Abstract: It has been identified in accordance with the present invention that NeM is essential for normal cardiovascular development by promoting proper formation of the heart and blood vessels. The present invention therefore provides therapeutic methods for treating cardiovascular disorders by employing a NeM protein or nucleic acid molecule.
FIELD OF THE INVENTION

The present invention relates in general to therapeutic methods for treating cardiovascular disorders. More specifically, the present invention relates to therapeutic treatments of cardiovascular disorders by employing the cell differentiation signaling protein Nelll, as well as functional derivatives thereof.

BACKGROUND OF THE INVENTION

Despite many available methods of treatment, cardiovascular disease is one of the major causes of death each year in the U.S. Thus, there is still a need for more effective agents to prevent and treat cardiac tissue injury, especially cardiac tissue injury resulting from ischemia/reperfusion.

The Nelll gene codes for a secreted trimeric protein that stimulates bone and cartilage precursor cells (osteoblasts and chondrocytes) to differentiate into mature bone and cartilage tissue (Zhang et al., 2002; Desai et al., 2006). Nell-I is a protein kinase C (PKC) β-binding protein. The Nelll cDNA and amino acid sequences from a variety of mammalian species, including human, rat and mouse, have been reported.
Overexpression of Nell I has been reported to cause premature fusion of the growing cranial bone fronts, resulting in craniosynostosis in humans and transgenic mice carrying a rat Nell transgene. A Nell I knock-out mouse was also shown to exhibit several bone- and cartilage-related defects. There has been no characterization, however, of the impact of Nell I, if any, on cardiovascular development.

SUMMARY OF THE INVENTION

It has been identified in accordance with the present invention that Nell I is essential for normal cardiovascular development by promoting proper formation of the heart and blood vessels. The present invention therefore provides therapeutic methods for treating cardiovascular disorders by employing a Nell I protein, functional derivatives thereof or nucleic acid molecule.

Cardiovascular disorders or conditions contemplated by the present invention are diseases that involve the heart or blood vessels (arteries and veins), including in particular myocardial infarction (or "MI"). By treating a cardiovascular disorder or condition with the present methodology, the disorder is prevented or is delayed; or alternatively, its progression is slowed down, the extent of the injury is reduced, and the recovery is accelerated.

In one embodiment, the present invention provides a method of treating a cardiovascular disorder by administering a Nell I protein or functional derivatives thereof to a subject in need of the treatment. Nell I proteins suitable for use in the present method include wild type Nell I proteins from any mammalian species, as well as functional derivatives thereof. Nell I proteins, as well as functional derivatives thereof, can be recombinant produced or purified from a mammalian body or tissue.

In another embodiment, the present invention provides a method of treating a cardiovascular disorder by administering a nucleic acid molecule encoding a Nell I protein to a subject in need of the treatment. The nucleic acid molecule can be provided in an expression vector, including viral vectors and non-viral vectors, suitable for effecting the expression of the Nell I protein in the targeted tissue or cells.
In accordance with the present invention, a Nell I protein, functional derivatives thereof, or nucleic acid molecule can be combined with an appropriate pharmaceutically acceptable carrier for administration. Administration can be conducted in any practical and convenient manner, including by ingestion, injection or implantation, for example.

In a specific embodiment, a Nelll protein, functional derivatives thereof, or Nell l-encoding nucleic acid molecule is used in combination with cell-based therapy for the repair and regeneration of damaged cardiac muscles and blood vessels. For example, a Nelll protein, functional derivatives thereof, or Nell l-encoding nucleic acid molecule can be administered together with cardiomyocytes for repopulation of cells in the injured site. Alternatively, a Nelll protein, functional derivatives thereof, or Nelll -encoding nucleic acid molecule can be administered together with stem cells isolated from adult bone marrow for regeneration of damaged cardiac muscles and blood vessels.

BRIEF DESCRIPTION OF THE DRAWINGS

The file of this patent application contains drawings executed in color.

Figures 1A-1B show the cardiovascular defects in mice without Nelll function (NellI<sup>6R</sup> mutation). Homozygote fetuses at E18 days of gestation (Top) show decreased blood circulation (arrows) and unexpanded lungs compared to heterozygotes (bottom) and wild type animals (not shown). Fetuses were unable to breathe after birth or after caesarean recovery.

Figures 2A-2B (in color) demonstrate that Nell 1 protein is required for blood vessel formation and establishment of a complex vascular network. The loss of Nelll function resulted in a significant reduction of the number of blood vessels and extensive branching of the vasculature in NellI<sup>6R</sup> mutants (Figure 2B) compared to (Figure 2A) normal fetuses. The decrease in blood vessel formation was observed throughout the fetal body.

Figures 3A-3B illustrate severe cardiovascular defects and neonatal lethality associated with the complete loss of Nelll function in the mouse. The cardiovascular defects resulting from the complete loss of Nelll function in Nelll<sup>6R</sup>
was associated with decreased blood circulation into the heart muscles and predominance of increased numbers of immature cardiomyocytes. The dense packing of smaller cardiomyocytes in the mutant (Figure 3B) was very apparent in the denser/darker staining with haematoxylin and eosin, compared to the wild type (Figure 3A). These cardiovascular defects are evident in E18.5 day fetuses recovered by caesarean.

Figures 4A-4C illustrate a strategy for treatment of heart muscle injury after MI in rodents using direct injection of stem cells or drugs to the border zone.

Figure 5 provides an alignment of the human (SEQ ID NO: 2) and murine (SEQ ID NO: 4) Nelll proteins. The functional domains of the human Nelll protein are found in the essentially same regions as those identified in the murine Nelll protein.

Figures 6A-6D. NELL1 Protein Treatment of Damaged Heart Tissue in Mice with Myocardial Infarction (MI). (6A) Untreated mouse hearts with MI due to the loss of blood supply from a ligation of the left anterior descending coronary artery had a readily visible creamy white looking damaged tissue on the surface of the heart (17 days post MI-induction). All Nelll protein treated hearts had lesser amount of damaged tissue as illustrated in Fig 7B to 7D. The damaged sections (outlined by blue lines) in controls were typically at least 50% while the treated hearts had barely visible (6B) to as high as 30% infarcts observed (6D).

Figures 7A-7F. Reduction of Damaged Heart Tissue Incurred From Myocardial Infarction in Nelll-treated Hearts. Longitudinal sections of normal hearts stained with either haematoxylin and eosin (7A) or masson-trichome (7B) show intense staining of the heart muscle and reveals a very compact organization of the muscle tissues in the right and left ventricles (rv and lv respectively), and the interventricular septum separating the two ventricular chambers (IVS). After a myocardial infarction event, the muscle tissues died due to a lack of oxygenated blood supply and the deterioration of the muscle architecture was evident by the large gaps in the tissue and the decreased intensity of the staining (7C; 17 days post-MI). Hearts with MI that were treated with the Nelll protein had lesser damage in the heart tissue from the surface to just before the
middle of the heart (7D and 7Is). In some hearts the improvement was manifested even deeper into the middle section of the heart (7F).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to therapeutic methods for treating cardiovascular conditions or disorders by employing the cell differentiation signaling protein Nelll, as well as functional derivatives thereof.

The present invention is based on the surprising discovery by the inventor that the Nelll protein is essential for normal cardiovascular development by promoting proper formation of the heart and blood vessels. The inventor discovered that loss of Nelll resulted in several tissue and organ changes typical of cardiac muscle injury, including heart enlargement, tissue hypertrophy, decreased blood vessel formation and blood circulation. The inventor observed that microscopic examination of Nelll-deficient hearts showed heart enlargement and cardiomyopathy, conditions associated with events of myocardial infarction ("MI"). Although the basic vasculature system was observed during embryo development even without a functional Nelll, the amount and complexity (branched network) was significantly reduced in Nelll mutants. The therapeutic application of Nelll for heart muscle regeneration is therefore dependent not only on the protein's abilities to signal muscle cell maturation, but also in its capabilities to support the construction of the highly branched vasculature that is required to sustain new heart muscle formation and maintenance of heart function. The inventor also observed that microarray experiments indicate that Nelll is essential for the proper formation of heart extracellular matrix, main structural components of heart muscle, and proper functioning of genes for heart metabolism and contraction.

Accordingly, the present invention provides methods for treating cardiovascular conditions or disorders by employing a Nelll protein, functional derivatives thereof, or Nelll nucleic acid molecules.

The term "condition," as used herein, refers to a disease or ailment. The term "disorder," as used herein, refers to a condition in which there is a disturbance of normal
functioning. The term "cardiovascular," as used herein, refers to the heart and/or blood vessels.

Accordingly, the term "cardiovascular condition" or "cardiovascular disorder", as used herein, refers to diseases or ailments that involve the heart, blood vessels (e.g., arteries and veins). Generally, such diseases or ailments result in an abnormality in the cardiac structure, cardiac muscle, and/or cardiac function. The cardiovascular condition or disorder can be acute or chronic.

The term "cardiovascular condition" or "cardiovascular disorder" can be used interchangeably throughout the specification. Examples of a cardiovascular disease include aneurysms, angina, atherosclerosis, cardiomyopathy, congestive heart failure, coronary artery disease, and myocardial infarction, among others. Further examples of cardiovascular conditions include, for instances, blood vessels that have been revascularized. Such patients generally have a stent placed in a blood vessel (e.g., artery, etc.)

A cardiovascular condition especially suitable for being treated with the method of the present invention is myocardial infarction (or "MI"). MI, also known as a "heart attack" or "heart failure", is a medical condition that occurs when the blood supply to a part of the heart is interrupted. MI is often caused by partial or complete occlusion of one or more of the coronary arteries, usually due to rupture of an atherosclerotic plaque. The occlusion of the coronary artery results in cardiac ischemia. The resulting ischemia or oxygen shortage causes damage and potential death of heart tissue.

The term "treating" or "treatment" a disease, as used herein, refers to preventing or delaying the onset of the disease, or when the disease does occur, retard the progression or ameliorate the symptoms of the disease, reduce the extent of tissue injury or damage, or promote recovery of the injured tissue and regeneration of new functional tissue or cells.

The subject suitable for receiving a treatment in accordance with the present invention includes any mammalian subject in need of the treatment. In one embodiment, the subject is a human subject. A subject in need of treatment includes both subjects who have been determined to have a higher risk of developing a cardiovascular disease, and
subjects who have a cardiovascular disease, as well as subjects who have recently experienced a cardiovascular event such as MI.

In one embodiment, the method of the present invention is achieved by administration of a Nelll protein to a subject in need of the treatment.

"A Nelll protein" as used herein, includes wild type (i.e., naturally occurring) Nelll proteins of any mammalian origin, such as human, murine, rat and the like. Preferred Nelll proteins for use in the present invention include human Nelll protein (SEQ ID NO: 2), murine Nelll protein (SEQ ID NO: 4), and rat Nelll protein (SEQ ID NO: 6).

"A Nelll protein" as used herein, also includes functional derivatives of a wild type Nelll protein. A "functional derivative" refers to a modified Nelll protein which has one or more amino acid substitutions, deletions or insertions as compared to a wild type Nelll protein, and which retains substantially the activity of a wild type Nelll protein. By "substantially" is meant at least 50%, at least 75%, or even at least 85% of the activity of a wild type Nelll protein. According to the present invention, in order for the functional derivative to substantially retain the activity or function of a wild type Nelll protein, the functional Nelll derivative shares a sequence identity with the wild type Nelll protein of at least 75%, at least 85%, at least 95% or even 99%.

The structure of Nelll proteins has been characterized (see, e.g., Kuroda et al., 1999a; Kuroda et al., 1999b, Desai et al., 2006). For example, the murine Nelll protein (SEQ ID NO: 4) is a protein of 810 amino acids, having a secretion signal peptide (amino acids # 1 to 16), an N-terminal TSP-like module (amino acids # 29 to 213), a Laminin G region (amino acids # 86 to 210), von Willebrand factor C domains (amino acids # 273 to 331 and 699 to 749), and a Ca²⁺-binding EGF-like domains (amino acids # 549 to 586).

The secretion signal peptide domain of Nelll protein is an amino acid sequence in the protein that is generally involved in transport of the protein to cell organelles where it is processed for secretion outside the cell. The N-terminal TSP-like module is generally associated with heparin binding, von Willebrand factor C domains are generally involved with oligomerization of Nelll. Laminin G domains of Nelll protein are generally involved in adherence of Nelll protein to specific cell types or other
extracellular matrix proteins. The interaction of such domains with their counterparts is
5 generally associated with, for example, processes such as differentiation, adhesion, cell
signaling or mediating specific cell-cell interactions in order to promote cell proliferation
and differentiation. The Ca$^{2+}$-binding EGF-like domains of Nell 1 binds protein kinase C
beta, which is typically involved in cell signaling pathways in growth and differentiation.

The amino acid sequence of Nell 1 protein is very highly conserved, especially
across mammalian species. For example, the murine Nell 1 protein shares about 93%
sequence identity with the human Nelll protein (SEQ ID NO: 2), which, in turn, shares
about 90% sequence identity with the rat Nelll protein (SEQ ID NO: 4). Those skilled in
the art can use any of the well-known molecular cloning techniques to generate Nell 1
derivatives having one or more amino acid substitutions, deletions or insertions, taking
into consideration the functional domains (e.g., secretion signal peptide sequence, N-
terminal TSP-like module, Laminin G region, von Willebrand factor C domain) of Nelll.
See, for example, Current Protocols in Molecular Cloning (Ausubel et al., John Wiley &
Sons, New York).

The minimum length of a Nelll functional derivative is typically at least about
10 amino acids residues in length, more typically at least about 20 amino acid residues in
length, even more typically at least about 30 amino acid residues in length, and still more
typically at least about 40 amino acid residues in length. As stated above, wild type
20 Nelll protein is approximately about 810 amino acid residues in length. A Nelll
functional derivative can be at most about 810 amino acid residues in length. For
example, a Nelll functional derivative can be at most at about 820, 805, 800, 790,
780, 750, 600, 650 600, 550, etc. amino acid residues in length.

Once a Nelll protein derivative is made, such protein can be tested to
determine whether such derivative retains substantially the activity or function of a wild
type Nelll protein. For example, the ability of a Nelll derivative to bind PKC beta can
be tested. Suitable assays for assessing the binding of Nelll to PKC beta is described in
e.g., Kuroda et al. (1999b). For example, protein-protein interaction can be analyzed by
using the yeast two-hybrid system. Briefly, a modified Nelll protein can be fused with
GAL4 activating domain and the regulatory domain of PKC can be fused with the GAL4 DNA-binding domain. The activity of β-galactosidase in yeast cells can be detected.

In addition, one can also test the ability of a Nell I derivative to stimulate differentiation of precursor cells, which are in the cardiomyocyte lineage, towards mature cardiomyocytes. Maturity of cardiomyocytes can be assessed cellularly (histology) and molecularly (expression of cardiac-specific proteins or extracellular matrix materials). Still further, a Nell I derivative can be tested for its ability to drive osteoblast precursors to mature bone cells, by detecting expression of late molecular bone markers or mineralization (i.e., calcium deposits). By comparing the activity of a Nell I derivative with that of a wild type Nell I protein in one or more of the assays such as those described above, one should be able to determine whether such derivative retains substantially the activity or function of a wild type Nell I protein.

A Nell I protein or functional derivative thereof may be prepared by methods that are well known in the art. One such method includes isolating or synthesizing DNA encoding the Nell I protein, and producing the recombinant protein by expressing the DNA, optionally in a recombinant vector, in a suitable host cell, including bacterial, yeast, insect or mammalian cells. Such suitable methods for synthesizing DNA are, for example, described by Caruthers et al. 1985. Science 230:281-285 and DNA Structure, Part A: Synthesis and Physical Analysis of DNA, Lilley, D. M. J. and Dahlberg, J. E. (Eds.), Methods Enzymol., 211, Academic Press, Inc., New York (1992).

Examples of suitable Nell I nucleic acid sequences include SEQ ID Nos: 1, 3, and 5. A Nell I protein or functional derivative may also be made synthetically, i.e. from individual amino acids, or semisynthetically, i.e. from oligopeptide units or a combination of oligopeptide units and individual amino acids. Suitable methods for synthesizing proteins are described by Stuart and Young in "Solid Phase Peptide Synthesis," Second Edition, Pierce Chemical Company (1984), Solid Phase Peptide Synthesis, Methods Enzymol., 289, Academic Press, Inc, New York (1997). Examples of suitable Nell I amino acid sequences include SEQ ID Nos: 2, 4, 6, and derivatives thereof.
In another embodiment, the method of the present invention is achieved by administration of a nucleic acid molecule encoding a Nell I protein or functional derivative to a subject in need of the treatment.

Suitable nucleic acid molecules for use in the present invention include nucleic acid molecules having a nucleotide sequence as set forth in SEQ ID NO: 1 (encoding the wild type human Ncll I protein), SEQ ID NO: 3 (encoding the wild type murine Nell I protein), and SEQ ID NO: 5 (encoding the rat wild type Nell I protein), as well as degenerate sequences thereof. As used herein, the term "degenerate sequence" refers to a sequence formed by replacing one or more codons in the nucleotide sequence encoding wild type Nelll protein with degenerate codes which encode the same amino acid residue (e.g., GAU and GAC triplets each encode the amino acid residue Asp).

In some embodiments, nucleic acid molecules for use in the methods of the present invention are provided in an expression vector. Expression vectors for use in the present methods include any appropriate gene therapy vectors, such as nonviral (e.g., plasmid vectors), retroviral, adenoviral, herpes simplex viral, adeno-associated viral, polio viruses and vaccinia vectors. Examples of retroviral vectors include, but are not limited to, Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV)-derived recombinant vectors. A Nelll -coding nucleotide sequence can be placed in an operable linkage to a promoter in the expression vector, wherein the promoter directs the expression of the Nelll protein in the targeted tissue or cells, and includes both a constitutive promoter and a tissue or cell-specific promoter.

A Nelll protein, functional derivative thereof or Nelll -encoding nucleic acid molecule can be combined with a pharmaceutically acceptable carrier and prepared in formulations suitable for administration to a subject by injections, implantations, inhalations, ingestions and the like. Pharmaceutically acceptable carriers are described hereinabove and include oils, water, saline solutions, gel, lipids, liposomes, resins, porous matrices, binders, fillers and the like, or combinations thereof. The carrier can be liquid, semi-solid, e.g. pastes, or solid carriers. Except insofar as any conventional media, agent, diluent or carrier is detrimental to the recipient or to the therapeutic effectiveness of the
active ingredients contained therein, its use the present invention is appropriate. Examples of carriers include oils, water, saline solutions, gel, lipids, liposomes, resins, porous matrices, binders, fillers, patches, and the like, or combinations thereof. The carrier can also be a controlled release matrix that allows optimum release of a Nelll protein or nucleic acid admixed therein.

The term "therapeutically effective amount" means the dose required to prevent or delay the onset, slow down the progression or ameliorate the symptoms of the disorder. Precise dosages depend on the disease state or condition being treated and other clinical factors, such as weight and condition of the subject, the subject's response to the therapy, the type of formulations and the route of administration. As a general rule, a suitable dose of a Nelll composition (i.e., including a Nelll protein or nucleic acid) for the administration to adult humans ranges from about 0.001 mg to about 20 mg per kilogram of body weight. In some embodiments, a suitable dose of a Nelll composition for the administration to adult humans is in the range of about 0.01 mg to about 5 mg per kilogram of body weight. However, the precise dosage to be therapeutically effective and non-detrimental can be determined by those skilled in the art.

A Nelll protein, functional derivative thereof, or nucleic acid molecule can be administered to the subject in any practical and convenient manner. Suitable routes of administration include the oral, nasal, topical, transdermal, and parenteral (e.g., intravenous, intraperitoneal, intradermal, subcutaneous or intramuscular) route. In addition, a Nelll protein, functional derivative thereof, or nucleic acid molecule can be introduced into the body, by injection or by surgical implantation or attachment, proximate to a preselected tissue or organ site such that the Nelll material is able to enter the site by direct diffusion. For example, a Nelll protein, functional derivative thereof, or nucleic acid can be provided in a patch or gel like substances, which, upon administration (by e.g., injection or implantation) can be taken up directly by tissues as a result of diffusing from a site of high concentration to one where there is very low level of the substance. IfNelll protein, functional derivative thereof, or nucleic acid molecule is administered locally, the formulation is such that the Nelll protein, functional derivative
thereof, or nucleic acid molecule does not diffuse and adversely affect surrounding organs.

Alternatively, a Nell I protein, or functional derivative thereof, can be administered directly to injured and damaged tissue (e.g., infarct and surrounding border zones). Such administration, can be applied, for example, to treat cardiovascular defects, thus minimizing heart muscle injury or stimulating tissue repair processes in the heart after MI.

Other delivery systems and methods include, but are not limited to: a) catheter-based devices that permit site specific drug delivery to the heart muscle, b) via a thorascopic opening (small minimally invasive wound in the thoracic cavity; similar to laparascopic methods) through which a scope and guided injection device containing Nelll protein, derivative thereof, or nucleic acid molecule is introduced, c) ultrasonic-based drug delivery methods (see, for example, Mayer et al., *Advanced Drug Delivery Reviews*, 2008, 60:1 177-1 192 and Bekeredjian et al., *Ultrasound in Medicine and Biology*, 2005, 31:687-691), and d) infusion into the pericardial space (see, for example, Xiao et al., *Am. J. Physiol, Heart Circ. Physiol*, 2008, 294:H12212-12218).

Important general considerations for design of delivery systems and compositions, and for routes of administration, for protein/peptide drugs may apply. For example, the appropriate delivery system for Nell1 protein and/or functional derivatives thereof will depend upon its particular nature, the particular clinical application, and the site of action.

Formulations for oral delivery or systemic delivery, for instance, may require certain considerations due to, for example, instability of Nelll protein and/or functional derivatives thereof in the gastrointestinal tract, or exposure of Nelll protein and/or functional derivatives thereof to proteases. Any method known to those skilled in the art can be utilized to address such considerations.

For example, for oral delivery, an absorption-enhancing agent can be utilized. A wide variety of absorption-enhancing agents have been investigated and/or applied in combination with protein compositions for oral delivery and for delivery by other routes (van Hoogdalem, Pharmac. Ther. 44, 407-43, 1989; Davis, J. Pharm. Pharmacol. 12
44(Suppl. 1), 186-90, 1992). Most commonly, typical enhancers fall into the general categories of (a) chelators, such as IiDTA, salicylates, and N-acyl derivatives of collagen, (b) surfactants, such as lauryl sulfate and polyoxyethylene-9-lauryl ether, (c) bile salts, such as glycholate and taurocholate, and derivatives, such as taurodihydrofusidate, (d) fatty acids, such as oleic acid and capric acid, and their derivatives, such as acylcarnitines, monoglycerides, and diglycerides, (e) non-surfactants, such as unsaturated cyclic ureas, (f) saponins, (g) cyclodextrins, and (h) phospholipids.

Alternatively, Nelll protein and/or functional derivative thereof, can be administered in combination with other drugs or substances that directly inhibit proteases and/or other potential sources of enzymatic degradation of proteins. Yet another alternative approach to prevent or delay gastrointestinal absorption of Nelll protein and/or functional derivative thereof is to incorporate them into a delivery system that is designed to protect the protein from contact with the proteolytic enzymes in the intestinal lumen and to release the Nelll protein and/or functional derivatives thereof at the site of cardiovascular injury. A more specific example of this strategy is the use of biodegradable microcapsules or microspheres, both to protect a protein from degradation, as well as to effect a prolonged release of active protein (see, for example, Deasy, in Microencapsulation and Related Processes, Swarbrick, ed., Marcell Dekker, Inc.: New York, 1984, pp. 1-60, 88-89, 208-11).

In a specific embodiment, a Nelll protein, functional derivative thereof, or nucleic acid molecule is administered to directly repair heart muscle after MI. Delivery can be performed via direct delivery to or near the injured heart muscle site (infarct and border zones) by injection, by catheter, via absorbable biomatrix (i.e. biocompatible porous) material, and the like, and combinations thereof. According to this embodiment, the Nelll composition is administered to the subject after the initial inflammatory responses subsides - usually within 72 hours, within 48 hours, within 36 hours, within 24 hours, or even within 18 hours of MI, in order to minimize the extent of the injury and achieve better therapeutic efficacy. There is a flood of inflammatory responses immediately after heart muscle injury. It is believed to be optimal to administer Nelll after this initial defensive response of the surrounding tissue. Regenerative processes,
which naturally begins after the inflammatory response slows down, are where Nell I is likely to work best.

Further according to the present invention, a NeIII protein, functional derivative thereof, or Nell I-encoding nucleic acid molecule can be used independently or in conjunction with additional therapeutic compositions useful for treating a cardiovascular condition.

In a specific embodiment, a NeIII protein, functional derivative thereof, or Nell I-encoding nucleic acid molecule is used together with stem cells for the repair and regeneration of damaged cardiac muscles and blood vessels.

Cell-based therapies for the repair and regeneration of damaged cardiac muscles and blood vessels utilize implantation of cells (such as cardiomyocytes), or introduction of stem cells isolated from adult bone marrow to develop new cardiac muscle in the area of implantation. See, e.g., Orlic et al., 2001; Rubart ct al., 2006; Ott et al., 2006; Rosenthal et al., 2006. Without being bound by theory, the use of Nell I increases the efficiency of cell-based therapies for the repair and regeneration of damaged cardiac muscles and blood vessels.

According to the present invention, a Nelll protein or nucleic acid molecule can be co-delivered with the appropriate cells, e.g., cardiomyocytes or adult stem cells, directly to the damaged sites of a subject using biological matrices or direct injection methods already in practice for cell-based therapies.

In another embodiment, a Nelll protein, functional derivative thereof, or Nelll -encoding nucleic acid molecule is used *in vitro* to stimulate or promote the development and differentiation of stem cells into cardiomyocytes useful for the repair and regeneration of damaged cardiac muscles and blood vessels. See, for instance, example 7.

This invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. The terms and expressions which have been employed in the present disclosure are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions
thereof. It is to be understood that various modifications are considered to be included within the scope of the invention. All the publications mentioned in the present disclosure are incorporated herein by reference.

5

Example 1

NeIII** Mutant Mouse

The $NeIII^{6R}$ mutant mouse was used in the experiments described in the following examples. Generation, breeding and maintenance of this mutant mouse is described in U.S. Published Application 2006/0053503, which is incorporated herein by reference. Briefly, the mutant mouse contains a recessive neonatal-lethal point mutation in the NeIII gene, originally induced by N-ethyl-N-nitrosourea (ENU). $NeIII^{6R}$ has T to A base change that converts a codon for cysteine into a premature stop codon (TGT to TGA; Cys(502)Ter), resulting in a severe truncation of the NeIII protein product and a marked reduction in steady state levels of the NeII transcript.

Example 2

Heart Defects in $NeUI^{6R}$ Mutant Mouse

Formalin-fixed specimens were analyzed by heart length and width measurements. These measurements were completed on wild type, heterozygous, and mutant mice at the 18.5-day embryonic stage. Further observations were made using standard histological methods (haematoxylin and cosin staining on mouse sagittal sections).

$NeII^{6R}$ mice were observed to have 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 was significantly greater compared to the width for wild type and heterozygous mice, this confirming presence of an abnormal heart phenotype in mutant mice.

Examination of the haematoxylin and eosin-stained slides showed dramatically reduced blood flow out of the heart. As shown in Figures 1-2, wild-type
and heterozygote mice showed arteries filled with blood whereas blood was not a very prominent feature in slides of mutant mice. Therefore, the loss of Nell 1 function resulted in a significant reduction of the number of blood vessels and extensive branching of the vasculature in mutants as compared to wild type fetuses. The decrease in blood vessel formation was observed throughout the fetal body.

In addition, a larger number of immature heart cells and lesser extracellular matrix were observed in mutant mice as compared to wild type mice (Figure 3A-3B). The dense packing of smaller cardiomyocytes in the mutant (Figure 3B) was very apparent in the denser/darker staining with haematoxylin and eosin, compared to the wild type (Figure 3A).
Table 1 Measurements of M7// Hearts Indicating Heart Enlargement

Measurement (mm) of 18.5 fetal heart width and length of NeU" heterozygotic and homozygotic mutant mice compared with wild-type lillennat ws. There is significant enlargement of fetal hearts in homozygotic mutant compared to the heterozygotes and normal mice.

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<th>No. of Fetuses</th>
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<td>10 Width</td>
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<td>17 Average</td>
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</tr>
<tr>
<td>50 No. of Fetuses</td>
<td>17</td>
<td>11</td>
<td>14</td>
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These above cardiovascular defects were evident in E18.5 day fetuses recovered by caesarean. Additionally, wild type and hetero/ygote mice had spongy lungs that filled their entire thoracic cavity, while mutant mice had compact, dense lungs. Mutant mice did not survive birth. The severity of the heart and blood vessel defects were likely to be the cause of the death of the fetuses during the birth process reported earlier (Desai et al., 2006). Fetuses that were recovered by caesarean were unable to breathe as depicted in the collapsed lung in the mutants.

**Example 3**

**ECM Genes Affected by Nelll Influence Heart Development**

A comprehensive gene expression analysis using public database (UCSC Genome Browser, Mouse Genome Informatics, Integrated Cartilage Gene Database, PubMed) was conducted to investigate the relationship between cardiovascular development and each of the 28 extracellular matrix (ECM) genes which were shown previously (Desai et al., 2006) to exhibit reduced expression in Nelll<sup>6R</sup> mutant mouse bodies. Of the 28 ECM genes studied, the bioinformatics analysis showed that the majority of genes with reduced expression in Nelll deficient mice are normally expressed in the heart (79% of the analyzed ECM genes; 22/28), blood vessels (71%; 20/28) and bone marrow (61%; 17/28) (See Table 2). The Mouse Genome Informatics database referenced several genes (Coll5al, Osf-2, Bmprla, Pkdl, Mfge8, Ptger4, Notch3) that have been mutated in mice and actually manifest abnormalities in cardiovascular development.

Mouse mutations in some of these genes display heart deformities commonly associated with heart enlargement, as shown in Table 3 below.
Table 2. Expression profile of genes in the NcIII pathway and association with mutant mouse phenotypes.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Expression</th>
<th># abnormal heart phenotype</th>
<th># total mutants</th>
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<td></td>
<td></td>
<td>heart</td>
<td>vascular</td>
<td>blood</td>
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<tr>
<td>Tnxb</td>
<td>tenascin</td>
<td>10</td>
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<tr>
<td>Prq4</td>
<td>proteoglycan 4</td>
<td>9</td>
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<td>Thbs3</td>
<td>thrombospondin 3</td>
<td>10</td>
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<td>10</td>
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<tr>
<td>Col5a3</td>
<td>collagen 5 alpha 3 subunit</td>
<td>10</td>
<td>10</td>
<td>10</td>
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<tr>
<td>Neurog2</td>
<td>neurogenin 2</td>
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<td>Col5a1</td>
<td>procollagen type V, alpha 1</td>
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<td>10</td>
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<td>Col6a1</td>
<td>procollagen Type VI, alpha 1</td>
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<td>procollagen type XV, alpha 1</td>
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<tr>
<td>Paccin3</td>
<td>PKC and casein kinase substrate in neurons 3</td>
<td>10</td>
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<td>Tnc</td>
<td>tenascin c</td>
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<td>procollagen type XII, alpha 1</td>
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<tr>
<td>Char</td>
<td>chondroadherin</td>
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<tr>
<td>Osr2-pending</td>
<td>osteoblast specific factor 2</td>
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<td>Col17a1</td>
<td>procollagen type XVII, alpha 1</td>
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<td>Ptkc</td>
<td>protein kinase C</td>
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<td>Ptkch</td>
<td>protein kinase C, eta symbol</td>
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<tr>
<td>Bk-pending</td>
<td>brain and kidney protein</td>
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<td>Ptk9</td>
<td>PTK9L protein tyrosine kinase 9-like</td>
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<td>Npdc1</td>
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<td>Pkd1</td>
<td>polycystic kidney disease 1 homolog</td>
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<td>Trilsf11tb</td>
<td>tumor necrosis factor (ligand)</td>
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<td>Mi6g8</td>
<td>milk fat globule-EGF factor 8 protein</td>
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<td>Matn3</td>
<td>matrilin 3, cartilage matrix protein</td>
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<td>Bmp7</td>
<td>bone morphogenetic protein type 7</td>
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<td>matrilin 2, cartilage matrix protein 2</td>
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<td>prostaglandin E receptor 4</td>
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<td># of Genes</td>
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<td>13</td>
</tr>
<tr>
<td>Percentage</td>
<td></td>
<td>79%</td>
<td>71%</td>
<td>46%</td>
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Table 3. Mutated genes causing heart defects associated with enlargement

<table>
<thead>
<tr>
<th>Gene</th>
<th>Defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col6al</td>
<td>Dilated descending aorta</td>
</tr>
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</table>
| Bmprla | Persistent truncus arteriosus  
Outflow tract formation abnormalities |
| Pkdl | Vascular leaks/ruptures  
Endocardial cushion defects  
Abnormal atrial septum morphology  
Double outlet right ventricle  
Abnormal septation |
| Bmp7 | Lack of endocardial cushion formation |
| Ptger4 | Dilated left ventricle  
Patent ductus arteriosus |
|         | Congestive heart failure |
Example 4
Gene Expression in NeIII Mutant Mouse

To define the involvement of NeIII in the known molecular pathways that
govern heart structure and function, a comprehensive gene expression analysis was
conducted in the entire mouse genome (~30,000 genes) of normal fetal hearts and those
dissected from NeIII 6R. This analysis consisted of 50 mutant fetal hearts separated into 4
pools of 10-13 hearts and 35 normal hearts separated into three pools of 10-12 hearts
(18.5 days of gestation). RNAs were extracted from the pooled tissues, processed for
microarray analysis on the Illumina Mouse V6 chips and scanned with Illumina
Beadstation 500GX. Data was analyzed with the BeadStudio software and Gene
Ontology Tree machine. At least 345 genes were identified that were differentially
expressed between normal and mutant samples (at p value=0.001 for the microarray
detection and differential p values; denotes a very high statistical significance).
Table 4 lists a representative sampling of genes influenced by NeI/U that already have established
functions in cardiovascular conditions. Table 4 also provides the literature references for
the specific studies that have demonstrated these gene functions.

Table 4 shows a number of genes in the NeI/U pathway that have been
implicated in the processes that ensue after heart failure. The ability of NeI/U to stimulate
proteins that control cell differentiation and proper secretion of the cardiac ECM strongly
suggests that this protein can restore proper ECM constitution and orientation in heart
muscle after a heart attack, thereby preventing or alleviating heart muscle damage and
subsequent loss of heart function (or death) resulting from MI.

Example 5

The data presented here were based on studies of the NeIII 6R mutant mouse.
Rodent NeI/U studies are believed to translate accurately to the human situation. The
complete mouse NeI/U coding sequence has been reported (Genbank Accession No.
AY622226; Desai et al, 2006). A comparison of this sequence with the most current
human NeIIl gene in the public genome databases (1k 'SC (igenome Browser and N('BI) indicates a very high homology of 87% gene sequence identity. The corresponding 810-amino acid residue polypeptides have a 93% identity in their amino acid sequences (Figure 5). When one considers conservative substitution of similar amino acids, the human and mouse NeIIl proteins are 97% conserved. This remarkable degree of gene and protein structure conservation suggests the conservation of functions and fundamental mechanisms of NeIIl-mediated pathways in human and mouse.

Example 6

Animal Model for Assessing Therapeutic Efficacy of NeIIl for MI

The efficacy of the NeIIl protein for regenerating cardiac muscle after damage induced by a myocardial infarction (MI) is tested in a widely used and accepted in vivo animal model. Myocardial infarction is induced in a murine in vivo model by blocking the main blood supply line to the left ventricle. The surgical procedures for generating this model are described in detail by several publications (Patten et al., 1998; Tarnavski et al., 2004; Ahn et al., 2004).

Briefly, mice are anesthetized, restrained in a supine position, and intubated with pure oxygen regulated by a small animal ventilator. A thoracotomy is performed under a dissecting scope, at the fourth or fifth intercostal space of the left side, between the heart and lung margins. The thoracic surgical hole is enlarged using retractors and the pericardial sac is gently torn with fine forceps.

The left anterior descending coronary artery (LAD) is visualized and ligated by passing a tapered microsurgical needle (1/4 circle, 140 microns) with a black silk monofilament suture (size 7 or 8) underneath the coronary artery and tying the suture to completely stop the blood flow in the artery. A small polyethylene tubing (PE 10) 2-3 mm is placed between the tie and the LAD to minimize cutting and severely injuring the artery.

Myocardial infarction is confirmed by observing for blanched or white appearance of the left vertical that correspond to the muscles that have lost blood supply
and the alteration of the wave pattern (pmnøuneed ST wave elevation) in an electrocardiogram. Since the LAD provides the blood supply to the left ventricle, this surgically-induced myocardial infarction will cause the death of myocardial tissue (necrosis) in the left bentricular wall and the anterior section of the interventricular section. The size of the myocardial infarction lesions/infarcts can be controlled by the exact position of the ligation along the LAD. Ligation at a high position (atrioventricular junction) will reduce blood flow to a larger area and make larger infarcts while ligations at lower areas will make medium or small lesions. Ligature position is kept constant for any given experimental group to keep the infarction size constant.

After myocardial infarction induction, the thoracic and skin wounds are sutured and mice are allowed to recover from anesthesia on a heating pad or with heat lamps.

To test the ability of Nell 1 to repair cardiac tissue damage due to an acute myocardial infarction event, purified Nelll protein are delivered directly into the surrounding tissue around the visible infarct and within the infarct. Direct delivery of Nelll protein is performed by reopening the original thoracic wound used to induce the infarct.

Nel1l and functional derivatives thereof containing EGF like domains and/or the von Willebrand like domain of Nelll are administered at 2-3 points along one side of the infarct border zone. In some animals, direct delivery of Nel1l protein is administered via microinjection, application of Nelll in a gel or microspray, via nanoparticles, or time-release patches. In others, it is administered via a Nelll protein expression vector (continuous delivery). Administration of Nelll is performed after the initial surge of inflammatory response triggered by cardiac damage and at the time heart tissue attempts innate regenerative mechanisms (approximately 4-5 hrs after MI). The effects of Nelll administration are evaluated by standard histology and immunohistochemistry techniques for detection of proteins associated with cardiac tissue regeneration (Orlic et al., 2001).
**Example 7**

*In vitro* Sleni Cell Therapy

A promising approach in the field of heart muscle regeneration after MI is the introduction of either embryonic or adult mesenchymal stem cells into the damaged heart.

However, data indicate that although new heart muscle cells can be regenerated that the new tissue may not necessarily display the full Junctional capacity of mature heart tissue (contractility).

To promote full Junctional capacity of mature heart tissue, Nelll protein and functional derivatives thereof containing EGF like domains and/or the von Willebrand like domain of Nelll are co-delivered with stem cells to the injured heart muscle using the same strategies currently in use for stem cell delivery.

**Example 8**

Animal Model for Assessing Therapeutic Efficacy of Nelll for Myocardial Ischemia and Reperfusion Injury

The efficacy of the Nelll for regenerating cardiac muscle after damage induced by myocardial ischemia and reperfusion injury is tested in a widely used and accepted *in vivo* animal model. Myocardial ischemia and reperfusion injury is induced in an *in vivo* murine model as follow:

1. After anesthesia, intubation and hook-up to a mouse ECG machine, the chest cavity of the mouse is opened at the intercostal space (usually 4th or 5th) and the opening is retracted to reveal the left side of the heart and to locate the LAD artery. The pericardial sac is torn gently with forceps and the LAD is positioned for easy access. All surgical steps are done under a dissecting microscope.

2. A tapered needle (1/4 circle 140 microns) with a size 8 silk or monofilament suture is partially passed underneath the artery. A small tubing 1-1.5" in length (e.g. polyethylene size 10 tubing) is placed on top and parallel to the LAD artery and perpendicular to the length of the needle. The suture is then pulled and a surgical tie is made such that the tubing is tied with the artery located beneath it.
3. The interruption of blood flow to the left ventricular heart muscles is easily visualized by a blanched or white appearance of the affected region (where infarct develops). The ECG will confirm the ischemia by the alteration of the wave pattern (e.g. ST segment elevation, T wave anomalies) compared to the normal pattern. The change indicates that the LAD is successfully ligated and restricted blood flow to the left ventricle has functionally induced an ischemic event.

4. The chest cavity and the skin are sutured such that one end of the tubing is sticking out of the thoracic area above the sutured skin. After the desired amount of time of ischemia, the tubing is gently pulled out to relax the knot/ligated suture thereby allowing reperfusion of blood into the affected area.

5. Reperfusion is indicated by the return of the ECG pattern to normal or near normal pattern. Different groups of mice with varying times of occlusion before reperfusion are made.

6. Varying concentrations of Nelll protein are administered via intraperitoneal injection or using a catheter device that is placed before the chest cavity is closed after LAD ligation and ischemia. The catheter device allows for controlled delivery so that Nelll protein can be delivered immediately after reperfusion or given time points after reperfusion is induced. In other models, Nelll protein is administered by reopening the surgical sutures and re-entry to the chest cavity and direct Nelll delivery by microinjection or gel patch.

Example 9
Animal Model for Assessing Therapeutic Efficacy of Nelll for Cardiac Hypertrophy

The use of Nelll protein as a therapeutic for cardiac hypertrophy is tested in a widely used and accepted in vivo animal model. Cardiac hypertrophy is generated by physical/surgical means [pressure-overload].

In the in vivo pressure overload animal model, the aorta of a mouse/rat or large animal is banded to reduce the diameter and thus the blood in the left ventricle builds up pressure and induces hypertrophy of the left ventricle (Tarnavski et al 2004). This type
of animal model mimics the human condition of aortic stenosis where the narrowing of the aortic valve restricts blood flow from the left ventricle to the aorta. The persistent increased pressure in the left ventricle leads to increase in muscle mass (hypertrophy) of the walls. This model is generated as follows:

1. Mice are anesthetized and a 5 mm transverse incision is made at the level of the left armpit, 2mm away from the sternal border. A small incision (5mm) is made at the 2nd intercostal space and opened with microretractors.

2. The thymus and fat covering the aortic area are pushed away and the pericardial sac is gently torn. The ascending portion of the aorta is located and bluntly dissected from the pulmonary trunk and forceps is placed underneath the ascending aorta.

3. A 7-0 silk suture is placed around the aorta and a loose knot is made. A 25 or 27 gauge needle (outer diameter of 0.51 mm) that is bent into an L shape is placed through the loose loop, positioned above and parallel to the aorta and a second knot is tied securely. The needle is retracted to yield a constricted aorta (60-80% constriction for a 27 gauge). Two more knots are tied.

4. The chest cavity is closed by suturing ribs and then the skin wound.

Nelll protein and functional derivatives thereof containing EGF like domains and/or the von Willebrand like domain of Nelll are administered as an injectable after the onset of hypertrophic changes and heart function anomalies detected by ECG. Times of administration are tested as one high dose after hypertrophy is diagnosed or at lower doses given multiple times (weekly) after hypertrophy is diagnosed. Efficacy of the treatment is evaluated by quantitative measurements of ventricular and heart size, physiological monitoring by ECG and other heart visualization tools, molecular markers for heart failure etc. as described earlier.
The use of Nell 1 protein as a therapeutic for cardiomyopathy is tested in a widely used and accepted *in vivo* animal model. The *in vivo* mouse model of cardiomyopathy is generated by gene-targeted approaches such as knock-outs or over-expression of a single gene, wherein the homozygous (two mutant gene copies) and/or heterozygotes (one mutant copy) can survive to the juvenile or adult stage. Suitable *in vivo* mouse models of cardiomyopathy contain knock-outs or over-expression of genes and pathways (e.g., (extracellular matrix and matricellular proteins, tenascins, thrombospondins, matrilins, etc.) that are controlled by the Nell 1 signaling protein. A specific example of an appropriate small animal model is the targeted knockout of the mouse *Nov* (*Ccn3*) gene reported by Heath *et al.* ([BMC Developmental Biology](https://academic.oup.com/bmcdevbiol/article/8/18/18))

Briefly, *Nov* (*Ccn3*) mutant mice are generated. Imaging of hearts by echocardiograms and electrocardiograms are conducted to determine heart function and presence of visible heart structure anomalies prior to treatment.

Nelll protein and functional derivatives thereof containing EGF like domains and/or the von Willebrand like domain of Nell 1 are administered by intraperitoneal injection to young *Nov* (*Ccn3*) mutant mice and corresponding controls during the first two months of life. Various dosages and timing regimens are tested. After treatment, heart function parameters are measured in Nelll-treated and controls during the time that untreated mutant mice show the severe symptoms of cardiomyopathy, generally at 4-5 months in *Nov* mice.

After cardiovascular functional/physiological studies, the mice are sacrificed and hearts are dissected and fixed for morphological and histological evaluation such as: total heart size, chamber sizes (especially left ventricle), heart valve structure, chordae tendinae, interventricular septum, heart muscle cell (cardiomyocyte) size and appearance, vessels going in and out of the heart etc.
The ability of Nell 1 protein to trigger cellular pathway(s) for regeneration of damaged heart muscle was demonstrated in an in vivo mouse model. A heart attack or myocardial infarction was generated in 4-5 month old adult mice (strain C57B1/6J) by surgically tying the left anterior descending (LAD) coronary artery, which is the main blood supply line to the left ventricle (LV) and the interventricular septum (IVS). The left ventricle pumps oxygenated blood through the aorta into the rest of the body while the IVS divides the right and left ventricles of the heart. LAD ligation in animal models results in the damage and subsequent death of the heart muscle tissue. Table 5 summarizes the results of treating mouse hearts with the purified human NELL1 protein on the third day post-MI event. The NELL1 protein was diluted in phosphate buffered saline (PBS) and was delivered directly onto the damaged heart muscle as a very concentrated microdrop, while the mice were under anaesthesia and intubation for about an hour. Three mice were treated with 312 ng and four mice with 624 ng purified NELL1 protein. Four mice underwent the same cardiac surgery but were given a microdrop of PBS on the damaged heart tissue and served as controls. In addition to these controls, over 20 MI mice were previously generated and studied to obtain consistency in MI surgical and post-surgical techniques. These earlier "controls" displayed the same characteristics as controls represented in Table 5. All treated and untreated mice were maintained for an additional 14 days before they were sacrificed to collect hearts and other major organs (a total of 17 days post-MI). Heart size measurements indicated slight increases in both heart width and depth in Nell 1-treated hearts. Remarkably ALL treated mice showed dramatically lesser visible areas of the infarcted tissue on the surface of the heart. In 6 out of 7 hearts the damaged tissue was only visible under the microscope after they were fixed in buffered formalin. Figure 6A-6D show the range of improvement observed in NELL1-treated hearts, from barely visible to about 30% infarct sizes in comparison to the usual 50-90% infarct sizes seen in controls. Figure 7A-7D present histological analysis of sectioned hearts stained with Masson-Trichome and further
confirmed that there is decreased damage at the cellular level in the NHLL.I-treated hearts compared to the controls. At 17 days posl-MI, heart muscle tissue is severely damaged such that huge gaps appear within the untreated heart muscle in the IeII ventricle to the interventricular septum. In contrast, there is a consistent and dramatic reduction in the amount of breakdown or damage observed in the heart muscle of treated mice. These data from an in vivo MI mouse model illustrates that clinical approaches that will enable delivery of Nell 1 protein directly onto damaged heart muscle will be effective in reducing the effects of an MI event.
<table>
<thead>
<tr>
<th>GENE and DESCRIPTION</th>
<th>UP (↑) OR DOWN (↓) REGULATION [p value ≤ 0.001]</th>
<th>ASSOCIATION WITH HEART DISORDERS AND DISEASES</th>
<th>REFERENCES</th>
</tr>
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<td>Tpm2; tropomyosin 2, beta</td>
<td>↑4.3</td>
<td>Cardiac-specific myofibrillogenesis; Cardiomyopathy</td>
<td>Denz et al., 2004</td>
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<tr>
<td>Dmn; desmuslin transcript variant 1</td>
<td>↑9.4</td>
<td>Hypertrophic Cardiomyopathy; heart failure</td>
<td>Mizuno et al., 2001</td>
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<tr>
<td>Acta1; skeletal muscle actin alpha 1</td>
<td>↑2.8</td>
<td>Hypertrophic cardiomyopathy; heart failure</td>
<td>Lim et al., 2001</td>
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<tr>
<td>Tpm1 tropomyosin alpha 1</td>
<td>↑4.8</td>
<td>Hypertrophic cardiomyopathy; heart failure</td>
<td>Wernicke et al., 2007; Kostin et al., 2007</td>
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<td>Lgals3; lectin, Galactose binding, soluble 3</td>
<td>↑2.6</td>
<td>Acute heart failure biomarker; excellent predictor of mortality within 60 days; increases in failure prone hypertrophied hearts; aortic stenosis; induces cardiac fibroblast proliferation, collagen deposition</td>
<td>Van Kimmenade et al., 2006; Sharma et al., 2004</td>
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<tr>
<td>Spp1 Secreted phosphoprotein 1 (osteopontin)</td>
<td>↑2.3</td>
<td>Heart contractility via control of ECM proteins Inflammation control in hypertrophy, myocardial infarction and heart failure, valvular stenosis</td>
<td>Okamoto. 2007; Singh et al.. 2007</td>
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<td>Phl1 Four and a half limb domains</td>
<td>↑1.3</td>
<td>Atrial fibrillation in cardiac arrhythmia; β-adrenergic induced cardiomyopathy and heart failure (β-blocker pathway); cardiac remodeling by transcriptional regulation and myofilament assembly</td>
<td>Chen et al.. 2007; Lim et al.. 2001</td>
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<td>Aqp1; aquaporin 1</td>
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<td>Myocardial edema</td>
<td>Egan et al.. 2006</td>
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<td>Il6st Interleukin 6 signal transducer</td>
<td>↑1.5</td>
<td>Cardiac hypertrophy</td>
<td>Terrell et al.. 2006; Coles et al.. 2007</td>
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<td>Tnc Tenascin c</td>
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<td>Tnxb Tenascin xb</td>
<td>↓1.8</td>
<td>Cardiac nerve sprouting after MI contributing to arrhythmia and sudden cardiac death</td>
<td>Lai et al., 2000</td>
</tr>
<tr>
<td>Igf1bp5 Insulin growth factor binding protein 5</td>
<td>↓1.3</td>
<td>Atrophy; Adaptive cardiac hypertrophy</td>
<td>Baurand et al., 2007</td>
</tr>
<tr>
<td>Fgl2 Fibrinogen-like protein</td>
<td>↓1.4</td>
<td>Acute congestive heart failure without structural abnormalities; contractile dysfunction and rhythm abnormalities</td>
<td>Mu et al., 2007</td>
</tr>
<tr>
<td>Ctgf; connective tissue growth factor</td>
<td>↓1.3</td>
<td>Excessive myocardial fibrosis and diastolic heart failure</td>
<td>Koitabashi et al., 2007</td>
</tr>
<tr>
<td>Dpt; dermotopontin</td>
<td>↓1.5</td>
<td>ECM remodeling in myocardial infarction</td>
<td>Takemoto et al., 2002</td>
</tr>
<tr>
<td>Ldlr; low density lipoprotein receptor</td>
<td>↓1.5</td>
<td>Heart failure</td>
<td>Weiss et al., 2006</td>
</tr>
<tr>
<td>Nppb Natriuretic peptide precursor type b</td>
<td>↓1.3</td>
<td>Cardiac fibrosis; Congestive heart failure and myocardial infarction; Biomarker for heart failure</td>
<td>Tamura et al., 2000 Hejmdal et al., 2007 Seferian et al., 2007 Doust et al., 2004</td>
</tr>
<tr>
<td>Nppa Natriuretic peptide precursor type a</td>
<td>↓1.5</td>
<td>Cardiac fibrosis; Congestive heart failure and myocardial infarction; Biomarker for heart failure</td>
<td>Tamura et al., 2000 Hejmdal et al., 2007 Seferian et al., 2007 Doust et al., 2004</td>
</tr>
<tr>
<td>Ttn Titin</td>
<td>↓1.4</td>
<td>Cardiac muscle dystrophies (contractility)</td>
<td>Fougerousse et al., 1998; Koatin et al., 2000</td>
</tr>
<tr>
<td>Cyr61 Cysteine rich protein 61</td>
<td>↓1.7</td>
<td>Inflammatory cardiomyopathy</td>
<td>Wittchen et al., 2007; Mo and Lau, 2006</td>
</tr>
<tr>
<td>Sgcβ Sarcoglycan</td>
<td></td>
<td>Cardiac muscle dystrophies</td>
<td>Fougerousse et al., 1998</td>
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31
<table>
<thead>
<tr>
<th>Mouse Number</th>
<th>Weight Change 17 day period</th>
<th>Heart length Top-Bottom (mm)</th>
<th>Heart Width Left-Right (mm)</th>
<th>Heart Depth Front-Back (mm)</th>
<th>Estimated Infarct Size 17 days post-MI (% left ventricle)</th>
</tr>
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<tbody>
<tr>
<td>Controls (PBS)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>m2589</td>
<td>0</td>
<td>8.32</td>
<td>5.84</td>
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<td>m2588</td>
<td>+1.2</td>
<td>8.55</td>
<td>6.20</td>
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<td>m2733</td>
<td>-0.9</td>
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<td>7.01</td>
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<tr>
<td>m2764</td>
<td>+1.1</td>
<td>8.52</td>
<td>6.09</td>
<td>5.57</td>
<td>90%</td>
</tr>
<tr>
<td>Average</td>
<td>+0.35</td>
<td>8.54</td>
<td>6.28</td>
<td>5.32</td>
<td>70%</td>
</tr>
<tr>
<td>Nell1 Protein Dose I (312 ng)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m2550</td>
<td>-3.2</td>
<td>8.42</td>
<td>7.41</td>
<td>6.19</td>
<td>Infarct hardly visible until fixation; ~16% faint area</td>
</tr>
<tr>
<td>m2597</td>
<td>-2.3</td>
<td>8.04</td>
<td>6.12</td>
<td>6.17</td>
<td>Infarct barely visible until fixation; 30% faint area</td>
</tr>
<tr>
<td>m2553</td>
<td>-2.3</td>
<td>9.21</td>
<td>6.44</td>
<td>5.52</td>
<td>Infarct hardly visible until fixation; 30% faint area</td>
</tr>
<tr>
<td>Average</td>
<td>-2.6</td>
<td>8.56</td>
<td>6.66</td>
<td>5.96</td>
<td>25%</td>
</tr>
<tr>
<td>Nell1 Protein Dose II (624 ng)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m2668</td>
<td>+0.1</td>
<td>8.51</td>
<td>6.55</td>
<td>5.65</td>
<td>Infarct hardly visible until fixation; 25% faint area</td>
</tr>
<tr>
<td>m2732</td>
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<td>8.94</td>
<td>6.44</td>
<td>5.73</td>
<td>Infarct hardly visible until fixation; 10% very small faint area</td>
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<tr>
<td>m2726</td>
<td>-2.7</td>
<td>8.50</td>
<td>6.90</td>
<td>5.94</td>
<td>Infarct hardly visible until fixation; very faint layer difficult to estimate</td>
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<tr>
<td>m2727</td>
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<td>8.42</td>
<td>6.95</td>
<td>6.26</td>
<td>Visible infarct at ~30%</td>
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<tr>
<td>Average</td>
<td>-0.75</td>
<td>8.59</td>
<td>6.71</td>
<td>5.90</td>
<td>16.3%</td>
</tr>
</tbody>
</table>
REFERENCES:


Lim DS et al. JAm Coll Cardiol 2001; 38:1 175-180


Mu J et al. Physiol Genomics Jun 5 2007 (Epub).
Rubart et al., Ann NY Acad Sci 2006; 1080: 34-48
Terrell et al. Shock 2006; 26: 226-234.
van Kimmenade et al. J. Am. Coll. Cardiol. 2006; 48: 1217-1224
WHAT IS CLAIMED IS:

1. A method of treating a cardiovascular disorder in a subject in need thereof comprising administering a Nell 1 protein to said subject.

2. The method of claim 1, wherein said Nelll protein comprises an amino acid sequence as set forth in any one of SEQ ID NO: 2, SEQ ID NO: 4 or SEQ ID NO: 6.

3. A method of treating a cardiovascular disorder in a subject in need thereof comprising administering a nucleic acid coding for a Nell 1 protein to said subject.

4. The method of claim 3, wherein said nucleic acid is an expression vector to effect expression of Nelll in said subject.

5. The method of claim 4, wherein said expression vector is a viral or non-viral vector.

6. The method of any one of claims 1-5, wherein said cardiovascular disorder is myocardial infarction, heart failure, cardiac ischemia, hypertrophy, or cardiomyopathy.

7. The method of claim 6, wherein said Nell 1 protein or said nucleic acid is administered systemically.

8. The method of claim 7, wherein said Nelll protein or said nucleic acid is administered by ingestion, injection or implantation.

9. The method of claim 6, wherein said Nelll protein or said nucleic acid is administered locally.

10. The method of claim 9, wherein said Nelll protein or said nucleic acid is administered by injection or implantation at or near the site of cardiac muscle damage.
11. The method of claim 6, wherein said Nell I protein or said nucleic acid is administered via catheter to or near the site of cardiac muscle damage.

12. The method of claim 6, wherein said Nelll protein or said nucleic acid is administered in conjunction with cells for the repair and regeneration of damaged cardiac muscles and blood vessels.

13. The method of claim 12, wherein said cells are cardiomyocytes.

14. The method of claim 12, wherein said cells are stem cells.

15. A method of treating myocardial infarction in a subject in need thereof comprising administering a Nell I protein to said subject.


17. A method of treating cardiac ischemia in a subject in need thereof comprising administering a Nelll protein to said subject.

18. A method of treating hypertrophy in a subject in need thereof comprising administering a Nelll protein to said subject.

19. A method of cardiomyopathy in a subject in need thereof comprising administering a Nelll protein to said subject.

20. The method of anyone of claims 15-19, wherein said Nell I protein is administered in conjunction with cells.

21. The method of claim 20, wherein the cells are cardiomyocytes.
22. The method of claim 20, wherein the cells are stem cells.
Figure 1A-1B
FIGURE 5

SUBSTITUTE SHEET (RULE 26)
**Figure 7A-7F**
INTERNATIONAL SEARCH REPORT

A CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 38/17; A61P 9/00 (2008.04)
USPC - 514/12; 530/350

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(8) - A61K 38/17, A61P 9/00 (2008 04)
USPC - 514/12, 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 514/12, 530/350

C DOCUMENTS CONSIDERED TO BE RELEVANT

Category* Citation of document, with indication, where appropriate, of the relevant passages Relevant to claim No

Y US 2006/0053503 A1 (CULIAT et al) 09 March 2006 (09 03 2006) SEQ ID NO 4, para [0030], [0031] 1-22


Y US 2006/0025367 A1 (SIMARI) 02 February 2006 (02 02 2006) para [0009], [001 1] 3-5, 7, 8 and 11


D Further documents are listed in the continuation of Box C

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"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" 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

"Z" document member of the same patent family

Date of the actual completion of the international search
09 December 2008 (09 12 2008)

Date of mailing of the international search report
02 JAN, 2009

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