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Cymbeline Culiat is a senior staff scientist at the Biological Sciences Division of Oak Ridge National Laboratory (ORNL). She obtained her Ph.D. in biomedical sciences from the ORNL-University of Tennessee at Knoxville School for Biomedical Sciences [currently the Genome Science and Technology program], where she demonstrated that deletions in the mouse 3 subunit of the type-A -aminobutyric acid receptor (Gabrb3) gene generates severe cleft palate. Her work suggested that a perturbation in Gabrb3 function is a strong genetic basis for this common human birth defect. Other geneticists later established the association between human GABRB3 and cleft palate. Dr. Culiat completed her post-doctoral training at ORNL as a recipient of the Department of Energy Alexander Hollaender Distinguished Postdoctoral Fellowship (1995-1999), before accepting her current position at ORNL. Dr. Culiat's research is focused on investigating the complex molecular pathways regulated by the cell signaling protein Nell1 during early bone, cartilage and cardiovascular development. Moreover, her lab also conducts high-throughput and large-scale DNA and RNA analyses to identify and characterize the phenotypic consequences of induced mutations and natural DNA sequence variations in the mouse genome that control hereditary traits and disorders.

CHARACTERIZING THE ROLE OF THE *NELL1* GENE IN CARDIOVASCULAR DEVELOPMENT

LEAH Y. LIU AND CYMBELINE CULIAT

ABSTRACT

Nell1^{6R} is a chemically-induced point mutation in a novel cell-signaling gene, *Nell1*, which results in truncation of the protein and degradation of the *Nell1*^{6R} transcript. Earlier studies revealed that loss of *Nell1* function reduces expression of numerous extracellular matrix (ECM) proteins required for differentiation of bone and cartilage precursor cells, thereby causing severe skull and spinal defects. Since skeletal and cardiovascular development are closely linked biological processes, this research focused on: a) examining *Nell1*^{6R} mutant mice for cardiovascular defects, b) determining *Nell1* expression in fetal and adult hearts, and c) establishing how ECM genes affected by *Nell1* influence heart development. Structural heart defects in *Nell1*^{6R} mutant fetuses were analyzed by heart length and width measurements and standard histological methods (haematoxylin and eosin staining). *Nell1* expression was assayed in fetal and adult hearts using reverse transcription polymerase chain reaction (RT-PCR). A comprehensive bioinformatics analysis using public databases (Stanford SOURCE Search, Integrated Cartilage Gene Database, Mouse Genome Informatics, and NCBI UniGene) was undertaken to investigate the relationship between cardiovascular development and each of twenty-eight genes affected by *Nell1*. *Nell1*-deficient mice have significantly enlarged hearts (particularly the heart width), dramatically reduced blood flow out of the heart and unexpanded lungs. Isolation of total RNAs from hearts of adult (control and heterozygote) and fetal (control and homozygous mutant) mice have been completed and RT-PCR assays are in progress. The bioinformatics analysis showed that the majority of genes with reduced expression in *Nell1*-deficient mice are normally expressed in the heart (79%; 22/28), blood vessels (71%; 20/28) and bone marrow (61%; 17/28). Moreover, mouse mutations in seven of these genes (*Col15a1, Osf-2, Bmpr1a, Pkd1, Mfge8, Ptger4, Col5a1*) manifest abnormalities in cardiovascular development. These data demonstrate for the first time that *Nell1* has a role in early mammalian cardiovascular development, mediated by its regulation of ECM proteins necessary for normal cell growth and differentiation. In addition, understanding the mechanisms by which *Nell1* and its associated ECM genes affect the cardiovascular system can provide future strategies for the treatment of heart and blood vessel defects.

INTRODUCTION

Nell1 is a gene that encodes a mammalian cell-signaling protein necessary for cell growth and differentiation. The *Nell1* protein is secreted in the cytoplasm and contains six epidermal growth factor-like (EGF-like) domains. These domains are segments of polypeptide common to more than 100 proteins with roles in cell proliferation and differentiation. In cell signaling pathways, *Nell1* is phosphorylated by protein kinase C-ß1 (PKC-ß1). PKC-ß1 is an enzyme that interacts with many different binding proteins in pathways that affect cell growth, differentiation, oncogenesis, and apoptosis. Because *Nell1* binds to PKC-ß1 via its EGF-like domains, *Nell1* seems to belong to a new class of cell-signaling ligand molecules with roles in cellular growth and development pathways [1]. *Nell1*'s over-expression in humans and mice causes craniosynostosis, the premature fusion of growing cranial sutures, which subsequently constrains growth in the brain [2,3]. This disorder is present in 1

of 3000 births in humans, and at the molecular level, seems to be caused by abnormalities in intramembranous ossification [4].

Nell1^{6R} is a point mutation induced by the common mutagen *N*-ethyl-*N*-nitrosurea, leading to a single base change in *Nell1*'s coding region (T→A) that results in the conversion of a cysteine codon to a premature stop codon (TG**T**→TG**A**). This mutation truncates the 810 amino acid polypeptide at amino acid residue #502 [4]. Consequently, the *Nell1* transcript is degraded and there is dramatically reduced expression of *Nell1* in mutant mice. Earlier studies revealed that loss of *Nell1* function reduces expression of numerous extracellular matrix (ECM) proteins required for the differentiation of bone and cartilage precursor cells. The reduced expression of ECM proteins leads to neonatal lethality and skeletal defects in the skull, ribcage and vertebral column in *Nell1*-deficient mice (Figure 1). Homozygote mutants (*Nell1*^{6R}/*Nell1*^{6R}) develop to late gestation (E18-19), but fetuses do not survive birth and those recovered by caesarean rescue are unable to breathe and nurse. There are no readily detectable phenotypic differences between heterozygote and wild-type mice [4].

Figure 1. Compared to wild-type mice (left), *Nell1*^{6R} mutants (right) display craniofacial and spinal curvature defects.

Skeletal and cardiovascular development are closely linked biological processes because the two systems share many common genes and molecular pathways. For example, in the human genetic disease Marfan syndrome, a single mutation in chromosome 15 reduces expression of the connective tissue protein fibrillin-1 and causes gross skeletal and cardiovascular abnormalities such as bone elongation, scoliosis, mitral valve abnormalities, and aortic dilation [5]. Prominent pathways in the bone such as matrix deposition and calcification have been identified in arterial calcification. In addition, the protein collagen I and certain phosphatases are common to both bone formation processes and atherosclerotic lesions [6]. Studies of diseased heart valves have revealed expression of bone proteins such as osteopontin [7] and even the presence of bone itself in the valves. Endochondral ossification, lamellar bone formation, and active bone remodeling have also been discovered in diseased heart valves, further verifying the molecular link between the skeletal and cardiovascular systems [8].

This research expanded upon earlier findings on the role of *Nell1* in the skeletal system and explored, for the first time, its impact on the cardiovascular system. During previous studies in *Nell1*^{6R}-deficient mice, the homozygote mutant hearts were observed to be noticeably larger than wild-type and heterozygote mutant hearts. However, this enlargement had never been quantified or verified to be statistically significant. In addition, it was unknown whether the suspected enlargement was caused by abnormal spine curvature and compression of intervertebral disc materials in mutants, which dramatically decreases the volume of the thoracic cavity and physically exerts pressure on the developing heart. Alternatively, the putative heart defect could be a direct effect of abnormal gene expression changes resulting from the loss of function of *Nell1*. This project was undertaken to accomplish the following specific objectives:

a) Examine *Nell1*^{6R} mutant mice for cardiovascular defects,

b) Determine *Nell1* expression in fetal and adult hearts, and

c) Establish whether genes with reduced expression in *Nell1*^{6R} mutant fetal bodies are known to influence cardiovascular development.

Previous studies on the role of the *Nell1* gene during skeletal development revealed new insights into the mechanisms behind osteoblast and chondrocyte differentiation and identified novel molecular targets for the treatment of cranial and spinal defects in humans [2, 3, 4]. Analyzing how the *Nell1* pathway influences the cardiovascular system provides an opportunity to elucidate the role of *Nell1* in heart and blood vessel development. This research is also of significant medical interest because certain structural and morphological abnormalities such as left ventricle thickening, mitral valve abnormalities, and outflow obstructions in the heart are associated with heart enlargement in humans [9].

METHODS OF ANALYSIS

The mice used in these experiments were maintained and bred at the Mammalian Genetics Research Facility at Oak Ridge National Laboratory (ORNL), using protocols approved under the ORNL Institutional Care and Use Committee.

Characterization of Cardiovascular Defects. To determine if structural heart defects in *Nell1*^{6R} mutant fetuses were significant enough to warrant further study, hearts were dissected from formalin-fixed specimens and were analyzed by heart length and width measurements. These measurements were completed on 14 wild-type (+/+), 11 heterozygous (+/*Nell1*^{6R}), and 17 homozygous mutant (*Nell1*^{6R}/*Nell1*^{6R}) fetuses at 18.5 days of gestation, using the Wild Heerbrugg M8 Stereozoom microscope grid ruler. Statistical analysis was completed using a two-tailed T-test and a p-value cutoff of 0.05.

Histological observations were made on haematoxylin and eosin stained mouse sagittal sections. These sections were cut from mice that were fixed in formalin and embedded in paraffin. Observations

were made at 8X on the Zeiss Stemi SV11 stereomicroscope microscope, and at higher magnification (up to 400X) on the Zeiss AxioSkop microscope. Haematoxylin and eosin staining shows general cell morphology, such as cell shape, presence of nuclei, and extracellular components. The fixation, staining, and sectioning were conducted by a commercial histology service (Ridge Microtome).

Gene Expression Assays. *Nell1* expression was assayed in adult (wild-type and hemizygous mutants) and fetal (wild type, heterozygous, and homozygous mutants) hearts and brains using reverse transcription polymerase chain reaction (RT-PCR). RNA was extracted from animals using standard phenol-chloroform techniques and Phase Lock Gel (Eppendorf) tubes for purification. To generate the cDNA product, the reverse transcription reaction was performed on samples using the RETROscript® Kit manufactured by Ambion. The cDNA was amplified by PCR using three primer sets for the *Nell1* gene (Desai et al., unpublished). The three primer sets necessary to cover the length of *Nell1* are as follows: 1) CTC138 and Jaya 3R covered approximately 30 bases of the untranslated region and exon 1 to exon 8, total length: 840 bases, 2) Jaya 3F and Jaya 5R covered half of exon 4 up to the first 40 bases of exon 13, total length: 850 bases, 3) Jaya 8F and Jaya 10R covered exon 16 to exon 20 and into the untranslated region, total size: 800 bases. The primer sequences are as follows:

CTC 138: 5'-CTGAAGCATTGGTTTCTTGC-3'
Jaya 3R: 5'-AGTTGACCAAGTCTCGTCTC-3'
Jaya 3F: 5'-GCCGATGGACAGTGGCACAA-3'
Jaya 5R: 5'-GTGTTGGCATGACAATAGTG-3'
Jaya 8F: 5'-ACCACTGTGAGTGCAGAAGC-3'
Jaya10R: 5'-CAACCCAAACGCCTTCCTC-3'

Comprehensive Bioinformatics Analysis. To investigate the relationship between cardiovascular development and each of twenty eight genes affected by *Nell1*, a comprehensive bioinformatics analysis using publicly accessible databases was undertaken. These genes were previously identified as those that showed reduced expression in mutant mouse bodies (E18) [4]. Stanford SOURCE, Integrated Cartilage Gene Database, and NCBI UniGene were used to search for the expression of ECM genes in tissues related to the cardiovascular system: heart, blood vessels, bone marrow, and blood [10, 11, 12]. In addition to expression data, disorders and abnormalities in humans that have previously been associated with the genes were noted. The Mouse Genome Informatics database was used to search for known mouse mutations in these genes and to investigate if these mutations resulted in cardiovascular defects [13].

RESULTS

Characterization of Cardiovascular Defects: *Nell1*-deficient mice had significantly enlarged hearts based on length and width measurements. As shown in Table 1, length measurements for all three genotypes did not differ significantly, however, based on the statistical T-Test, the width measurements for mutant mice (*Nell1*^{6R}/*Nell1*^{6R}) were significantly greater than the width measurements for wild type and heterozygote mutant mice, thus confirming the presence of an abnormal heart phenotype in *Nell1*-deficient mice.

	Homozygote *Nell1*^{6R}/*Nell16R*	Heterozygote +/ Nell1^{6R}	Wild-type +/+
Width	3.3	2.8	2.7
	2.5	2.8	1.8
	2.8	2.3	2.8
	2.3	2.7	2.5
	2.8	2.8	2.7
	3.0	2.5	2.3
	3.2	2.5	2.2
	3.0	2.5	2.5
	2.8	2.2	2.8
	2.8	2.5	2.7
	3.3	2.2	2.7
	3.0	-	2.2
	3.0	-	2.5
	2.5	-	2.3
	2.5	-	-
	3.0	-	-
	2.5	-	-
No. of Fetuses	17	11	14
Average	2.853	2.530	2.476
Length	3.2	3.7	2.7
	2.8	3.2	2.7
	2.8	2.8	3.3
	2.7	3.2	3.0
	3.0	3.2	3.3
	3.2	3.0	3.0
	3.2	3.0	2.5
	3.3	3.3	3.0
	3.0	2.8	3.3
	2.8	3.0	3.2
	3.2	2.8	3.3
	3.2	-	2.7
	3.2	-	2.8
	2.8	-	3.0
	2.5	-	-
	3.0	-	-
	2.8	-	-
No. of Fetuses	17	11	14
Average	2.984	3.091	2.988

T-Test p-values			
	Mutant-Wildtype	**Mutant-Heterozygote**	**Heterozygote-Wildtype**
Width	0.0012442891	0.0046893426	0.6143698331
Length	0.9351530349	0.2470911230	0.3514701862

Table 1. Measurements (mm) of E18.5 fetal heart width and length of *Nell1*^{6R} heterozygote and homozygote mutant mice compared with wild-type littermates..

Observations on haematoxylin and eosin-stained, sectioned specimens showed additional morphological anomalies in the cardiovascular system at the organ and cellular level. At low magnification, dramatically reduced blood flow out of the heart was observed in mutant mice. Sections from wild-type and heterozygote mice distinctively showed bright red, blood-filled arteries whereas the sections from mutant mice showed a scant amount of blood.

Another prominent difference between normal and mutant mice was the variation in lung density and size. Wild-type and heterozygote mice had spongy lungs which occupied the entire thoracic cavity space while mutant mice have dark, compact, dense lungs (Figure 2A and 2B).

The slides were also observed at higher magnification to detect differences in cellular morphology. Images of the heart apex in heterozygote and mutant mice depicted differences in cell density. The cells were more closely packed in mutant hearts, revealing a higher density of cellular nuclei (Figure 2C and 2D), whereas there was more space between cells in the heterozygote. It was difficult to detect a marked heart width enlargement in mutant mice using just these histological observations because the orientation of the heart in the thoracic cavity differs with each animal, and only one plane of the mouse can be examined at one time. In addition, comparable sections were not available for all specimens because the sections were cut into the heart at different planes.

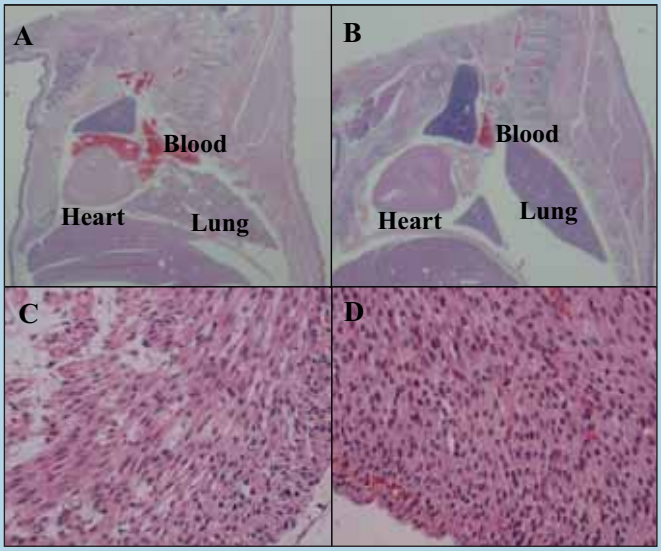

Figure 2. Heterozygote (A) and mutant (B) mice show differences in blood flow and lung expansion (8X magnification). Heart muscle in the heterozygote (C) has fewer nucleated cells per unit area compared to mutant (D; 400X magnification).

Gene Expression Assays. Isolation of total RNAs from the hearts and brains of adult (control and heterozygote) and fetal (wild-type, heterozygote, and homozygous mutant) mice have been completed and RT-PCR assays are in progress.

Comprehensive Bioinformatics Analysis. The comprehensive bioinformatics analysis showed that of twenty-eight genes studied, 79%, 71%, and 61% were normally expressed in heart, blood vessels and bone marrow, respectively. Table 2 summarizes the expression profile of these genes in the various tissues of the cardiovascular system. The database searches resulted in more specific expression data for 12 of the genes, such as expression at particular stages of development, increased or decreased expression in certain disease states, or localized expression in one particular tissue type such as cardiomyocytes (Table 3). Twenty genes (71%) had recorded mouse mutations according to the Mouse Genome Informatics database. This database also referenced seven genes (*Col15a1, Osf-2, Bmpr1a, Pkd1, Mfge8, Ptger4, Col5a1*) that manifest certain abnormalities in cardiovascular development commonly associated with heart enlargement, as shown in the Table 4.

DISCUSSION

These data demonstrate for the first time that *Nell1* has a role in early mammalian cardiovascular development, mediated by its regulation of ECM proteins necessary for normal cardiovascular growth and differentiation. Observations of heart abnormalities are consistent with earlier studies on the skeletal effects of *Nell1*-deficiency. As in the skeletal system, the loss of *Nell1* function seems to disrupt the balance between cell proliferation and differentiation in the cardiovascular system.

The confirmation of a heart enlargement phenotype in *Nell1*-deficient mice, and the dramatic differences in blood flow and lung density between normal and mutant mice show clear cardiovascular defects associated with the reduced expression of *Nell1*. The lung phenotype is probably due to the inability of mutant mice to survive birth or open their mouths. As a consequence, mutant mice cannot breathe when they are born and therefore have unexpanded lungs compared to the spongy, air-filled lungs of the normal phenotype.

Histological analysis suggests that heart enlargement could be due to hypertrophy of the myocardium, indicated by the higher density of nucleated cells in the cardiac muscle of mutant mice. During development there is a delicate balance of cell differentiation and proliferation in the body. Since *Nell1* is necessary for cell differentiation, the absence of *Nell1* reduces cell differentiation and cell proliferation therefore becomes the predominant mechanism. This observation in the heart is consistent with observations in bone and cartilage tissue [4].

Heart enlargement is commonly caused by structural defects or disease states that force the heart to work harder. To compensate for this extra work, the cardiac muscle may increase in size. The detailed expression data in Table 3 shows expression in specific structures of the cardiovascular system, or specific stages of development when mutations may affect the ability of the heart to pump and direct blood efficiently. Many of the structures showing expression for the ECM proteins are some of the same ones affected by the disease phenotypes listed in Table 4, which are defects known to be associated with heart enlargement.

Because of the association between *Nell1* and the ECM proteins, the observed abnormalities are probably not caused by mere compression of the thoracic cavity as a result of vertebral column defects. The data from the bioinformatics analysis shows many possible cardiovascular defects associated with the reduced expression of *Nell1* and ECM proteins. Further work would involve verifying the expression of ECM genes in the cardiovascular system by staining mouse sections with antibodies specific to ECM proteins. Once RT-PCR experiments are completed and the resultant PCR products are sequenced, *Nell1* expression in fetal and/or adult hearts can be determined. In addition, *Nell1* and ECM genes can be researched with the goal of establishing a direct or indirect causal relationship with the heart defects mentioned in Table 3. Even if *Nell1* is not found to be expressed in the heart, it may still cause heart

Gene Symbol	Gene Name	Expression					
		heart	vascular	blood	bone marrow	# abnormal heart phenotype[13]	# total mutants[13]
Tnxb	tenascin	10	10	11	11		2
Prg4	proteoglycan 4	33	12	12			1
Thbs3	thrombospondin 3	10	10	12			2
Col5a3	collagen 5 alpha 3 subunit						
Neurog2	neurogenin 2						5
Col5a1	procollagen type V, alpha 1	10	10	10	10	1	1
Col6a1	procollagen Type VI, aloha 1	10	16	12	10		1
Col15a1	procollagen type XV, alpha 1	10	19	10	12	1	1
Pacsin3	PKC and casein kinase substrate in neurons 3	10			10		
Tnc	tenascin c	10	10	21	11		3
Col12a1	procollagen type XII, alpha 1	10	12		10		
Chad	chondroadherin	15	15				
Osf2-pending	osteoblast specific factor 2	10	10		10	1	2
Col17a1	procollagen type XVII alpha 1						
Prkcc	protein kinase C						2
Prkch	protein kinase C, eta symbol	10	10	10	10		1
Bk-pending	brain and kidney protein						
Ptk9l	PTK9L protein tyrosine kinase 9-like	10	10	10	10		
Npdc1	neural proliferation, differentiation and control gene	10	10				1
Bmpr1a	bone morphogenetic protein receptor type 1a	10	12		10	2	4
Pkd1	polycystic kidney disease I homolog	10	27	10	12	7	12
Tnfrsf11b	tumor necrosis factor (ligand)	10	34		12		3
Mfge8	milk fat globule-EGF factor 8 protein	10	12	10	10	1	5
Matn3	matrilin 3, cartilage matrix protein	28					1
Bmp7	bone morphogenetic protein type 7	10			10		8
Matn2	matrilin 2, cartilage matrix protein 2	10	10	10	10		2
Ptger4	prostaglandin E receptor 4		10	10	10	3	4
Notch3	notch gene homolog 3	10	30				4
	# of Genes	22	20	13	17	7	20
	Percentage	79%	71%	46%	61%	25%	71%

*Compiled from references 10, 11, 12, 13, 15, 16, 19, 21, 27, 28, 30, 33, 34. Superscripted numbers in table refer to specific references.

Table 2. Expression profile of genes in the *Nell1* pathway and association with mutant mouse phenotypes.

abnormalities by acting on the associated ECM genes from elsewhere in the body through paracrine mechanisms. The identification of the role of *Nell1*-mediated pathways in cardiovascular development can provide potential drug or cell-based strategies for treating heart and blood vessel defects.

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Gene	Detailed Expression	Expression in Cardiac Developmental Processes	Associated Disorders
Tnxb	cardiac muscle[11]		
Prg4			Jacobs Syndrome (pericarditis)[11, 39]
Col6a1	mitral valve[40] pericardium[40]		abdominal aortic aneurysm disease[16] congenital heart defects in Down Syndrome[17] dilated descending aorta[18]
Col15a1			cardiac muscle degeneration[19] collapsed capillaries[19]
Tnc	expression only in disease states[20, 22]	neovascularization[20] migrates in response to cardiac growth factors[20] stromal cell dependent erythropoiesis[21]	
Postn (Osf2)	vascular injury increases expression[37] overexpression in myocardium in heart failure patients[38]	endocardial cushion development[35] valve formation[35] developing endothelium of ventricular trabeculae[36] outflow tract development[36] valve leaflet development[36] chordae tendinae development[36]	
Prkch	expressed during ischemia (in myocardial infarction)[23]		
Bmpr1a	cardiomyocytes[14]	signaling in cardiac organogenesis[24] outflow tract development[25] ventricular myocardium development[25] annulus fibrosus development[26]	
Pkd1		myoelastic arterial organization[27]	
Tnfrs11b			calcification of aorta and renal arteries[34]
Bmp7	myocardium[14]	atrioventricular cushion development[14] outflow tract development[14] endocardial cushion formation[14] septation[14]	
Notch3	arteries (but not veins)[30] arterial pericytes[32]		increased chance of myocardial infarction[29] cerebral arteriopathy (CADASIL)[29] degeneration of vascular smooth muscle cells[31]

*Compiled from references 11, 14, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 29, 30, 31, 32, 34, 35, 36, 37, 38, 39, 40. Superscripted numbers in table refer to specific references.

Table 3. Detailed expression of *Nell1*-pathway genes in the cardiovascular system.

Gene	Defect
Col15a1	Abnormal heart capillaries
Osf-2	Discontinuity in valve leaflets
Bmpr1a	Persistent truncus arteriosus Mitral valve prolapse Abnormal cardiomyocyte apoptosis
Pkd1	Vascular leaks/ruptures Endocardial cushion defects Abnormal atrial septum morphology Abnormal septation
Mfge8	Abnormal neovascularization
Ptger4	Dilated left ventricle Patent ductus arteriosus Congestive heart failure
Col5a1	Abnormal vasculature

*Compiled from reference 13.

Table 4. Mutations in genes of the *Nell1* pathway generate defects in mice associated with heart enlargement.

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