Abstract:
The present invention provides methods for repairing damaged tissue using nanoparticles for the elution of growth factors for recovery and regeneration of tissue.
NANOPARTICLES FOR THE ELUTION OF GROWTH FACTORS FOR RECOVERY AND REGENERATION OF ORGANS

Field of the Invention

[0001] The present invention relates to the use of nanoparticles for the elution of growth factors for recovery and regeneration of tissue.

Background of the Invention

[0002] A major challenge in engineering tissues is mimicking the complex cellular organization and function of the native tissues of the human body. Tissue structure and function are very highly interrelated so that cellular and macromolecular organization of the tissue often brings about mechanical and biological functionality. For example, it is the circumferential arrangement of smooth muscle fiber layers that allows for change in the caliber in the lumen of blood vessels (Fawcett, 1986); the wickerwork pattern of collagen fibers in the skin give it mechanical strength (Alberts, 1994); the polygonal phenotype and complex arrangement of hepatocytes are essential for proper liver function (Boron, 2003); and the spiraling parallel arrangements of myocytes in the ventricle eject blood (Streeter, 1979; Sommer, 1995). Without proper cellular organization, an artificial tissue does not function adequately.

[0003] Many approaches to regain, for example, cardiac function depend on making layers of bundles of contractile myocytes in vitro with the intention of surgical implantation. Seeding of cardiac cells randomly into matrices or hydrogels fails because the cells are isolated from each other promoting cellular atrophy and apoptosis. The surviving cells are few in number and not electrically connected resulting in poor force production (Langer and Vacanti, 1993). Rat neonatal cells from primary culture have been grown on surfaces adhering to peptides stamped in parallel lines but are not all electrically connected laterally (Reinecke, 1999) and have all the disadvantages of two-dimensional culture. Another 2D approach involves releasing a monolayer of cells by temperature sensitive chemistry allowing the randomly oriented myocytes to contract (Shimizu, 2003). Multiple layers were tightly stacked and implanted by 'poly-surgery' to generate a thicker construct. Repeat surgeries at daily intervals would not be acceptable for human application.
A more promising approach involves myocytes grown on collagen networks that are mechanically paced to form 3D electrically connected strips that have been grafted in vivo (Zimmerman, 2002). This is appealing because it recreates trabeculae ensheathed with fibroblasts and endothelium ~100μm in diameter. However, it is not easy to see how this approach could be scaled up for surgery. The recent exterior "chain-mail jacket" approach (Zimmerman, 2006) is fraught with practical difficulties for use in human patients. This model has an outer layer of connective tissue that might well cause fibrosis and prevent the myocytes of the graft from connecting directly with the healthy heart of the host. Without a good electrical connection the electrocardiogram would show incomplete synchronous activity. The cell source also remains an unresolved challenge.

There are inadequate local repair mechanisms within the heart to deal with physical and free radical damage following ischemic injury or mechanical overload. This problem is exacerbated by a wave of programmed death (apoptosis) of susceptible myocytes resulting in contractile dysfunction (Cheng, 1996; Kajstura, 1996). As in many cell types, IGF-I can inhibit apoptosis (Muta, 1993; Rodriguez, 1992; Beurke, 1995) and thus, delivery of IGF-I to the stressed heart may prevent myocyte cell death and improve cardiac function. However, data from animal studies have been equivocal, and data from human clinical trials have not demonstrated any significant effects of either administration of the mature IGF-I or growth hormone (Deurr, 1996; Fazio, 1996). This result may not be too surprising since there is no evidence showing increased secretion of growth hormone or elevation of systemic IGF-I levels following myocardial infarcts. Furthermore, there are limitations to this approach due to the bioavailability in the heart because IGF-I binds very rapidly to proteins in the systemic circulation. However, over-expression of the IGF-I gene in the heart has proven beneficial in eliciting cardiac hyperplasia (Reiss, 1996), inhibiting apoptosis (Li, 1997) and preventing dilation in a transgenic mouse model of cardiomyopathy (Welch, 2002). Consequently, these salutary effects were attributed to the IGF-I produced by the muscle even though the cDNAs used could not be spliced to produce muscle specific isoforms.

Thus there exists a need in the art to develop materials and methods for improving tissue regeneration in not only cardiac tissue but other tissue as well.
Summary of the Invention

[0007] The present invention provides a method for repairing damaged tissue comprising the step of administering a composition comprising non-degradable or biodegradable nanoparticles (NP) associated with a releasable growth factor and a carrier in an amount and over a time effective to repair damaged tissue. In certain aspects, the growth factor is releasable over a predetermined amount of time.

[0008] In one embodiment, the nanoparticle is inert. In other embodiments, the nanoparticle is porous. In various embodiments, the carrier is aqueous, saline or a buffer.

[0009] In certain aspects, the nanoparticle is synthesized from one or more phospholipids, one or more polymers, or one or more copolymers. In one aspect, the polymer is polylactic acid (PLA), polyglycolic acid (PGA), or poly(e-caprolactone) (PCL). In another aspect, the copolymer is poly(lactide-co-glycolide) (PLGA) or poly(lactide-co-glycolide) poly(e-caprolactone) (PLGA/PCL).

[0010] In one embodiment, the nanoparticle is on average, between 1 nm and 1,000 nm in diameter.

[0011] In another embodiment, the growth factor is a protein in the insulin-like growth factor (IGF) family of proteins. In still another embodiment, the growth factor is an isoform of the IGF family of proteins. In another aspect, the growth factor is an E-domain peptide of the IGF family of proteins. In still another aspect, the growth factor is an Ea, Eb, or Ec domain peptide of the IGF family of proteins. In yet another aspect, the growth factor is mechano-growth factor (MGF).

[0012] In various embodiments, the MGF is stabilized or native. In other embodiments, the growth factor is an E-domain peptide of MGF or a biologically active fragment of MGF.

[0013] In one aspect, the growth factor is intercalated within said nanoparticle. In another aspect, the growth factor is covalently attached to said nanoparticle through a cleavable covalent linkage.

[0014] In one embodiment, the growth factor is associated with the nanoparticle at a surface density of between 10 pmol/cm² and 75 pmol/cm².
In other embodiments, the composition is administered in a single dose or a plurality of doses over a period of time. In still other embodiments, the composition is administered by injection into coronary vasculature. In another embodiments, the composition is administered by injection into the ventricular wall.

In one embodiment, the damaged tissue is muscle tissue or arises from a muscular disorder. In another embodiment, the damaged tissue is cardiac tissue, cardiac muscle tissue or arises from trauma. In still another embodiment, the damaged tissue is neuronal tissue or arises from a neurological disorder. In another aspect, the damaged tissue is neuroendocrine tissue or arises from a hormonal disorder such as diabetes.

In various aspects, the damaged tissue is selected from the group consisting of skeletal, bone, tendon and connective tissue or arises from growth abnormalities, osteoporosis, fractures, and ischemic damage due to peripheral vascular disease.

**Brief Description of the Drawings**

[0018] Fig 1. shows monodisperse, aggregate-free, nanoparticles.

[0019] Fig 2. shows tight control over processing parameters.

[0020] Fig 3. shows encapsulation efficiency.

[0021] Fig 4. shows peptide release control over several days.

[0022] Fig 5. shows NRVM grown on MGF E-domain eluting sheets.

[0023] Fig 6. shows immature ES-CMs grown on flat surface are able to divide.

[0024] Fig 7. shows mouse stem cells and MGF.

[0025] Fig 8. shows quantification of MGF and IGF-I isoform expression in the mouse heart following myocardial infarct using real time RT-PCR.

[0026] Fig 9. shows pressure-volume loops derived from instrumented mice and typical hemodynamic parameter derived from the P-V loop analysis.

[0027] Fig 10. shows FACS analysis of myocytes.

[0028] Fig 11. shows expansion of stem cells in mouse heart with MGF treatment after myocardial infarction.
Fig 12. shows FACS analysis of the side population after MGF treatment.

Fig 13. shows results from treatment of infarcted sheep with MGF.

**Detailed Description of the Invention**

Integrating the E-domain of MGF in a nanoparticle (NP) peptide delivery device offers many advantages as an innovative cardiac therapy. Although peptide therapy is the treatment of choice for many diseases, innovations in NP-based delivery – an area that integrates bioengineering, nanotechnology, materials science and cell biology – shows promise to revolutionize the treatment of organ specific diseases. It is advantageous to design drug and peptide delivery systems that reproducibly and safely carry drugs to their point of action at the required level, thus controlling the pharmacokinetic parameters.

The present invention provides a method for repairing damaged tissue by administering a composition of a nanoparticle associated with a releasable growth factor in an amount and over a time effective to repair target damaged tissue. In various embodiments of the invention, the growth factor is releasable over time or over a predetermined amount of time.

In certain instances, nanoparticles that are inert are beneficial. Alternatively, nanoparticles can be synthesized to be porous as a means to increase surface area. Also, depending on their intended use, nanoparticles are either non-degradable, partially biodegradable, wholly biodegradable, or combinations and/or mixtures thereof.

In various aspects, the nanoparticles are synthesized from one or more polymers, one or more copolymers, one or more block polymers (including di-block polymers, tri-block polymers, and/or higher multi-block polymers), as well as combinations thereof. Useful polymers include but are not limited to poly(methyl methacrylate), polylactic acid, polyglycolic acid, poly(lactide-co-glycolide), polycaprolactone, and elatin/caprolactone, collagen-GAG, collagen, fibrin, poly(anhydrides), poly(hydroxy acids), poly(ortho esters), poly(propylfumerates), polyamides, polyamino acids, polyacetals, biodegradable polycyanoacrylates, biodegradable polyurethanes and polysaccharides, polypyrrol, polyanilines, polythiophene, polystyrene, polyesters, non-biodegradable polyurethanes, polyureas,
poly(ethylene vinyl acetate), polypropylene, polyethylene, polycarbonates, poly(ethylene oxide), polydioxanone, "pseudo-polyamino acid" polymer based on tyrosine, tyrosine-derived polycarbonate poly(DTE-co-DT carbonate), polyethylene, tyrosine-derived polyarylate, polyanhydride, trimethylene carbonate, poly(β-hydroxybutyrate), poly(g-ethyl glutamate), poly(DTH iminocarbonate), poly(bisphenol A iminocarbonate), poly(ortho ester), polycyanoacrylate, and polyphosphazene, a modified polysaccharide (cellulose, chitin, dextran) a modified protein, casein- and soy-based biodegradable thermoplastics, collagen, polyhydroxybutyrate (PHB), multiblock copolymers of poly(ethylene oxide) (PEO) and poly(butylene terephthalate) (PBT), polyrotaxanes (which are polymers comprising cyclic compounds that are threaded onto linear polymeric chains capped with bulky end groups). In other aspects, the microrods are formed from one or more phospholipids. 2-methacryloyloxyethyl phosphorylcholine (MPC), one or more cationic polymers (poly(a-[4-aminobutyl]-L-glycolic acid), or one or more silicone-urethane copolymers. In still other aspects, microrods in the scaffold are formed from co-polymers of any of the above, mixtures of the above, and/or adducts of the above. The worker of ordinary skill will readily appreciate that any other known polymer is suitable for making nanoparticles of the instant compositions.

[0035] Poly(oactide-co-glycolide) (PLGA) is an FDA-approved biocompatible and biodegradable copolymer of poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) that can be formulated into nanoparticles. PLGA for drug delivery usually consists of the amorphous racemer D,L-PLA, which allows for more homogeneous drug dispersions (Jain 2001). The PLA:PGA monomer ratio dictates the physical and chemical characteristics of the copolymer. For example, the PLA methyl group (CH3) renders the copolymer more hydrophobic and less crystalline than PGA, and thus it degrades slower.

[0036] During PLGA degradation, the ester bonds of the polymer backbone are hydrolyzed to form soluble oligomers and monomers that then enter the citric acid cycle. The degradation rate depends on the PLA:PGA ratio, and the fastest PLGA microsphere degradation occurs with 50:50 ratios (Shive, 1997). For delivery of a day or more, PLGA is an ideal material for devices because it is FDA approved, can release incorporated drug molecules by diffusion and by matrix degradation, and its by-products are non-toxic. PLGA nanoparticles for drug delivery are interesting
because they are biocompatible, allow controlled release and encapsulation of several types of therapeutic molecules (small molecules, proteins, peptides, DNA) (Soppinath, 2001). Encapsulating potent drug molecules within the NP protects poorly soluble and unstable drugs from the biological milieu. PLGA nanoparticles are also small enough for capillary penetration, cellular internalization and endosomal escape (Panyam, 2002; Muller, 2004; Brannon-Peppas, 2004; Panyam, 2003; Kumar, 2001), so intracellular targets are readily accessible to released molecules. Thus, MGF-loaded PLGA nanoparticles are an exciting novel application of bionanotechnology.

[0037] Because nanoparticles of the invention are synthesized, physical properties of the nanoparticles can be designed and controlled. In one aspect of the composition, nanoparticles are on average, each about 0.01, about 0.05, about 0.1, about 0.5, about 1, about 5, about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 55, about 60, about 65, about 70, about 75, about 80, about 85, about 90, about 95, about 100, about 105, about 110, about 115, about 120, about 125, about 130, about 135, about 140, about 145, about 150, about 155, about 160, about 165, about 170, about 175, about 180, about 185, about 190, about 195, about 200, about 205, about 210, about 215, about 220, about 225, about 230, about 235, about 240, about 245, about 250, about 255, about 260, about 265, about 270, about 275, about 280, about 285, about 290, about 295, about 300, about 305, about 310, about 315, about 320, about 325, about 330, about 335, about 340, about 345, about 350, about 355, about 360, about 365, about 370, about 375, about 380, about 385, about 390, about 395, about 400, about 405, about 410, about 415, about 420, about 425, about 430, about 435, about 440, about 445, about 450, about 455, about 460, about 465, about 470, about 475, about 480, about 485, about 490, about 495, about 500, about 505, about 510, about 515, about 520, about 525, about 530, about 535, about 540, about 545, about 550, about 555, about 560, about 565, about 570, about 575, about 580, about 585, about 590, about 595, about 600, about 605, about 610, about 615, about 620, about 625, about 630, about 635, about 640, about 645, about 650, about 655, about 660, about 665, about 670, about 675, about 680, about 685, about 690, about 695, about 700, about 705, about 710, about 715, about 720, about 725, about 730, about 735, about 740, about 745, about 750, about 755, about 760, about 765, about 770, about 775, about 780, about 785, about 790, about 795, about 800,
about 805, about 810, about 815, about 820, about 825, about 830, about 835, about 840, about 845, about 850, about 855, about 860, about 865, about 870, about 875, about 880, about 885, about 890, about 895, about 900, about 905, about 910, about 915, about 920, about 925, about 930, about 935, about 940, about 945, about 950, about 955, about 960, about 965, about 970, about 975, about 980, about 985, about 990, about 995, or about 1000 or more nanometers in diameter.

[0038] In various aspects the composition comprising the nanoparticles also includes a carrier. The nature of the carrier, if present, is in certain aspects dictated by the intended use of the nanoparticles, and in other aspects, the means by which the nanoparticles are placed in a target tissue. In various aspects, therefore, the carrier is simply water, and in one aspect pharmaceutical grade water. In other aspects, the carrier is a buffer, and in certain aspect, the buffer is pharmaceutically acceptable. Buffers in the composition include, but are not limited to, saline, glycine, histidine, glutamate, succinate, phosphate, acetate, aspartate, or combinations of any two or more buffers. The worker of skill in the art will appreciate any pharmaceutically acceptable buffer is contemplated for use with the NP as a carrier.

[0039] In various embodiments, the composition comprising the nanoparticles also includes a growth factor. In one aspect, the growth factor is a protein in the IGF family of proteins. In one embodiment, the growth factor is mechano-growth factor (MGF) or a biologically active fragment thereof, including, but not limited to a MGF E-domain peptide or a biologically active fragment thereof. In yet another aspect, the IGF E-domain peptide is the Ea, Eb or Ec domain or a biologically active fragment thereof.

[0040] The E-domain of MGF is produced by the heart within days after stress but not bound by circulating binding proteins and thus is available to potentiate the actions of local IGF-I. MGF is up-regulated in skeletal muscle under conditions of increased growth (Yang 1996, 2002; McCoy 1999), and it is a splice variant of IGF-I only produced by the tissue at times of stress. While IGF-I functions as an endocrine factor secreted by the liver, it also functions as a paracrine/autocrine growth factor expressed in non-hepatic tissues mediating regenerative processes thus having the potential for undesirable effects for therapeutic application (Russell, 1985; Vetter, 1986). Multiple IGF-I isoforms are expressed in different tissues that arise by alternate splicing from a common translated preprohormone. The preprohormone is
cleaved by cellular endoproteases leaving the prohormone (mature 70 amino acid peptide + E-domains), which is further cleaved to yield identical mature peptides from all isoforms but different E-domains (Foyt, 1991). One isoform, the IGF-IEa, has a similar structure to the major endocrine form produced by the liver and has been given various abbreviations including (confusingly) muscle-liver type IGF-I and muscle IGF-I. Another, IGF-IEb, or MGF is produced in stressed skeletal muscle. In the rodent, MGF (IGF-IEb) has a 52 base pair insert from exon 5, which causes a reading frame shift at the carboxyl end encoding a unique 24 amino acid E-domain which distinguishes it from the predominant IGF-IEa isoform (Shimatsu, 1987). Note, rodent Eb is named Ec in humans. Stabilized MGF E-domain is chemically modified to increase the molecule's stability, preserve its activity, or resistance to degradation. Conversely, native MGF E-domain refers to non-stabilized or not otherwise modified MGF.

[0041] In various aspects, the growth factor is associated with the nanoparticles by covalent interaction, and in other aspects the growth factor is associated with the nanoparticles by non-covalent association. In a covalent interaction, one or more growth factors are directly attached to the nanoparticles through any suitable means. Alternatively, one or more growth factor is attached to the nanoparticle through a space or linker that has no biological activity itself, or through a second growth factor which possesses the same or a different biological activity compared to the first growth factor. In still another aspect, the growth factor is elutable from the NP. Elutable in various aspects means that the growth factor can be separated from the nanoparticles through, for example, simply diffusion, cleavage of a covalent bond, dissociation or some other type of interaction. The growth factor, in various aspects, is released in a controlled manner and in other aspect, the release is bolus in nature.

[0042] In one embodiment of the invention, the growth factor is associated with the nanoparticle at a surface density of at least 10 pmol/cm², at least 15 pmol/cm², at least 20 pmol/cm², at least 25 pmol/cm², at least 30 pmol/cm², at least 35 pmol/cm², at least 40 pmol/cm², at least 45 pmol/cm², at least 50 pmol/cm², at least 55 pmol/cm², at least 60 pmol/cm², at least 65 pmol/cm², at least 70 pmol/cm², or at least 75 pmol/cm².

[0043] Several traditional and novel fabrication methodologies easily achieve the sub-micron size constraint for polymeric nanoparticles. A major advantage of polymeric nanoparticles over microparticles is their enhanced cellular uptake (Desai
Many cell types take up PLGA nanoparticles in a concentration, size and surface coating-dependent manner (Bivas-Benita, 2004; Elamanchili, 2004; Win, 2005). This enhanced uptake, receptor-mediated endocytosis, and the ability to escape the endosomal pathway (Panyam, 2002) may enhance the efficacy of drugs that have cytoplasmic target. Loading into PLGA nanoparticles and subsequent release through diffusion and polymer erosion are important parameters that ultimately determine the utility of PLGA nanoparticles as drug delivery devices. As in drug-loaded PLGA nanoparticles, our MGF peptide molecules in the PLGA NP core are protected from enzymatic and chemical degradation until they are released from the matrix. This protection within the NP permits the delivery of insoluble drugs and, most importantly, increases the chances that our otherwise unstable peptide can be delivered to its site of action. Finally, PLGA's biocompatibility and degradation into natural by-products is not affected by its formulation into nanoparticles, so PLGA nanoparticles may safely release encapsulated drugs or peptides over a long period of time (Sahoo, 2003). Thus, PLGA nanoparticles constitute promising biocompatible delivery devices because they protect the peptide from the biological environment and enable sustained therapeutic release to intra- and extra-cellular targets.

In other embodiments of the invention, the NP composition is administered in a single dose or in a plurality of doses over a period of time. Other aspects, compositions comprising the nanoparticles of the invention are designed based on the intended use of the nanoparticles, for example, with respect to the target tissue or cell type and/or the route or means of administration. In certain aspects, the route or means of administration is an overriding factor is preparation of the scaffold or a composition comprising a NP. Administration of the NP compositions may be accomplished in a number of ways including, but not limited to, surgical implantation, injection, injection into coronary vasculature or into the ventricular wall, parenteral delivery, including intramuscular, subcutaneous, intramedullary as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, intraocular, intestinal administration, topical, inhalation, transdermal, transmucosal, buccal, pulmonary, sublingual, oral, rectal or vaginal. Osmotic mini-pumps and timed-released pellets or other depot forms of administration may also be used.
[0045] Methods are also contemplated by the present invention. In one embodiment, a method for repairing damaged tissue comprising the step of administering an aforementioned composition to damaged tissue in an amount and over a time effective to stimulate tissue repair, is provided. Methods of the invention are useful for the treatment of disorders such as, but not limited to, muscular disorders such as, but not limited to all forms of muscular dystrophy, atrophy, cachexia, sarcopenia and weakness of urethral sphincter muscle. In another aspect, the damaged tissue is cardiac tissue, wherein the method are useful for the treatment of, among other disorders, cells damaged by trauma, cardiomyopathies, ischemic cardiac injury, myocardial infarction, congestive heart failure, coronary artery disease or other damage. In yet another aspect, the damaged tissue is neuronal tissue, and methods are useful for the treatment of, among other disorders, neurological disorders (loss of neurons due to damage or maintenance of CNS, stroke), and neurodegenerative disorders (ALS, Parkinson's Alzheimer's). In still another aspect, the damaged tissue is neuroendocrine tissue, and methods of the invention are useful for the treatment of, among other disorders, hormonal disorders (diabetes). In yet another aspect, the damaged tissue is skeletal, bone, tendon and connective tissue,. And methods of the invention are useful for the treatment of, among other disorders, growth abnormalities, osteoporosis, and fractures.

[0046] Ischemic damage due to peripheral vascular disease affects many of the tissues in body mentioned above, as well as others. All tissues damaged by ischemic disease would benefit from treatments with the invention.

[0047] The invention provides methods for use of the NP compositions for veterinary uses. In animal husbandry, the administration of drugs to animals involves a great deal of handling which is laborious for the handler and stressful for the animals. Often animals must be brought in from long distances making prolonged or frequent treatment difficult if not prohibitive. In some cases the stress levels caused by handling stock can impair the performance of the treatment, this is particularly true in deer. Many drugs are given in slow release capsules that can cause problems when still remaining in the animal at slaughter. (United States Patent 6,669,682)

[0048] Many formulations are designed for long release to reduce stock handling requirements but concentrations often fall below the effective therapeutic levels long before the next dose is administered thus increasing the risk of drug resistance.
Bacterial drag resistance is a growing problem that affects both animals and humans and commonly arises from the ineffective administration of drug treatments. Parasite drug resistance is now a major problem particularly with anti-parasite drugs such as anthelminthics. (United States Patent 6,669,682)

[0049] Accordingly, the present invention contemplates uses of the NP compositions for growth factor delivery in veterinary applications.

[0050] The condition of skin tissue is affected by factors such as humidity, ultraviolet rays, cosmetic compositions, aging, diseases, stress and eating habits. As the result, various skin troubles can arise. The skin also becomes less resilient with age as illustrated by the formation of wrinkles. Aging is generally associated with the thinning and general degradation of skin. As the skin naturally ages, there is a reduction in the number of cells and blood vessels that supply the skin. There is also a flattening of the dermal-epidermal junction that results in weaker mechanical resistance of this junction. As a consequence, older persons are more susceptible to blister formation in cases of mechanical trauma or disease processes. (United States Patent 7,196,162).

[0051] The skin also contains an elaborate network of elastin fibers that is responsible for maintaining its elastic properties. With excessive exposure to sunlight the elastic fiber system becomes hyperplastic, disorganized and ultimately disrupted. This process is known as actinic elastosis and it is the principal cause of wrinkling, discoloration and laxity of the skin in the exposed areas of the body. As new fibroblasts, endothelial cells and keratinocytes form, the skin can repair itself. However, the skin becomes less able to do so as it ages. Therefore, agents that can accelerate the growth and repair of prematurely aged skin are needed. (United States Patent 7,196,162)

[0052] Accordingly, the invention contemplates methods for treating symptoms of aging where the NP compositions are administered in an amount and over a time effective to reverse or treat the symptoms of aging.

[0053] The compositions of the invention can also be used to treat anabolic disorders including but not limited to, andropause, adipoprogenital syndrome, functional metrorragia, fibroma and endometriosis as well as asthenia, osteoporosis, senescence and metabolic perturbations after prolonged treatment with
corticotheraphy. Methods are contemplated for treating anabolic disorders by
administering an anabolically effective amount of the microrod scaffold over a time
effective to reverse the effects of the anabolic disorder (United States Patent
4,431,640).

Example 1

Release mechanism of MGF-loaded E-domain Nanoparticles in vitro

[0054] In recent years, several PLGA NP synthetic techniques have been
developed. However, only a limited number of techniques are suitable for the
encapsulation of drugs and peptides, particularly those that are poorly water-soluble.
In addition, achieving adequate peptide loading and physiological kinetics has been
non-trivial. MGF is hydrophobic and thus soluble in organic solvent — acetonitrile —
as opposed to water. Therefore, a simple method to encapsulate this peptide in <200
nm, uniformly sized nanoparticles is desirable. Nanoprecipitation (interfacial
deposition, solvent diffusion) is an NP synthesis method that is relatively simple to
perform and yields appropriately sized particles (Fessi, 1989). The nanoprecipitation
method is advantageous because the surfactants, high shearing rates and toxic solvents
required in multiple emulsion-based techniques are not necessary (Bilati, 2005;
Panyam, 2003). Surfactants, while not required, are included during
nanoprecipitation primarily to enhance the stability of the nanoparticles (Dong, 2004).
Nanoprecipitation is based on the interfacial turbulence between unequilibrated liquid
phases that results from surface tension gradients, a phenomenon known as the
Marangoni effect (Jelcic, 2004). Although nanoprecipitation approaches have been
described previously, the use of this method to deliver and target small peptides to the
cardiocascular system in vivo has not been investigated.

[0055] To form nanoparticles, the polymer and MGF are dissolved in a water-
miscible solvent (S), typically acetone or acetonitrile and this solution is then
dispersed into a stirred, aqueous non-solvent (NS). The concentration gradient of the
S at the liquid interface induces interfacial turbulence and surface tension gradients,
eventually resulting in nanoscale emulsion droplets (Murakami 2000). Subsequently,
there is rapid mutual diffusion of the S and NS into and out of the droplets coupled
with solvent evaporation. The diffusion significantly decreases the droplet sizes and
PLGA solubility, thus causing the polymer to precipitate and form nanoparticles
(Choi, 2002). Drugs or proteins dissolved in the S are incorporated in the precipitated PLGA nanoparticles. This process routinely results in biodegradable nanoparticles <200 ran diameters with narrow distributions. Due to its simplicity and efficiency, we modify the nanoprecipitation technique to formulate MGF-PLGA nanoparticles as therapeutic delivery devices. Recently, we have refined the nanoprecipitation method to encapsulate hydrophobic small molecules or peptides (Nehilla and Desai, NSTI conference, 2006). This is a novel approach that may have significant therapeutic implications in terms of peptide stabilization and targeting.

[0056] Much of the focus on nanoprecipitation has been in the formulation of poly(ester) (e.g., PLA, PGA, PLGA) and PEG-poly(ester) nanoparticles. PEG is a highly hydrophilic, biocompatible polymer that acts like a surfactant and resists protein adsorption (Uhrich, 1999). PEG-poly(ester) copolymers result in 'stealth' nanoparticles with the poly(ester) core surrounded by surface-exposed PEG chains. This structure inhibits opsonization and recognition by body defense systems due to steric repulsion and PEG's 'brush-like' motions (Soppimath, 2001; Dong, 2004). The amphiphilic nature of these PEG-polyester copolymers eliminates the need for surfactants in some NP synthetic techniques. Although opsonization is not a major concern for the in vitro cell model, injection of nanoparticles to the human heart may require pegylation for stabilization. Therefore, the use of PLGA-PEG chains or surfactants in the nanoprecipitation method is initially avoided, but have the capability to do so.

[0057] MGF is poorly water-soluble and rapidly metabolized, which severely limits the peptide efficacy if injected directly. If MGF can be protected from rapid degradation in a PLGA NP, appropriately presented to the cell of interest, and delivered with physiological release kinetics, then such an approach can be useful for targeted MGF therapy. Alternative routes of MGF administration are limited due to hydrophobicity and negligible bioavailability. While numerous NP approaches have been developed, these approaches can reduce MGF bioactivity due to harsh solvents and processing times, and often result in poor encapsulation efficiencies due to the chemical structure and properties of MGF. The NP size allows cells to internalize them so that the MGF payload is in close proximity to intracellular enzymes and structures. Thus, PLGA nanoparticles provide the ability to better modulate intracellular targets involved in cellular regulation and function.
A. Synthesis of MGF E-domain peptide PLGA Nanoparticles via the nanoprecipitation method

[0058] Although nanoprecipitation simply involves the mixing of different solutions, the formulation details must be optimized for nanoparticles of the appropriate size, stability, encapsulation efficiency and MGF release. Microspheres synthesized from low molecular weight PLGA chains with free carboxyl end groups ("uncapped") degrade faster than those containing high molecular weight, capped PLGA chains (Tracy 1999). Also, the PLA:PGA copolymer ratio influences the final NP size and drug release rate (Fonseca 2002). With these formulation aspects in mind, the NP size and MGF encapsulation is optimized by using different ratios (50:50 and 85:15) of low molecular weight, uncapped PLGA for nanoprecipitation. PLGA is soluble in water-miscible (acetone) and organic (ethyl acetate, dimethyl formamide) solvents. Ubiquinone is a ringed compound with a long hydrocarbon side chain so its solubility and hydrophobicity is similar to that of PLGA. Nanoprecipitation of PLGA occurs in NS, like DI water, alcohols or mixtures of these two (Redhead 2001, Govender 1999). In addition to the PLGA copolymer ratio, the NP size and encapsulation efficiency depend on the chemical characteristics of the S and NS, the S/NS ratio, the amount of PLGA and MGF in the S and the presence of surfactants in the NS (Redhead 2001, Fonseca 2002). Therefore, these formulation parameters can also be manipulated for optimal NP sizes and MGF loading. PLGA nanoparticles have been synthesized via nanoprecipitation without surfactants, but we may add poloxamers or poloxamines (PPO and PEO copolymers) to the NS in order to stabilize the nanoparticles and prevent sedimentation or precipitation (Chorny, 2002; Bilali, 2005; Redhead, 2001). However, our preliminary work indicates that precipitation is not a problem. Growth factor nanoscale therapeutic delivery devices should be feasible by modifying the nanoprecipitation formulation parameters as our preliminary data illustrates.

B. Characterization of the biophysical properties of the Nanoparticles

[0059] Physical characterization of the PLGA nanoparticles is necessary to evaluate the synthesis efficacy. Commonly reported NP parameters include particle size, distribution, morphology and polydispersity index. These properties are analyzed in this work by dynamic light scattering with a particle size analyzer. In addition, tapping-mode topography atomic force microscopy (AFM) has been used to
examine the diameter, height and morphology of PLGA nanoparticles (Dong, 2004; Mu, 2003). Topography and phase mode AFM are also performed for more complete physical characterization of the PLGA nanoparticles, which are adsorbed on atomically flat mica substrates. Phase AFM is an underutilized technique that offers enhanced contrast of surface details for improved imaging of NP morphology (Nehilla, 2005). By combining light scattering and AFM, the physical properties of the PLGA nanoparticles may be quickly and completely characterized.

C. Encapsulation efficiency, MGF E-domain loading and acellular release kinetics

[0060] The incorporation (encapsulation) efficiency measures the amount of peptide loaded into PLGA nanoparticles in relation to the initial amount of peptide in the solvent during NP synthesis. To measure the encapsulation efficiency, MGF-loaded PLGA nanoparticles are solubilized in appropriate solvents, which liberate the incorporated MGF molecules. The released MGF is analyzed using a customized Enzyme Linked Immunosorbent assay (ELISA). This parameter is necessary in order to calculate the equivalency of NP and MGF doses.

[0061] To measure growth factor release in a cell-free system, MGF-loaded PLGA nanoparticles are typically mixed in aqueous solutions (Chorny, 2002; Dong, 2004) or dialysis bags (Liu, 2005; Yoo, 2001), and the released MGF molecules in solution are analyzed appropriately. The MGF-loaded PLGA nanoparticles are suspended in PBS, collect the supernatant and measure the MGF released over time by ELISA, as described above. A FITC-conjugated MGF is also tested which can be visually observed in the nanoparticles and measured spectrophotometrically. If MGF is released rapidly and independent of PLGA degradation, the MGF may be directly conjugated to PLGA chains before NP synthesis. Conjugation of proteins to PLGA chains has been used to improve encapsulation efficiency and prolong drug release (Yoo, 2001).

[0062] The aforementioned methods have been employed as follows.

Monodisperse, aggregate-free, Nanoparticles

[0063] Carboxylic-acid end group, PLGA nanoparticles were successfully synthesized via nanoprecipitation. In these formulations, nearly monodisperse populations of nanoparticles with diameters between 100 nm and 200 nm were
achieved. AFM images suggested spherical NP morphology (Fig 1). NP sizes were modulated by varying formulation parameters, such as the PLGA mass in acetone and the solvent:non-solvent volumetric ratio. Acetonitrile had low background UV absorbance and that it can dissolve the lyophilized nanoparticles.

**Control over processing parameters**

Our process allows for control over NP diameter and stability (Fig 2). Higher inherent viscosity PLGA resulted in significantly larger and more heterogeneous nanoparticles, p<0.05. The pH of the aqueous non-solvent did not affect the nanoparticles, P<0.05. Also, there was no significant difference in the diameter or polydispersity of nanoparticles synthesized in acetone and acetonitrile.

To calculate the yield, encapsulation efficiency and loading, nanoparticles were separated from unencapsulated drug and free PLGA by ultracentrifugation. The NP pellet was washed, sonicated and lyophilized. The mass of the NP powder was measured before dissolving the nanoparticles in acetonitrile for absorbance measurements.

**Encapsulation efficiency of greater than 50% for hydrophobic drugs**

Figure 3 shows the effect of solvent on encapsulation efficiency of a hydrophobic drug in PLGA-COOH nanoparticles. Due to the higher encapsulation efficiency and because it is compatible with MGF, acetonitrile was chosen as the solvent of choice for future experiments. Using acetonitrile, encapsulation efficiencies of 60 to 80% were typically achieved. This meant that for an initial drug concentration of 1 mg/ml, MGF loading of -500 μg/ml nanoparticles was achieved.

**Peptide release was controlled over a period of several days.** Figure 4 shows a typical release profile from peptide-containing nanoparticles in a biopolymer matrix. The cell culture assays confirm a functional effect of the MGF released from the MGF loaded polymeric films.
Example 2

Beneficial effects from MGF release on cultured cardiac cells and stem cells in environmental conditions that mimic the failing or infarcted heart

[0067] In order to confirm the bioavailability and biological action of localized MGF NP delivery to neonatal rat ventricular myocytes (NRVM) and mouse embryonic stem cells (mES), both the natural and a stable form of the MGF E-domain, 24 amino acids, that act as a paracrine/autocrine growth factors for muscle are employed. It is likely that MGF has additional benefits in poorly oxygenated regions in addition to the normal physiologic conditions that are present in the well-perfused regions of the ventricle. Therefore, the prototype PLGA nanoparticles on NRVM is also tested under hypoxic conditions. Thus, the conditions present in the ventricle in vitro are mimicked, such as cell type, temperature, oxygenation and composition of the media.

Beneficial effects to myocytes of MGF release from Nanoparticles

[0068] Natural or stable MGF is first loaded into prototype PLGA nanoparticles, and then added to a Petri dish of normal, or hypoxic NRVM in order to model cardiac ischemia, and assess eventual effectiveness for the heart. The maximum load MGF that each <200nm NP can carry will be used, varying the number of nanoparticles delivered. A monolayer of NRVM is grown and introduced to variable quantities of the natural or stable MGF-loaded nanoparticles of different formulations into the supernatant media, with the necessary controls. Empty nanoparticles are used to control for cytotoxicity by the PLGA vehicle. Use of the peptide in solution or delivered via the NP permits evaluation of the effect of the time-release properties of the NP MGF level. Dishes without cells are used to confirm characteristic release properties after storage. NRVM are harvested at day 1 or day 7 for assays of gene and protein levels to test for gene expression, apoptosis and proliferation by biological evaluation methods described below. Five different cell isolations are used for statistical evaluations.
NRVM culture and NP loading of MGF

[0069] The NRVM are isolated from the newborn rat ventricles, plated and allowed two days to adjust and re-establish spontaneous beating (Boateng, 2003). 100ng/ml bolus of stabilized or natural MGF E-domain peptide is first pipetted directly to the media, or the required number of nanoparticles loaded is added to attain a similar total load. Media is not removed so any accumulated MGF stays in the dish but fresh media is added at day 3 to keep the cells healthy. Nanoparticles are distributed in varying quantities to approximately two million NRVM per dish in culture. Calculations and pilot data suggest that a ratio of 1 to 10 nanoparticles per myocyte is expected to be sufficient. To induce hypoxic conditions, cells are incubated in a humidified chamber (Billups-Rothenberg Inc) with 5% CO₂, 1% O₂ and the remainder balanced with N₂ for 12 hrs at 37°C. Thus, for a single bolus of native or stable MGF, results are compared between simple addition of MGF to the media or with time-release MGF delivery from nanoparticles.

Time profile

[0070] The natural MGF peptide degrades rapidly in vitro with the majority being lost by 30 minutes and all by two hours in plasma at 37°C. Thus, the natural MGF delivered in vivo would degrade before it reached its destination in underperfused regions of the heart. FITC labeled MGF in the nanoparticles is used to characterize the time course of MGF release. Fluorometric measurements of FITC levels in the supernatant media are taken every hour for 6 hours and less frequently for the next 24 hours. During this time the media is not changed. As a control, nanoparticles alone are used to confirm the profile of MGF release seen in the acellular experiments described above. Thus, time profiles are collected for natural or stable MGF-nanoparticles with and without NRVM growing. This permits determination of the effect of NRVM on the MGF level that is equivalent to the interstitial spaces of the heart.
Tracking of NP distribution within NRVM

MGF normally crosses into the myocyte and acts internally. Nanoparticles may stay in the media, settle on the surface of the cells, or be internalized yet MGF is released regardless of their location and is effective in producing cellular responses in vitro. However, only those in plasma degraded close to the cell, adhered to the surface, or internalized are comparable to the therapeutic delivery we seek. Thus, it is necessary to characterize endocytosis and the adherence to the cell surface. Quantum dots (QD) are used as indicators of NP behavior to track their density and location inside or on the NRVM with confocal microscopy. 5-15 run QDs (ITK Carboxyl, 525-green or 655-red, Invitrogen) are applied to cardiomyocytes in varying concentrations in order to investigate the binding, trafficking and distribution of these nanoparticles over a 24 hour time period, sampling at 1,3,6,12, and 24 hours using the Z-stack feature of the LSM confocal scope to assess QD number (brightness) within and on the upper membranes of the NRVM. QDs readily enter NRVM in large numbers over a few hours.

Biologic evaluations are assessed at day 1 and day 7 after MGF addition to detect short term and long-term effects. Sensitive analytical techniques (described in the following subsections) are selected that provide well-established indices of cardiac responses.

Physiological growth

Quantitative real time RT-PCR is used to assess changes in muscle gene expression normalized to the housekeeping genes (such as GAPDH or L7) with SYBR Green detection in the LightCycler thermocycler (Roche Diagnostics). Total RNA is extracted from cells using Trizol and 100 ng of total RNA is used in each RT-PCR reaction. Quantification of the RT-PCR reaction is based upon a series of in vitro transcribed mRNA standards prepared for each gene and run along side to develop a standard curve as previously published (Goldspink, 2004). Primers for both the alpha and beta myosin heavy chains are used as indices of contractile protein expression, and the α-subunit of L-type calcium channel for rhythmicity of beating.
Pathophysiological growth

[0074] Overload of the heart triggers the release of the atrial natriuretic factor (ANF) that the circulation takes to the kidney where excretion of water and salt is regulated to decrease blood volume, and cardiac load. Thus, ANF upregulation is concomitant with cardiac stress, and is the most widely used index of overload leading to hypertrophy and failure. RT-PCR is used to monitor niRNA levels of ANF.

Apoptosis

[0075] Overstress to cardiac cells initiates the undesirable pathways for apoptosis that is protected by members of the IGF-I family. The effect of MGF on enhanced survival and recovery is assessed by using Western blots. A number of different acrylamide/bisacrylamide ratios are used to optimally separate the extracted proteins. Proteins are transferred to nitrocellulose and probed with specific antibodies using standard western blotting techniques (Goldspink 2004). Antibodies for activated Caspase-3 C (18kD cleavage fragment), Bax and Bcl-2 (Cell Signaling) are used.

Cytotoxicity

[0076] Propidium iodide (PI) can be used to assess dead cells. PI intercalates into double-stranded nucleic acids. It is excluded by viable cells but can penetrate cell membranes of dying or dead cells. PI is dissolved in buffer at a concentration of 1 μg/ml. To assess viability of cell sample, 2 μL of the PI stock solution are added to each well and mixed well. Samples are kept in solution at 4°C protected from light until analysis on the flow cytometer.

Beneficial effects to cardiac stem cells of MGF E-domain release from Nanoparticles

[0077] Since IGF-I has a potent effect on many stem cells, particularly those in skeletal and cardiac muscle, it is likely that MGF, its family member, is also active. Therefore MGF E-domain is tested on mouse embryonic stem cells from the well-established R1 ES cell line that can be grown indefinitely to derive a heterogeneous cell population, including cardiomyocytes (ESCM), see reviews (Boheler, 2002; Caspi and Gepstein, 2004; Wobus and Boheler, 2005). For normal heart development to proceed, complex interactions must take place among cardiac-restricted transcription factors to regulate early processes of commitment and differentiation, and to promote maturation (Garcia-Martinez and Schoenwolf, 1993; Olson and
Schneider, 2003; Kelly and Buckingham, 2002). Early cardiac lineage markers, Nkx2.5, GATA-4, IsI-I are used after MGF treatment. Molecular markers for maturation of the contractile phenotype are TnI contractile isoforms.

[0078] MGF loaded nanoparticles is compared with unloaded nanoparticles. These experiments are used to determine the time course of cardiac lineage commitment, maturation of cells and protection against apoptosis in hypoxic conditions. The cell heterogeneity requires that we select powerful assays that can be done on small samples or individual cells. Methods of conventional biochemistry cannot be sensibly applied to tiny amounts of tissue in a heterogeneous population. However, gene expression is attainable with RT-PCR with specific primers. Traditional immunochemistry also gives individual ES cell information. Confocal microscopy of QDs is used to confirm internalization by the stem cells. ESCM is studied with or without MGF to determine the effects on their proliferative properties, apoptosis, differentiation and maturation ability. At least five different cultures are assessed to provide sufficient data for statistical analysis.

**Mouse ES cell derived cardiomyocytes**

[0079] R1 ES cell derived cardiomyocytes are used for these experiments (Wobus, 2002; Boheler, 2003). The wild type R1 cell line cells are used to form an embryoid body by the hanging drop method and suspended for 2 days. Cells are dispersed and plated and after 2-3 days, clusters of beating cells are dissected, dispersed by 15-20 minutes in collagenase, spun and replated on gelatin coated dishes. These are allowed to attach, and additional media is added. Cells are cultivated and observed over the following week, and used over 2–4 weeks. The R1 cell line produces a heterogeneous population.

**Myocyte lineage**

[0080] In order to assess a shift of a subpopulation towards the myocyte lineage, PCR analysis is used for early cardiac lineage markers, Nkx2.5, GATA-4 IsI-I, for cells at various time points after exposure to different formulations of MGF. Comparisons are made at 2 and 7 days after MGF treatment. Individual cells are identified to determine the proportion that are muscle-positive (ESCM) in the whole population. The heterogeneous cells are fixed, permeabilized and stained and used to compare cells with or without MGF nanoparticles over the experimental time course.
DNA synthesis in cells is quantified by BrDU incorporation and cells are co-stained with muscle-specific markers (desmin and oactinin) to identify the ESCM subpopulation. Random fields are selected for counts of total BrDU stained cells and the proportion that are also muscle positive. The ratio is expected to increase with MGF treatment due to the beneficial shift in the proportion of cardiomyocytes.

**Contractile maturation of ESCM**

[0081] The maturation level of ESCMs is assessed using quantitative RT-PCR to determine the levels of cTnl and ssTnl isoform expression in ESCMs at 2 and 7 days after treatment with MGF. Contracting ESCMs undergo a Troponin I (TnI) isoform transformation from the slow-skeletal (ss) isoform to the cardiac(c) isoform early in cardiogenesis, as they do in natural vertebrate development (Schiaffino et al., 1996). Only ssTnl is present in ESCMs one-day after spontaneous contraction begins. However, as the duration of spontaneous contraction increases, ESCMs mature and the cTnl isoform is expressed. Without MGF it takes 20 days after the onset of spontaneous contraction for all of the ESCMs to stain positive for the cTnl isoform (Westfall, 1996). Some cells are also fixed, permeabilized and stained and traditional immunochemistry is used to compare cells with or without MGF therapy to observe the TnI isoform transformation.

**Proliferation of mES progeny**

[0082] Cytometry of lysed cells gives only a rough, unreliable index of cell number in the various conditions. Therefore, to assess the overall proliferation of cells with or without MGF, the WST-I colorimetric assay is used (Roche Applied Science, Manheim, Germany). The stable tetrazolium salt WST-I (slightly red) is cleaved to a soluble formazan (dark red) by a complex cellular mechanism that occurs primarily at the cell surface. This bioreduction is largely dependent on the glycolytic production ofNAD(P)H by mitochondrial-succinate-tetrazolium-reductase system in viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture for 4 h and is quantified with an ELISA reader at 450nm and 630nm. The measured absorbance (abs450- abs630) directly correlates to the number of viable cells.
Assessment of apoptosis

[0083] Western blot is used for activity of Caspase-C by 18kD cleavage fragment, annexin V and PI staining. Stem cell proliferation and cytotoxicity of ESCM in the presence/absence of MGF nanoparticles is determined by cell number assessed by propidium iodide.

[0084] The aforementioned methods have been employed as follows.

Time course of MGF E-domain degradation

[0085] In order to administer the peptide in vivo, the unique, highly basic 24 amino acids from the C-terminal of the MGF E-domain was amidated (C-term) and specific arginines switched to the D-stereoisomer. To determine the effect of stabilization, the time course of degradation of both the natural and stabilized E-domain peptides was examined. Peptide (1mg) was incubated with human plasma at 37°C for various times and the extent of degradation examined by blotting with a custom made E-domain specific antibody. The data show that there was almost complete degradation of the natural peptide by 2 hours, whereas the stabilized peptide shows little or no degradation by 24 hrs.

MGF release by stress in culture

[0086] NRVM in culture showed that a 1 Hz cycle 20% strain was the required stimulus, while the 10% strain at 1 Hz is insufficient. Thus, the expression of MGF from overstressed myocytes in vitro occurred over the first 48 hours after extreme work.

NRVM response to MGF

[0087] The bio-effectiveness of MGF on NRVM was demonstrated by growing them in culture on a thin film of a copolymer of polycaprolactone (PCL) and gelatin films (10% w/v). The stable MGF E-domain peptide as used with NRVM plated at high density (1-2 million cells) on pieces containing about 50 ng MGF per dish. NRVM were viable, and had abundant RNA and protein for analysis. NRVM beating was spontaneous and vigorous, confirming physiologic function. The media was changed at day 1 to remove debris from the cell isolation process, not changed for the remaining days but additional media is added on day 3. At day 7 cells were lysed for protein and RNA. Analysis of physiological gene expression by quantitative real time
RT-PCR showed an increase in the expression of α-myosin heavy chain (α-MHC) isoform and a significant increase in the L-type Ca2+-channel (α-subunit) in NRVM grown on MGF E-domain eluting PCL sheets compared to PCL without peptide for 7 days (Fig 5). This demonstrates the bio-effectiveness of the MGF E-domain peptide on NRVM since we see upregulation of mature muscle proteins.

**Hypoxic stress of NRVM**

[0088] To determine whether MGF has beneficial effects on myocytes in the ischemic region of an animal, NRVM was exposed to hypoxia. Cells were grown for 7 days on the sheets containing 50 ng stabilized MGF E-domain peptide per dish, or without the MGF. One set of each was made hypoxic for 12 hrs in a humidified chamber (Billups-Rothenberg Inc). Apoptosis was measured using Western blots for activity of Caspase-C by 18kD cleavage fragment shows protection by MGF.

**Cell division in ES cells**

[0089] 5-bromo-2-deoxyuridine, BrDU, incorporation was used to identify cells undergoing DNA replication. In the heterogeneous mESCs of the R1 line, most of the cells underwent rapid proliferation and show BrDU incorporation. ES cells were double-labeled with α-actinin antibody to identify cells expressing myocyte specific protein, thus distinguishing ESCMs from non-myocytes (Fig 6). The majority of dividing ES cells (BrDU+) were non-myocytes, but a few immature ESCM were still dividing (identified as both BrDU+ and α-actinin +), while many ESCMs were only α-actinin positive and have no sign of ongoing cell division.

**MGF treatment of stem cells**

[0090] Mouse ESCs were differentiated (7-9 days) and the spontaneously beating regions dissected. ESCMs were grown for 7 days with 100 ng stabilized MGF E-domain per dish, or without the MGF. Sufficient mRNA was isolated for RT-PCR analysis of the contractile isoforms (Fig 7).
Example 3

Physiological, cellular and molecular gains with delivery of NP MGF therapy to infarcted mouse hearts without harmful side effects

[0091] The overall aim is to prevent early dilatation of the heart following acute myocardial infarction by improving systolic and diastolic performance of the ventricle and to create a microenvironment sufficient to support endogenous cardiac stem cell engraftment in order to rescue the dilated failing heart. These changes elicit remodeling of the heart and lead to long-term improvement in ventricular function. Accordingly, the natural MGF is compared with the stabilized peptide, which has salutary effects in mice and sheep when delivered after myocardial infarction. The rationale for using the stabilized peptide in the first place was to prevent degradation. Sustained administration of stable MGF E-domain systemically in the mouse via osmotic pumps over several weeks is beneficial to cardiac structure and function. However, there are serious concerns about other potential sites of action of the stabilized MGF peptide in the circulation. This is particularly relevant in females where an abundance of experimental and epidemiological evidence has suggested that the insulin like growth factor system is a treatment target in patients with HER2 positive breast cancers (Nahta, 2005). Clinically, therefore, sustained use of the stable from and a single intervention with a degradable peptide is preferable. Evaluation of the release natural MGF peptide from NP encapsulation may prove beneficial and minimize any further untoward effects of the stabilized version. The time dependent release of the MGF E-domain from the biodegradable nanoparticles provides a means by which the peptide can be administered over the one-day period of time similar to the expression dynamics of the endogenous molecule. Therefore, this ensures biological delivery and exposure without the need for repeated surgical interventions. In sheep, a single intervention of stabilized MGF soon after infarct did indeed protect the heart from the expected decline in function. Use of a single MGF delivery in the chronic stage of sheep or mouse has not yet been evaluated on cardiac remodeling and function.

[0092] The site and timing of administration is controllable. As described below, MGF E-domain nanoparticles are injected into the coronary vasculature during an acute MI as a model for a human soon after a heart attack to find if this is sufficient to prevent the onset of remodeling and decompensation to heart failure. MGF E-domain
nanoparticles are also injected into the ventricular wall in mouse hearts that are progressing into failure as a model for treatment efficacy in long-term chronic heart failure humans by an approach that could be used during a surgical intervention such as coronary artery bypass. This explores whether MGF can improve systolic and diastolic performance of the ventricle of the dilated heart, which would serve to mechanically unload the heart and decrease the need for transplantation. At both the acute and the chronic models, we perform a complete analysis of the cardiac hemodynamic geometric, biochemical and molecular data. These variables are determined relative to administration and the actions of the stabilized peptide without nanoparticles, which we predict serve as a positive control. Likewise, we predict the natural peptide serve as a negative control in the absence of the nanoparticles. Finally, the importance of stem cells in the recovery processes is examined either by increasing the pool or preventing the cell death, thus leading to engraftment and ultimately regeneration of the damaged myocardium, thereby effecting complete repair of the heart.

A. Prevention of the onset of pathologic hypertrophy and early dilation of hearts following myocardial infarction

[0093] Treatment of hearts by coronary artery administration of nanoparticles containing the MGF E-domain peptide during the acute phase of an MI prevents apoptosis of cardiac myocytes in the "area at risk" surrounding the infarct. Controlled delivery and release will provide an efficacious means for supplying the MGF E-domain peptide to potentiate the actions of the paracrine/autocrine IGF-I and prevent apoptosis. Promoting cell survival and preventing cell death in cardiac myocytes means more are viable and thus the need to normalize wall stress is minimized, thereby preventing hypertrophy.

[0094] The experimental approach is to administer the MGF encapsulated nanoparticles during the induction of the MI and determine the effects at 2 and 10 weeks post-infarct. These time points are critical in the mouse model since they represent distinct measurable events that without treatment, herald the onset of the hypertrophic response (2 wks) and the transition to failure (10 wks). Both males and female mice are proposed due to the different hypertrophic response associated with gender (males develop eccentric hypertrophy and females develop concentric hypertrophy) plus they represent two different risk groups in the patient population.
The time points selected for analysis are based on our preliminary data and those previously used to study the adaptations of the cardiovascular system in response to myocardial infarction in mice.

Following the stated time points (2 and 10 weeks post MI), the mice within each cohort are systematically analyzed to ensure correspondence between, hemodynamic, geometric and biochemical data collection. The methodological approaches detailed below are first for functional assessment in vivo and then for the molecular and biochemical studies.

1. Analysis of cardiac function is derived from the pressure-volume loops in instrumented animals.

2. Echocardiography is used to examine both structural and functional changes within each group.

3. Following physiological measurements, hearts are removed for determination of the heart weight to body weight ratio.

4. In one subset of the mice, myocytes are isolated for analysis of the extent of apoptosis using FACS. RNA is extracted for analysis of gene expression using real time RT-PCR and protein expression evaluated using Western blot analysis.

5. In the other subset, ventricular tissue is used for quantification of the infarct size, analysis of apoptosis using Tunnel staining and immunohistochemical staining. Nanoparticles with QDs are injected in a few animals to track their distribution in sectioned tissue.

6. In addition tissues (spleen, liver, skeletal muscle, lungs) are removed, fixed in 10% buffered formalin and sent for histopathology screening at the University of Illinois Veterinary Diagnostics laboratory.

Myocardial infarct

Mice are anesthetized with methoxyflurane inhaled in a closed chamber and intubated with an 18-gauge angiocatheter. Surgical anesthesia is maintained using 0.5% isoflurane delivered through a vaporizer with a mixture of 95% oxygen/5% carbon dioxide connected in series to a rodent ventilator with the stroke volume set at 0.2 to 0.4 ml and a respiration rate of 125 breaths/min. A left
thoracotomy is performed to expose the heart and the pericardium ruptured. The heart is exteriorized and the left coronary artery ligated to produce myocardial infarction.

**Administration of the MGF Nanoparticles**

[00103] The body temperature of the mouse is cooled to 18°C and the descending aorta cross-clamped distal to the left subclavian artery according to a previously published technique (Logeart, 2000). 10μl of MGF-nanoparticles carrying 100 ng load suspended in saline and 1 µg/kg histamine is injected into the ventricular cavity to direct delivery through the coronary vasculature. The aorta remains clamped for 30 sec prolonging circulation through the coronary vasculature after which the clamp is released and the mouse is gradually warmed to 37°C and the incision closed. A single dose is administered 10 minutes following coronary ligation. The heart is replaced, a chest tube is placed in the wound and the thoracotomy closed in three layers (intercostal muscles, pectoral muscles, and skin) followed by evacuation of the chest cavity and removal of the tube. Animals are allowed to recover in a heated cage before being returned to the animal facility.

**Echocardiography**

[00104J] Echocardiography is used to examine the geometric changes of the heart. Transthoracic two-dimensional targeted M-mode and pulsed-wave Doppler echocardiography is performed with a 15-MHz linear array transducer attached to a Sequoia C256 system. Images of the left ventricle, LV, are taken from the parasternal short axis view at the level of the papillary muscles and LV internal dimensions are measured at the end of diastole and systole according to the American Society of Echocardiography leading-edge method on the M-mode tracings (Sahn, 1978). The LV fractional shortening (FS, %) is calculated from digital images as (LV end-diastolic dimension - LV end-systolic dimension) / LV end-diastolic dimension x 100. Velocity of LV circumferential fiber shortening (Vcf, sec-1) is calculated as FS/LVET, where LVET = LV ejection time derived from the Doppler recordings from the ascending aorta. Stroke volume (SV) is calculated as product of main pulmonary artery (MPA) mean velocity time integral (VTI) and corresponding MPA mean cross-sectional area. Multiplying SV by heart rate yields cardiac output (CO). All calculations are made from at least three consecutive cardiac cycles (Goldspink, 2004).
PressurE-volume Loop Analysis

Cardiac functional studies in vivo require insertion of a 1.4 French pressure-conductance catheter (SPR-839, Millar Instruments, Houston TX) into the right carotid artery and fed retrogradely into the left ventricle (Goldspink, 2004). Following these procedures, mice are euthanized with an overdose of isoflurane (2%) the hearts removed for determination of the heart weight to body weight ratio, followed by subsequent molecular and biochemical analysis. Mice are divided into two subsets (6 in each group). In one group, cardiac myocytes are freshly isolated and processed for the analysis of apoptosis using FACS plus gene and protein expression. In the remaining group, hearts are fixed and used for quantification of infarct size, analysis of apoptosis with the tunnel staining.

Quantitative RT-PCR

As for NRVM, gene expression is assessed by real-time quantitative RT-PCR for myosin, actin and troponin isoforms. Total RNA is extracted using Trizol and a 100 ng of total RNA is used in each RT-PCR reaction (Goldspink, 2004).

Infarct quantification

The abdominal aorta is cannulated, the heart(s) arrested in diastole with KCl, and then perfused with 10% (vol/vol) formalin at a pressure equal to the in vivo measured end-diastolic pressure. The left ventricular intracavitary axis is measured, and three transverse slices from the base, mid-region, and apex are fixed and embedded in paraffin for sectioning. Sections are incubated with triphenyltetrazolium chloride (TTC) (20 mins, 37°C), which permits clearer discrimination of the various regions. The tissue in the center of the infarct zone appears white, whereas the healthy tissue distal to the infarct zone has the typical deep red appearance. The mid-section is used to measure left ventricular thickness, chamber diameter, and volume. Infarct size is determined by planimetry of the scarred portion of the ventricle and expressed as a percent of scar/viable myocardium yielding the extent of fibrosis.

Apoptosis

Assessment of the extent of apoptosis in situ is made on sectioned tissue with TUNEL staining and quantified with FACS analysis of isolated cardiac myocytes using binding of Annexin V and propidium iodide. TUNEL staining (CardiacTACS, Trevigen, Inc) is based on DNA end labeling using terminal
deoxynucleotidyl transferase (TdT) with a modified nucleotide that is subsequently detected via a colorimetric detection system. This allows detection of DNA fragmentation within the cardiac myocytes rather than the whole heart. Sections are examined using transmitted light microscopy and digital images captured for quantification. Myocyte isolation is necessary for FACS analysis. Mice are heparinized (5000 units/kg b.w.) and anesthetized with methoxyflurane. Hearts are quickly removed and put into ice cold, nominally Ca2+-free control solution (in mM: NaCl 133.5, KCl4, NaH2PO4 1.2, MgSO4 1.2, HEPES 10 and glucose 11). The aorta is cannulated and the heart is mounted in a Langendorff perfusion system and perfused at a pressure of 60 cm H2O for 5 min with Ca2+-free control solution containing bovine serum albumin (1 mg/ml; Sigma), and subsequently for 8-15 min with the same solution containing 25 µM Ca2+ together with specially selected collagenase (Boehringer; type D; 0.39 U/mg; 0.30 mg/ml) bubbled with 100 % O2 at 37°C. The perfusion is stopped when the heart is well digested or coronary flow increases dramatically. The hearts are removed, transferred to a small dish containing control solution with 100 µM Ca2+, the ventricles minced and the pieces gently triturated with glass pipettes (10 mins). The cell suspension is filtered through a 70µm mesh and the cells permitted to settle under gravity for 3 to 7 min. The supernatant fraction is removed and the cells are resuspended in 400 µL binding buffer (BB) (Vybrant Apoptosis Assay, Molecular Probes). Annexin V Pacific Blue (5 µL) is added and incubated for 20 mins at RT before the addition of 2 µL propidium iodide (50 µg/ml) incubated for 1 min RT and flow cytometry is performed using MoFlo (Cytomation, Inc) equipped with triple lasers.

Statistics and sample size estimates
[00109] Based upon preliminary studies, changes are expected in the mean hemodynamic measurements (e.g. +/- dp/dt) to be approximately 25% in experimental groups (sham vs. ext group for each treatment) with a standard deviation of 10-12%. These differences are detected with a sample of 8 animals in each group. With this sample size, the power of test for the two-sided hypothesis is 0.8, when alpha is set at 0.05. However, a greater number of animals are required for the post functional analysis (biochemical, cell isolations) therefore, the final number increased to 12. Data are expressed as mean ± standard error (SEM). Differences are tested for statistical significance (P<0.05) using t-test, paired or unpaired, two-way analysis of
variance (ANOVA), followed by post-hoc analysis to test for significance within the group where appropriate.

Improving systolic and diastolic performance of the ventricle of the dilated heart

[00110] Treatment of hearts by injection directly into the myocardium of nanoparticles with the MGF E-domain peptide during the chronic phase of an MI is associated with improved systolic and diastolic function. We hypothesize that controlled delivery provides an efficacious means for supplying the MGF E-domain peptide to promote physiologic hypertrophy and improved function. Promoting physiologic hypertrophy due to the pleotropic effects of IGF-I may increase both systolic and diastolic performance, unload the dilated ventricle preventing further decline in function and failure.

[00111] Cardiac dilation (an increase in the dimension chamber of the ventricle relative to the thickness of the wall) is an end-stage phenotype associated with heart failure due to a number of etiologies of which ischemic heart disease is just one. Those patients whose cardiac disease has progressed despite being optimized on conventional medical management represent the largest group of patients receiving heart transplants. At a functional level, the thinning of the ventricles (decreased maximum force or systolic dysfunction) or stiffening of the tissue (inability to relax, diastolic dysfunction) is associated with loss of pump function. Consequently, the heart fails to eject the appropriate volume from the chamber during each cycle causing an accumulation of blood, which increases the intraventricular pressure thus promoting the expansion of the chamber. This precipitous situation continues and is characterized by transitions of heart failure in patients (New York Class stages I-IV) with no means to reversal. At present, widely used therapies are directed at prolonging the patient’s time at any one stage thereby slowing the rate of transition to failure. Therefore, the approach outlined below is directed at determining whether NP delivery of the MGF E-domain is a viable in vivo therapy for what would be a considerably larger patient population than the acute MI population.

[00112] Mice reproducibly show signs of decompensation to failure 10-weeks post-infarct making this a useful model for progression to failure in chronic human disease. The experimental approach is to administer the MGF carrier nanoparticles directly
into the ventricles during the transition to failure (10 weeks) and determine whether there is functional improvement and reverse remodeling 2 and 10 weeks post treatment during the progression of heart failure. To evaluate the efficacy of the MGF NP prototypes identified initially, comparisons are made to the stabilized and natural peptides administered via the same route without encapsulation the nanoparticles. All biochemical analyses are conducted on the same number of hearts used for hemodynamic analysis so additional mice are not required.

[0013] Following the stated time points (10MI+2 and 10MI+10 weeks treatment), the mice within each cohort are systematically analyzed to ensure correspondence between, hemodynamic, geometric and biochemical data collection. Methodological approaches are the same as described above, except as noted below.

[0014] 1. Analysis of cardiac function is derived from the pressure-volume loops in instrumented animals.

[0015] 2. Echocardiography is used to examine both structural and functional changes within each group.

[0016] 3. Following physiological measurements, hearts are removed for determination of the heart weight to body weight ratio.

[0017] 4. RNA is extracted for analysis of gene expression using real time RT-PCR and protein expression using Western blot analysis.

**Administration of the MGF Nanoparticles 10 weeks post-MI**

[0018] To administer the nano-MGF therapy in vivo a left lateral incision is used to expose the second intercostal space and to open the thorax in anesthetized mice. The body temperature of the mouse is cooled to 18°C and the descending aorta cross-clamped distal to the left subclavian artery according to a previously published technique. Ten μl of MGF- nanoparticles suspended in saline and 1 μg/kg histamine is injected directly into the ventricular wall. A single dose (equivalent to 100ng of E-domain peptide) is administered 10 weeks following coronary ligation and the effects 2 and 10 weeks post treatment.
Statistics and sample size estimates

Based upon preliminary studies, we expect changes in the mean hemodynamic measurements (e.g. +/- dp/dt) to be approximately 25% in experimental groups (sham vs. expt group for each treatment) with a standard deviation of 10-12%. These differences can be detected with a sample of 8 animals in each group. With this sample size, the power of test for the two-sided hypothesis is 0.8, when alpha is set at 0.05. All biochemical analysis is conducted on the same number of hearts used for hemodynamic analysis so additional mice are not required.

Manipulation of the stem cell microenvironment and support endogenous cardiac stem cell engraftment

Treatment of hearts with trophic agents following infarction induces proliferation of cells with early cardiac lineage markers. NP delivery of MGF E-domain peptide may induce proliferation of the cardiac stem cell pool and regenerates new cardiac muscle cells. This combined approach to stem cell therapy using MGF E-domain delivery rescues the failing dilated heart.

The approach in the instant sub-section investigates whether the nano-MGF therapy is sufficient to expand and sustain the resident cardiac stem cell population as well as to promote their differentiation and regeneration into myocytes. As such, this novel observation and innovative application of the nano-MGF therapy may provide a means by which resident cardiac stem cells may be supported for therapeutic use in cardiac muscle regeneration. To evaluate the efficacy of the nano-MGF prototypes identified initially we make comparisons to the stabilized and natural peptides administered via the same route without encapsulation within the nanoparticles. The model proposed is the same as noted in above (administer the treatments during the infarct into the coronary vasculature). The effects of MGF delivered on the cardiac stem cell population size are assessed at 48 hours post-treatment, and the expression of cardiac specific lineage markers at 2 weeks post-treatment. Additional mice at the 48 hour time point (4 mice/group) are required for immunohistochemical analysis, whereas tissue sections already exist for the 2-week group from the previous study described above.

Following the MI and treatment at the 48h or 2 weeks time points, the mice within each cohort are processed and systematically analyzed as follows.
[00123] 1. Immunohistochemical analysis on sections examines the expression of stem cell markers and expression of cardiac lineage specific proteins. (4 hearts/group for 48 hr and 2 weeks).

[00124] 2. Resident stem cells are isolated from the cardiac myocyte depleted cell suspensions prepared by collagenase digestion of the heart (6 hearts/group).

[00125] 3. The size and presence of the side population are determined by the Hoechst dye exclusion technique.

[00126] 4. To identify the size and specific subpopulation of stem cells within the heart, cells are incubated with antibodies against resident stem markers (Sca-1 and c-kit), hematopoietic markers (CD45), bone marrow markers (CD34) and cell adhesion markers (CD31) and subject to FACS analysis.

[00127] Except for the procedures noted below, the methodological approaches are the same as already detailed.

**Immunohistochemistry**

[00128] Sections are blocked with serum before incubation with primary antibodies to label stem cell markers (Sca-1, c-kit, CD45, CD34, CD31, (Pharmingen) cell cycling (Ki67 and BrdU), lineage specific proteins (Nkx2.5, GATA4, IsI-I), cTnl, ssTnl, smooth muscle actin, and Von Willebrand factor. Confocal microscopy is used to visualize fluorescent conjugated secondary antibodies.

**Stem Cell isolation and Identification**

[00129] Stem cells are isolated along with cardiac myocytes via collagenase digestion, but the isolate is then depleted of myocytes by centrifugation at 3,000 rpms for 10 minutes. Cell suspensions are incubated with 5/µg/ml Hoechst 33342 stain (Sigma) for 90mins at 370C in DMEM plus 2% fetal calf serum and 10mmol/L HEPES, with (control) and w/out 0.1mM verapamil (Sigma). Propidium iodide (2µg/ml) is added to exclude dead cells. Cells are washed in cold HBSS before cell surface antigen staining (40C for 30 mins) with fluorochrome conjugated monoclonal rat anti-mouse antibodies. Flow cytometry is performed using MoFlo (Cytomation, Inc) equipped with triple lasers. Hoechst dye is excited at 350nm using a multiline UV laser and emission collected at 405nm (Blue) and 660nm (Red). Phycoerythrin (PE) and PI are detected using 488nm agarose laser. A 610 DMSP (610 nm short
pass dichroic mirror) is used to separate the emission wavelengths. The number of side population cells are expressed as a percentage of the isolate and the number of stem cells are expressed as a percent of the side population (Pfister, 2005).

[00130] The aforementioned methods have been employed as follows.

### Isoforms of IGF-I family

[00131] Increased expression of the IGF-IEb (MGF E-domain) occurred within 24 hours following myocardial infarct at a time when the predominant IGF-IEa isoform does not change (Fig 8). The investigation into the actions of the unique Eb-domain of MGF in vitro (cell proliferation and gene expression studies) suggested that the E-domain peptide does have independent biological activity, but also potentiates the actions of IGF-I (i.e. mature peptide). Therefore, it is proposed that by providing more Eb-domain, the actions of IGF-I produced by the heart are enhanced following stress or a myocardial infarct (MI). Studies delivered a stabilized synthetic peptide corresponding to the Eb-domain of MGF systemically via a mini-osmotic pump implanted in mice that have received an MI.

#### Delivery of stable MGF E-domain peptide to mouse hearts post-infarct

[00132] To evaluate the potential benefits of the MGF E-domain peptide, the stabilized peptide was delivered systemically (2 weeks) via mini-osmotic pumps implanted prior (12 hrs) to coronary artery ligation in mice. Four groups of mice were used: Sham, MI, Sham+MGF (E-domain peptide, 1mg/Kg/day), and MI+MGF. At 2 weeks post-infarct, cardiac function was evaluated by pressure-volume analysis in situ, along with assessment of cardiac mass index and changes in gene expression (Fig 9). Analysis of cardiac function showed no significant differences in the hemodynamic parameters between the Sham and Sham+MGF groups. However, there was a 63% decrease in Emax and a 32% decrease in dP/dtmax with MI, which was ameliorated in the MI+MGF group. Other hemodynamic parameters (LVSP, both +/- dP/dt, Tau) were also preserved in the MI+MGF group, and were significantly different in the MI group (Fig 9B). Analyses of gene expression in these hearts showed significant increases in both ANF and α-MHC mRNA expression was associated with an increased heart to body weight ratio in the MI group, indicative of hypertrophy. Conversely, there was no increase in the heart weight to body weight ratio in the MI+MGF group, which is associated with the inhibition of the
hypertrophy/fetal gene program. This demonstrated that there was improved contractile function with 2-week systemic application of the stabilized E-domain peptide in infarcted mice. The improvement at 2 weeks was associated with decreased expression of the pathophysiological gene program and prevention of hypertrophic remodeling of the heart.

**Effect of MGF E-domain peptide on apoptosis in mouse heart post-infarct**

[00133] To determine whether an inhibition of apoptosis explains the functional improvement seen in the mouse heart post-MI with MGF E-domain peptide treatment, adult cardiac myocytes were isolated by collagenase digestion and stained with propidium iodide (PI) and annexin V (AV). The extent of apoptosis was examined using flow cytometry (FACS). Staining revealed a greater percent of necrotic and apoptotic myocytes isolated from the untreated 2 week MI group compared to the 2 week MI+MGF treated group, which showed a greater number of viable myocytes (Fig 10). These data showed that systemic administration of the MGF E-domain peptide during the acute phase of a myocardial infarct protects the myocardium by preventing apoptosis, which in turn, may contribute to preserving contractile function and prevent pathologic remodeling of the heart.

**Expansion of cardiac stem cell with MGF E-domain peptide treatment to stressed mouse heart**

[00134] Further evaluation using immuno-histochemical analysis of the mouse hearts treated with the E-domain peptide revealed a large number of small troponin I staining positive cells in the septum and viable ventricular tissue but not in the infarct region (Fig 11). Conversely, these cells were not detected in sham-operated hearts at all. Further characterization of these cells showed that the expression of the muscle lineage protein (TnI) was also co-expressed with the cardiac specific transcription factor (Nkx2.5) and a cardiac progenitor cell marker (IsI-I) in the nuclei. In order to isolate and characterize these cells, hearts were digested with collagenase and the resultant cells were subjected to a series of pre-plating steps in order to enrich for this population of cells. Gene expression analysis confirmed that the second pre-plating step was enriched for cells expressing the slow skeletal isoform of TnI, which is normally expressed during embryonic development, and the cardiac transcription
factor GATA 4. The adult isoforms of myosin heavy chain (oMHC) and cardiac TnI (cTnI) were not expressed in cells present in the second pre-plating step indicating a lack of contamination with differentiated cardiac myocytes (Fig HB).

Cardiac lineage side population with MGF

[00135] Given the appearance of numerous small cells expressing cardiac lineage proteins in the hearts treated with the E-domain peptide, the sufficiency of E-domain peptide to either expand the resident population of stem cells or act as a mobilization/homing factor attracting stem cells to the heart was determined. Resident stem cells found within the heart reside within a side population of cells that efflux Hoechst stain and were sensitive to verapamil treatment (Asakura, 2002). The analysis revealed a side population of cells that effluxed Hoechst and is sensitive to verapamil treatment (Fig 12). These cells usually constitute 0.03% of the total digest (Oh 2003). However, the side population was increased over five-fold to 0.16% of the total digest in mice treated with the MGF E-domain peptide for 48 hrs. These data suggested that there was expansion of the stem cell pool within the hearts of mice treated with MGF E-domain peptide.

MGF effects on sheep after myocardial infarction

[00136] While these data derived from the small animal models showed that E-domain peptide treatment is beneficial, it was also determined whether the E-domain peptide protects cardiac myocytes by reducing the incidence of apoptosis in the border regions of an infarct in vivo, in a more clinically relevant large animal model. Infarcts were induced by injecting microspheres (10 μm) into the left circumflex coronary artery of 24 female sheep. Ten minutes post-MI, one of four treatments (n=6 per group) are delivered via the perfusion catheter: Vehicle (saline), 200 nM recombinant IGF-I, 200 nM synthetic MGF E-domain peptide, or 200 nM full MGF (IGF-1+E-domain peptide). On day 8, hearts were imaged using M-mode echocardiography to evaluate left ventricular ejection fraction, LVEF, and the hearts were harvested and perfused with 0.15% Evans blue dye, stained with TTC, and assessed (Fig 13). A significant reduction in the area ‘at-risk’ was found with the E-domain peptide and full MGF treatments (48% and 63% respectively). In addition, the E-domain peptide increased the viable area by 3-fold (E-domain alone) or by 3.5-fold (full MGF) compared to controls. This is associated with an increase in the
ejection fraction in the E-domain and full MGF treated groups compared with control Ml or rIGF-1 treated.
References:


Klinz, F. W. Bloch, K. Addicks and J. Hescheler, Inhibition of phosphatidylinositol-3-kinase blocks development of functional embryonic cardiomyocytes, Exp. Cell
Liu JP, Baker J, Perkins AS, Robertson EJ, and Efstratiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and Type 1 IGF receptor (Igflr). Cell. 75 (1993) 59-72.
Martin CM, Meeson AP, Robertson SM, Hawke TJ, Richardson JA, Bates S, Goetsch SC, Gallardo TD, Garry DJ. Persistent expression of the ATP-binding cassette


McDevitt TC, Laflamme MA, and Murry CE. Proliferation of cardiomyocytes derived from human embryonic stem cells is mediated via the IGF/PI 3-kinase/Akt signaling pathway. J. Molecular and Cellular Cardiology. 39:6 (2005) 865-873.


to trastuzumab resistance of breast cancer
Nehilla and Desai, Formulation parameters and characterization of drug-loaded biodegradable Polymeric Nanoparticles, NSTI conference poster presentation, Boston, MA 2006.
Panyam, J., W.Z. Zhou, S. Prabha, S.K. Sahoo, V. Labhasetwar. Rapid endo-
Olson EN, Schneider MD Sizing up the heart: development redux in disease. Genes Dev. 2003 Aug 15;17(16):1937-56.


Siegfried JM, Kasprzyk PG, Treston AM, Mulshine JL, Quinn, KA, Cuttitta F. A mitogenic peptide amide encoded within the E peptide domain of the insulin-like growth factor IIB prohormone. Proc Natl Acad Sci U S A. 1992 Sep 1;89(17):8107


Westfall MV, Samuelson LC, Metzger JM. Troponin I isoform expression is developmentally regulated in differentiating embryonic stem cell-derived cardiac myocytes. Dev Dyn. 1996


Yang SY, Goldspink G. Different roles of the IGF-I Ec peptide (MGF) and mature IGF-I in myoblast proliferation and differentiation. FEBS Lett. 2002 Jul 3;522(1-3):156-60.


Ziolo MT, Martin JL, Bossuyt J, Bers DM, Pogwizd SM. Adenoviral gene transfer of mutant phospholamban rescues contractile dysfunction in failing rabbit myocytes

We Claim:

1. A method for repairing damaged tissue comprising the step of administering a composition comprising a nanoparticle associated with a releasable growth factor and a carrier in an amount and over a time effective to repair damaged tissue.

2. The method according to claim 1 wherein said growth factor is releasable over time.

3. The method according to claim 2 wherein said growth factor is releasable over a predetermined amount of time.

4. The method according to claim 1 wherein said nanoparticle is biodegradable.

5. The method according to claim 1 wherein said nanoparticle is inert.

6. The method according to claim 1 wherein said nanoparticle is porous.

7. The method according to claim 1 wherein said carrier is aqueous.

8. The method according to claim 1 wherein said carrier is saline.

9. The method according to claim 1 wherein said carrier is a buffer.

10. The method according to claim 1 wherein said nanoparticle is synthesized from one or more polymers.
11. The method according to claim 1 wherein said nanoparticle is synthesized from one or more copolymers.

12. The method according to claim 10 wherein said polymer is polylactic acid (PLA), polyglycolic acid (PGA), or poly(e-caprolactone) (PCL).

13. The method according to claim 11 wherein said copolymer is poly(lactide-co-glycolide) (PLGA) or poly(lactide-co-glycolide) poly(ε-caprolactone) (PLGA/PCL).

14. The method according to claim 1 wherein said nanoparticle is on average, between 1 nm and 1,000 nm in diameter.

15. The method according to claim 1 wherein said growth factor is an insulin-like growth factor (IGF) protein.

16. The method according to claim 15 wherein said growth factor is an E-domain peptide of an IGF protein.

17. The method according to claim 15 wherein said growth factor is an Ea, Eb, or Ec domain peptide of an IGF protein.

18. The method according to claim 15 wherein said growth factor is mechano-growth factor (MGF).

19. The method according to claim 15 wherein said growth factor is an E-domain peptide of mechano-growth factor (MGF).

20. The method according to claim 18 wherein said MGF has a stabilized E-domain.
21. The method according to claim 18 wherein said MGF has a native E-domain.

22. The method according to claim 15 wherein said growth factor is a biologically active fragment of mechano-growth factor (MGF).
Fig 1. Monodisperse, aggregate-free, NPs. Phase AFM image A. of PLGA-COOH NPs demonstrates spherical morphology, sub-micron size and absence of large aggregates; B. typical nanoprecipitation parameters yield sub-200nm, nearly monodisperse NPs.
**Fig 2. Tight control over processing parameters.** A. NP diameters increased as the mass of PLGA-COOH dissolved in the solvent increased (significance at 150 mg, p<0.05) but there was no difference in polydispersity index, PDI. B. The NP diameters and PDI were stable across most non-solvent:solvent volumetric ratios.
**Fig 3.** Encapsulation efficiency. PLGA-COOH NPs (0.4 dL/g) synthesized using acetone or acetonitrile as the solvent. *; t-test, p<0.05.
**Fig 4.** *Peptide release control over several days.* Protein release from biodegradable thin films shows first phase release (initial slope) in 24 hours followed by sustained release over several days (corresponding to physiological MGF uptake).
Fig 5. NRVM grown on MGF E-domain eluting sheets. Note, increased L-type channel and αMHC. Data are means ± SE, *P<0.05, n=3.
Fig 6. Immature ES-CMs are able to divide. Cardiac muscle cells are identified by α-actinin (red). BrDU incorporation labels nucleus for recent DNA replication (green).
Fig 7. *Mouse stem cells and MGF.* RT-PCR detects L-type channels, myosin heavy chains, and tropinins.
Figure 8. Quantification of MGF and IGF-1 isoform expression in the mouse heart following myocardial infarct using real time RT-PCR. A. Expression of the MGF splice variant (550bp) is increased within 24hrs following an infarct. B. IGF-1Ea transcript (500bp), the most predominant transcript, is unchanged 24hrs post-infarct. Data are means ± SE, *P<0.05, n=4.
Fig 9. A. Representative Pressure-volume loops derived from instrumented mice 2 weeks-post MI with and without systemic delivery of MGF E-domain peptide. B. Typical hemodynamic parameter derived from the P-V loop analysis. Data are means ± SE. * = P < 0.05, n=5.
Fig 10. FACS analysis of myocytes. Cells isolated with mouse hearts 2 week post-MI, stained with Annexin V and propidium iodide with and without MGF E-domain treatment. Data are means ± SD.
Fig 11. A. Expansion of stem cells. Immunohistochemical analysis revealing the presence of numerous small cells staining positive for troponin I within the healthy myocardium away from the infarct zone. B. Gene expression analysis of RNA extracted cells present in the 2nd pre-plating step.
Fig 12. FACS analysis of the side population after MGF. Characterization of side population cells found in the heart following systemic application of the stabilized MGF E-domain peptide for 48 hrs, based on the efflux of Hoechst stain and sensitivity to verapamil.
Fig 13. Treatment of infarcted sheep with MGF. A. Representative 1 cm thick transverse ring of heart muscle at day 8 showing demarcation of the infarct, uptake of Evans blue dye in the ischemic area ("At-risk") and TTC staining of the viable myocardium. B. Analysis of the percentage of myocardium compromised by infarct 8 days post-infarct, n = 6 per group. C. Improvement of ejection fraction in sheep treated with MGF E-domain peptide. Asterisks indicate significant differences from controls (P<0.05).
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K9/51 A61K38/30

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)

A61K

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

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

EPO-Internal, EMBASE, WPI Data, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>EP 0 896 825 A (SULZER INNOTEC AG [CH]) 17 February 1999 (1999-02-17) paragraphs [0001], [0028], [0042] [0047]</td>
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Further documents are listed in the continuation of Box C. See patent family annex.

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<td>WO 03/066082 A (UNIV LONDON [GB]; TRUSTEES OF THE UNIVERSITY OF [US]; GOLDSPIK GEOFF) 14 August 2003 (2003-08-14)</td>
<td>1-22</td>
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<tr>
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<td>EP 1928434 A2</td>
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<td>WO 9908728 A1</td>
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<td></td>
<td></td>
<td>US 6582471 B1</td>
</tr>
<tr>
<td>WO 2005072417 A</td>
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<td>US 2003203038 A1</td>
<td>30-10-2003</td>
<td>NONE</td>
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<tr>
<td>US 2007122388 A1</td>
<td>31-05-2007</td>
<td>NONE</td>
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<tr>
<td>WO 03066082 A</td>
<td>14-08-2003</td>
<td>AU 2003244511 A1</td>
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<td>EP 1480669 A1</td>
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<td>US 2008039390 A1</td>
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