation for pulmonary hypertension**

One consistent finding among these IIA-/- mice with premature death is increased right ventricular wall thickness. Combination of hypercellularity and hypertrophy may explain this finding. Right ventricular hypertrophy may be a manifestation of primary myocardial disease, or secondary to pulmonary hypertension. To rule out pulmonary hypertension, pulmonary artery pressure is measured directly by inserting a single-lumen catheter (0.25mm O.D.) with a curved tip from the right jugular vein through the right atrium, tricuspid valve, and right ventricle into the main pulmonary artery. Lung sections are examined for microscopic changes associated with pulmonary hypertension, such as medial hypertrophy of airway vasculature and pulmonary fibrosis.

**Determination of the cellular and molecular defects in the hearts of adult ColIIA-/- mice.**

Murine cardiomyocytes continue to proliferate at a low rate during first three weeks after birth. More recent morphometric analysis has confirmed that mouse cardiomyocytes continue to divide for the first 4 postnatal days and increase in the volume between days 5 and 14. After day 14, individual cardiomyocytes continue to increase in volume, albeit at a slower rate, and finally reaches the adult size at 3 months of age. It is possible that this transition point from proliferation to developmental growth has shifted in 191 ColIIA-/- mice. Therefore, this part of study focuses on adult mice at 2, 5, and 8 months of age. If cardiomyocyte hypertrophy is a prominent feature, the hearts are examined from earlier time points to determine when pathological hypertrophy starts after birth. All the reagents for sections (1), (2), and (4) are commercially available. Antibodies for the characterization of the extracellular matrix proteins are either commercially available or available through our overseas colleagues.
1) Cardiomyocyte hypertrophy
   a) Morphometric analysis
      i) Increased wall thickness could be due to increase in
cardiomyocyte size (hypertrophy), increase in density
(hyperplasia) of myocytes and/or nonmyocytes, and/or increase
in interstitial space from infiltrative process. To determine
whether myocyte hypertrophy is present, HA/- mice and their
sex-matched wild type littenates will be sacrificed at 2 months,
the body and heart weights, and tibial lengths determined. The
LV or RV/body weight ratio and LV/RV ratio will be determined.
For histological analysis, 1 mL of cadmium chloride 0.1 M will be
infused into the inferior cava to achieve cardiac arrest in diastole
and the animal will be retrogradely perfused with phosphate
buffered saline at 100 mmHg followed by perfusion with 3.7% formalin. The arrested heart will be fixed for 2 hours to 24 hours
in 4% paraformaldehyde and embedded in paraffin for sectioning.

      ii) To determine the density and size of cardiomyocytes, heart
sections will be processed for double immunofluorescent
labeling with FITC-laminin antibody, which outlines the cardiac
sarcolemma, and propidium iodide (PI). To determine the
dimensions and volume of individual cardiomyocytes from left
and right ventricles, individual cardiomyocytes will be isolated
using the method developed by Leu and co-workers. Briefly, a
strip of myocardium from either left or right ventricle will be tied
to a board and collagenase digestion will be carried out in the
presence of verapamil, a calcium channel blocker. By triturating
gently, individual cardiomyocytes are released as rod-shaped
cells. These cells will be plated on gelatin-coated slides and
processed for immunofluorescence labeling with sarcomeric
alpha-actin antibody. The arrangement as well as density of sarcomeres per cell will be determined. This method also allows
selective isolation of cardiomyocytes from right ventricles and
will be extremely useful in assessing the presence of cellular hypertrophy in these cells.

iii) The cellular volume is measured using laser confocal microscopy and 3-D image reconstruction program.

5  b) Alterations in contractile proteins
The levels of myosin heavy chain, myosin light chain, tropomyosin, troponin T, and actin are analyzed by immunoblotting of heart lysates.

c) Reactivation of "fetal" genes
Pressure-overload induced cardiac hypertrophy is associated with reactivation of "fetal" genes. Therefore, the expression of ANP, BNP, α-skeletal actin, β-MHC, and SERCA by quantitative RT-PCR.

d) Hypertrophic signaling pathways
Mitogen-activated protein (MAP) kinases are one of the major mediators of cardiac hypertrophy. Activation of MAPK pathway, which involves phosphorylation of p38 MAPK. ERK1/2, and JNK, is determined by Western blot analysis.

e) Apoptosis
One possible explanation for the postmortem findings is that cardiomyocyte apoptosis and cell cycle regulation are altered. Hypertrophy may occur as a compensatory reaction to apoptosis. To determine whether apoptosis is enhanced in adult IIAd/− mice, in situ TUNEL staining will be done on the heart sections of two-month old mice.

25  2) Hyperplasia
a) Myocyte vs. nonmyocyte
If PI staining indicates increased density, we will identify the proliferating fibroblasts, myofibroblasts, and cardiomyocytes by dual labeling with vimentin, smooth muscle alpha-actin, or sarcomeric alpha-actin antibodies, respectively.

30  b) Cardiomyocyte hyperplasia
   i) In case of cardiomyocyte hyperplasia, the question is whether the cardiomyocyte DNA synthesis and mitoses are enhanced in the adult 191ColIIa/− mice. BrdU is injected intraperitoneally
into 2-month old mice 4 hours before euthanasia and the hearts will be processed for indirect immunofluorescent labeling with anti-BrdU and anti-sarcomeric MHC (MF20) antibodies. In addition, mitotic cells is identified by using phospho-histone H3 monoclonal antibody.

ii) If cardiomyocyte hyperplasia is present, a permanent cell line is isolated and established by enzymatic dissociation of cardiomyocytes from adult IIa-/- hearts. Such a cell line is a useful tool to study the cell cycle regulation incardiomyocytes.

3) Alterations in the cardiac extracellular matrix

Given the presence of left and/or right ventricular hypertrophy, it is imperative to determine whether there is any change in collagen total content, isoforms, and distribution in the heart. Because alterations in the composition of cardiac extracellular matrix may be present at birth or may be in response to mechanical stress, both newborn and adult myocardiums are examined. Antibodies for the characterization of the extracellular matrix proteins are either commercially available or available through our overseas colleagues.

a) Collagen content and isoforms

i) Total collagen content in the left ventricle, septum, and right ventricle are determined separately by measuring the hydroxyproline concentration.

ii) Spatial distribution and protein expression levels of types I-V, and VI are determined by immunohistochemistry.

iii) Distribution patterns of type I and III collagen by Sirius red-polarization method. When the heart section is stained with Sirius red, type I collagen appears as thick, strongly birefringent, yellow or red fibers while type III collagen appears as thin, weakly birefringent green fibers.

iv) Transmission electron microscopy confirms the findings from Sirius Red method and allows for the examination of the relationship between various components of extracellular matrix and surrounding cells at the highest resolution.

v) To determine whether types I and III collagen content is altered because of type IIA deletion, quantitative real-time TaqMan RT-PCR is
performed at various time points. As mRNA may not reflect protein levels or even expression, Western Blot analysis is performed from cardiac lysates to correlate the expression levels between the transcript and protein.

vi) To determine whether there is abnormal ectopic expression of type IIB procollagen in the postnatal hearts, Western blot analysis is performed using type II collagen antibody. In the IIa-/- mice, immunoreactive bands indicate the exclusive presence of IIB procollagen.

b) **Distribution of non-collagen components**

The distribution and expression levels of laminin, fibrillins, proteoglycans (biglycan, decorin, and fibromodulin) and fibronectin is examined by immunohistochemistry, in situ hybridization, Western blots, and quantitative RT-PCR.

4) **Integrity of TGF-β/BMP signaling pathways in adult myocardium**

a) **TGF-β isoform expression**

Recent human and animal studies have shown that myocardial TGF-β1-3 is increased in the setting of cardiac hypertrophy and myocardial fibrosis. Therefore, if hypertrophy or fibrosis is present, quantitative PCR is performed to determine the time course and magnitude-of TGF-β elevation as well as immunohistochemistry to localize TGF-β isoform expression in the interstitium.

b) Because the BMP2 and TGF-β1 binding cysteine-rich domain is lost as a result of targeted deletion of ColIIA gene, it is mechanistically important to determine whether the adult cardiac pathology is a consequence of derangement in TGF-β/BMP signaling pathways.

i) Protein expression level, phosphorylation status of Smads 1/5/8 or 2/3, and nuclear translocation is determined by Western blot analysis of cardiac cytoplasmic and nuclear isolates with the antibodies against total or phosphorylated forms of Smads 1/5/8 or Smads2/3.

ii) Immunohistochemistry with the same sets of phospho-Smads antibodies are performed on adult cardiac sections from IIa-/- and +/- littermates to demonstrate nuclear translocation of
phospho-Smads 1/5/8 or phospho-Smads2/3 and Co-Smad4 complex. Analysis will focus on the prevalence of nuclear co-localization in relation to the presence of myocardial hypertrophy/hyperplasia. If no nuclear co-localization is detected, the expression levels of type I and II receptors (TβR-II, BMPR-I1, ALK 1-2,3,5, and 6), Co-Smad 4, and inhibitory Smads 6 and 7 in the hearts of affected and non-affected adult 191 ColIIA-I- mice are determined by quantitative PCR and immunoblotting.

In addition to potential defects at the intracellular level, access of TGF-β 1 and BMP-2 ligands to their specific receptors may be affected by a large family of proteins known as ligand traps.

5) **Microarray analysis to define the mechanisms of cardiomyopathy**

In order to understand the molecular events leading to these cardiac defects, microarray technology is used to analyze the pattern of gene expression in 191 ColIIA-I- hearts. In addition to alteration in TGF/BMP signaling pathways, many classes of genes are affected in 191 ColIIA-I- hearts, including genes encoding for extracellular matrix proteins, cell cycle regulators, contractile and cytoskeletal proteins, ion channels, and muscle-specific transcription factors. The microarray technology is chosen over the candidate gene approach because the former allows genome-wide screening for alteration in many mRNAs simultaneously in a highly efficient and quantitative manner. Microarray analysis in this instance can determine the complex interaction between the extracellular matrix and cardiomyocytes. Time-dependent changes in gene expression profiles are of interest as well as chamber-specific changes. Gene expression profiles are examined at three different time points: Embryonic day 15.5, neonatal period 1 week, and young adult period (2 months). These three time points are chosen because at E15.5, cardiac morphogenesis has completed and there is a clear anatomical separation of the left ventricle from right ventricle. There is a major hemodynamic alteration at birth and isoform switching occurs in major contractile proteins in response to this hemodynamic shift. However, because
murine cardiomyocytes continue to proliferate at a low rate during first three weeks after birth, the gene expression profile is examined beyond this transition point from proliferation to developmental growth. Because chamber specification (i.e., left versus right ventricles) is transcriptionally regulated by different sets of genes and because there is prominent right ventricular hypertrophy in 191 ColIIA-/- mice, the gene expression profiles is compared between the left and right ventricles at these three time points. (Example: Table 2)

<table>
<thead>
<tr>
<th>Group</th>
<th>Embryonic Day 15.5</th>
<th>Postnatal Day 3</th>
<th>Adult 2 month</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Left ventricle</td>
<td>Left ventricle</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>191Co/Ila/-</td>
<td>Left ventricle</td>
<td>Left ventricle</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>Wild type</td>
<td>Right ventricle</td>
<td>Right ventricle</td>
<td>Right ventricle</td>
</tr>
<tr>
<td>191 Co/I IA/-</td>
<td>Right ventricle</td>
<td>Right ventricle</td>
<td>Right ventricle</td>
</tr>
</tbody>
</table>

Thus, this invention provides a method for producing a mutant procollagen IIA (Col2A-1) transgenic mouse comprising the steps of: a) introducing into mouse embryonic stem cells a gene-targeting vector comprising a mutant Col2A-1 transgene which is to replace a corresponding endogenous homologous gene by homologous recombination in the mouse embryonic stem cell; b) selecting for the mouse embryonic stem cells of step (a) which contain the mutant Col2A-1 transgene; c) introducing the mouse embryonic stem cells selected in step (b) into mouse blastocysts or aggregating the mouse embryonic stem cells selected in step (b) with mouse morula; d) transplanting the mouse blastocysts or aggravated morula of step (c) into a pseudopregnant mouse, thereby forming an embryo; e) allowing the embryo of step (d) to develop to term, thereby producing a chimeric founder transgenic mouse; and f) crossing the chimeric founder transgenic mouse of step (e) with a wildtype mouse, thereby producing a mutant Col2A-1 transgenic mouse. The mutant Col2A transgene lacks exon 2 which encodes a cysteine-rich binding domain for BMP-2 or TGF-β1. This invention further provides the above-described transgenic mouse.
This invention further provides a method of producing a null mutant mouse (IIA-/-) comprising backcrossing of the mutant Col2A-1 transgenic mouse with an inbred strain (C57BL6) of mouse. The null mutant mouse displays multiple cardiovascular malformations, features of human congenital heart defects or defects in patterning and morphogenesis of the heart similar to those associated with loss of function of the BMP, TGFβ and nodal signaling pathways. The complex heart defects include ventricular septal defect (VSD), patent ductus arteriosus (PDA), persistent truncus arteriosus (PTA), right ventricular hypertrophy, right aortic arch with or without anomalous origins of subclavian arteries, and the Double Outlet Right Ventricle (DORV) where aorta and pulmonary arteries arise wholly or in great part from the right ventricle, with associated VSD, transposition of great arteries (TOGA), anomalous coronary arteries, and endocardial cusion defects with primum atrial septal defect. This line of mutant mice represents a novel genetic model of Taussig-Bing anomaly, a rare but severe complex cyanotic heart disease in humans. This invention further provides the above-described transgenic mouse. The above-described transgenic mouse may be a strain of mouse designated 191-IIA or a transgenic mouse with similar phenotypes. Such phenotypes include hypertrophy of left or right ventricles, cardiomyocytes enlargement and disarray, hypercellularity with atypical nuclei, valvular fibrosis, and absence of extensive myocardial necrosis outflow tract abnormality, and branchial arch anomalies.

This invention further provides a method of determining agents for treatments of cardiac disorders using 191-IIA mice comprising the steps of: a) administering the agent to a first group of adult 191-IIA mice; b) administering a placebo to a second adult 191-IIA mice; c) measuring one or more of the following symptoms for the group of step (a) and the group of step (b): cardiac hypertrophy, cardiac hyperplasia, valvular fibrosis, cardiac function, serial blood pressures and heart, malignant arrhythmias and time-dependent changes in gene expression profiles in right and left ventricles; and d) comparing the measurements of step (c) in order to determine whether the agent treats the cardiac disorder. The treatments may be pharmaceutical and gene therapies that are targeted at the BMP/TGF related molecules and induced Smad signaling pathways on cellular hypertrophy. The agent
can be naturally occurring compounds or their purified derivatives, synthetic compounds, ligand traps, BMP or TGF agonists, receptor antagonists, Smad family proteins, or viral or non-viral vectors.

This invention also provides a cell line derived from the heart tissues of a 191IIA mouse or those of IIAs-/- mouse embryos.

This invention also provides a method of using procollagen IIA or its derivatives to titrate the expression level of a key cardiac transcription factors such as Nkx2.5 and GATA4 in embryonic cardiomyocytes.
References

What Is Claimed:

1. A method for producing a mutant procollagen IIA (Col2A-1) transgenic mouse comprising the steps of:
   a) introducing into mouse embryonic stem cells a gene-targeting vector comprising a mutant Col2A-1 transgene which is to replace a corresponding endogenous homologous gene by homologous recombination in the mouse embryonic stem cell;
   b) selecting for the mouse embryonic stem cells of step (a) which contain the mutant Col2A-1 transgene;
   c) introducing the mouse embryonic stem cells selected in step (b) into mouse blastocysts or aggregating the mouse embryonic stem cells selected in step (b) with mouse morula;
   d) transplanting the mouse blastocysts or aggravated morula of step (c) into a pseudopregnant mouse, thereby forming an embryo;
   e) allowing the embryo of step (d) to develop to term, thereby producing a chimeric founder transgenic mouse; and
   f) crossing the chimeric founder transgenic mouse of step (e) with a wildtype mouse, thereby producing a mutant Col2A-1 transgenic mouse.

2. A method according to claim 1 wherein the mutant Col2A transgene lacks exon 2 which encodes a cysteine-rich binding domain for BMP-2 or TGF-β1.

3. A method for producing a null mutant mouse (IIA-/−) comprising backcrossing of the transgenic mouse of claim 1 with an outbred strain of mouse.

4. A method according to claim 2 wherein the null mutant mouse displays multiple cardiovascular malformations, features of human congenital heart defects or defects in patterning and morphogenesis of the heart similar to those associated with loss of function of the BMP, TGFβ and nodal signaling pathways.

5. A method according to claim 4, wherein the heart defects include ventricular septal defect (VSD), patent ductus arteriosus (PDA), persistent truncus arteriosus
(PTA), right ventricular hypertrophy, right aortic arch with or without anomalous origins of subclavian arteries, and the Double Outlet Right Ventricle (DORV) where aorta and pulmonary arteries arise wholly or in great part from the right ventricle, with associated VSD, transposition of great arteries (TOGA), anomalous coronary arteries, and endocardial cusion defects with primum atrial septal defect.

6. A transgenic mouse according to the method set forth in claim 1.

7. A transgenic mouse according to the method set forth in claim 3.

8. A transgenic mouse according to claim 7 wherein the transgenic mouse is a strain designated 191-IIA.

9. A method of screening agents for treatment of a selected cardiac disorder comprising the steps of:
   
a) providing a 191-IIA mouse displaying symptoms associated with the selected cardiac disorder;
   
b) obtaining a first measurement of one or more of the symptoms;
   
c) administering an agent to the 191IIA mouse;
   
d) obtaining a second measurement of the symptoms measured in step (b); and
   
e) comparing the first measurement of step (b) with the second measurement of step (d) in order to determine whether the agent treats the selected cardiac disorder.

10. A method according to claim 9 wherein the treatment may comprise a pharmaceutical and gene therapy that is targeted at the BMP/TGF related molecules and induced Smad signaling pathways on cellular hypertrophy.

11. A method according to claim 9 wherein the agent can be naturally occurring compounds or their purified derivatives, synthetic compounds, ligand traps, BMP or TGF agonists, receptor antagonists, Smad family proteins, or viral or non-viral vectors.
12. A method according to claim 9, wherein the selected cardiac disorders include
sudden cardiac death, cardiomegaly, cardiomyocyte hypertrophy, cellular
disarray, pulmonary hypertension with right ventricular hypertrophy, and valvular
fibrosis.

13. A cell line derived from the heart tissues of a 19IIA mouse.

14. A method according to claim 5 where absence of procollagen IIA protein
production leads to decreased expression of NKx2-5 and GATA-4 in
embryonic cardiomyocytes, two important transcription factors that control
cardiac morphogenesis and of which mutations have been identified in human
congenital heart disease.

15. A method according to claim 14 where procollagen IIA protein is used to
modulate the expression level of transcription factors such as NKx2-5 and
GATA-4 and other genes regulated by BMP/TGFβ signaling, thereby alters
the patterning of cardiovascular structure.

16. A method according to claim 5 where absence of procollagen IIA protein
production leads to decreased Smad1 phosphorylation and nuclear
translocation, two features that are central to BMP2-induced intracellular
signal transduction.
Figure 1. Pedigree of 191ColllA-/- mice

- Female ○ - Male □
- Dead newborn ⬤
- Died of natural causes; age at death 20 1d, 30 Dec 02
- Sacrificed ⬨
- Date of birth indicated at left upper corner

191.3 male (3 months)
191.11 male (6 months)
191.73 male (9 months)
191.75 male (12 months)

5 mm
Figure 2. H&E stainings of 191.1 heart
Figure 3. H&E stainings of 191.3 heart
Figure 4. H&E stainings of older 191ColIIA-/- mice
Figure 5. Normal expression pattern of IIA mRNA and generation of IIA-/- mice
Figure 6. Cardiovascular Defects in Newborn ColIIA⁺ Mutants
Figure 7. Defective BMP signaling in IIA-/- embryos
Figure 8. Type IIA - *Nkx2.5* cross-depandant regulatory loop

A

B

*Col2a1* mRNAs
Figure 8. Type II A - Nkx2.5 cross-dependant regulatory loop cont’d

C

Rat

Human

Dog

Cow

Opossum

-7 -5 -3 0 3 5 7 9 11 kb

D

Col2a1 locus

A

B

C

Col2a1

pA

SRF

mouse
TGTCATTCACCACTGCACCACTGGCCATGCTGGCGTGTTCTAGCCCTTCACAGAAAGCTGGGCTTCC
rat
GTGGTTAATCCCGAGCGGAGCTAGGCGACCTATCGCAGTTGCGGCTGAGGTGAGTGCCTTGGACG
human
GTGGATCCACCCGCGCCCTGCGGCTGGGTCTTACCCTTCTTCAGAAAGCTGGGCTTCC
dog
GTCGGCTTCACCTAGCCCTGGCGGGGCTGGGTCTTACCCTTCTTCAGAAAGCTGGGCTTCC
cow
GTCGGCTTCACCTAGCCCTGGCGGGGCTGGGTCTTACCCTTCTTCAGAAAGCTGGGCTTCC
opossum
GGGGTTGAGGAGGCTGGGCGGCTGGGGCTGGGTCTTACCCTTCTTCAGAAAGCTGGGCTTCC
consensus
TTACATATTCCCATTGGGGGGTTCTGCTGGAGGTGAGTGCCTTGACCCTTCTTCAGAAAGCTGGGCTTCC

-4434

GATA

Nkx2.5

mouse
CTCTCTCTCTCTACGTGATTTATATAGCCTTGGATGAGCTGGAGAAGAGCTGGAGATCTTTCTT
rat
TTTTTTATCTAGCCTTGGATGAGCTGGAGAAGAGCTGGAGATCTTTCTT
human
TCATTTTATCTAGCCTTGGATGAGCTGGAGAAGAGCTGGAGATCTTTCTT
dog
TCATTTTATCTAGCCTTGGATGAGCTGGAGAAGAGCTGGAGATCTTTCTT
cow
ACACCTTTATCTAGCCTTGGATGAGCTGGAGAAGAGCTGGAGATCTTTCTT
opossum
TCATTTTATCTAGCCTTGGATGAGCTGGAGAAGAGCTGGAGATCTTTCTT
consensus

-4374

GATA

AT. T. T. T. T

mouse
TACAGTTATACACATATATATATATATACACCCTTGGATGAGCTGGAGAAGAGCTGGAGATCTTTCTT
rat
TACAGTTATACACATATATATATATATACACCCTTGGATGAGCTGGAGAAGAGCTGGAGATCTTTCTT
human
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dog
TACAGTTATACACATATATATATATATACACCCTTGGATGAGCTGGAGAAGAGCTGGAGATCTTTCTT
cow
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opossum
TACAGTTATACACATATATATATATATACACCCTTGGATGAGCTGGAGAAGAGCTGGAGATCTTTCTT
consensus

-4315

9/11
Figure 8. Type IIA - Nkx2.5 cross-dependant regulatory loop cont'd

E

Col2a1

\[73.6\text{kb} -16.5\text{kb} -9.5\text{kb} -4.2\text{kb} \]

[ +1 ST ]

\[\text{Col2a1} \quad 33\text{kb} \]

pA

bSY1

[ +1 ST ]

Delete Nkx2.5 site

bSY1\text{Nkx2.5}

NcoI

[ +1 ST ]

pA

33\text{kb}
Figure 9. Summary of roles of IIA procollagen in Nkx2.5 regulatory loop during cardiovascular morphogenesis

Cardiomyocytes in the looping heart, E9.0 onward

- BMP receptors
- S1: BMP-responsive smad phosphorylation
- S4: Co-smad 4
- myofibril
- IIA procollagen
- BMP2/BMP4
- Nkx2.5 gene
- Col2a1 gene

*Configuration of the IIA procollagen complex in the extracellular matrix – as BMP-IIA procollagen or BMP-IIA N-propeptide is unknown. Additional possible interactions in the complex are with proteases e.g. tolloid, furin-like, spc4 which may cleave to facilitate BMP interaction with its receptor.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC*: C12N5/00, C12N15/00, A61K48/00, A61P9/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC*: C12N, A61K, A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

GenBank, EMBL, DDBJ, PDB, PIR, WPI, JAP, CNPAT

Dual models, congenital heart defects, hypertrophic cardiomyopathy, transgenic mouse, gene knockout, cardiovascular, procollagen IIA,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
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</table>

☐ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim (S) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 21.Sep 2005 (21.09.2005)

Date of mailing of the international search report 20.OCT 2005 (20.10.2005)

Name and mailing address of the ISA/CN
6 Xitucheng Rd., Xinmeng Bridge, Haidian District, 100088 Beijing, China

Facsimile No. 86-10-62019451

Authorized officer Wang PengFei

Telephone No. 86-10-62085299

Form PCT/ISA/210 (second sheet) (April 2005)
Form PCT/ISA/210 (continuation of second sheet) (April 2005)
**INTERNATIONAL SEARCH REPORT**

**Box No. II**  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:6-8
   because they relate to subject matter not required to be searched by this Authority, namely:
   animal varieties.

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III**  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on protest**  ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.
<table>
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<th>Patent Documents referred in the Report</th>
<th>Publication Date</th>
<th>Patent Family</th>
<th>Publication Date</th>
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<td>WO92223333A</td>
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