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Novel Polymer Carriers and Gene Constructs for Treatment of Myocardial Ischemia and Infarction

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Abstract

The number one cause of mortality in the US is cardiovascular related disease. Future predictions do not see a reduction in this rate especially with the continued rise in obesity [1,2]. Even so, potential molecular therapeutic targets for cardiac gene delivery are in no short supply thanks to continuing advances in molecular cardiology. However, efficient and safe delivery remains a bottleneck in clinical gene therapy [3]

Keywords

myocardium; angiogenesis; non-viral gene delivery; ischemia

Viral vectors are looked upon favorably for their high transduction efficiency, although their ability to elicit toxic immune responses remains [4]. However, this high transduction does not necessarily translate into improved efficacy [5]. Naked DNA remains the preferred method of DNA delivery to cardiac myocardium and has been explored extensively in clinical trials. The results from these trials have demonstrated efficacy in regards to secondary end-points of reduced symptomology and perfusion, but have failed to establish significant angiogenesis or an increase in myocardial function [6]. This may be due in part to reduced transfection efficiency but can also be attributed to use of suboptimal candidate genes.

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Currently, polymeric non-viral gene delivery to cardiac myocardium remains underrepresented. In the past decade several advances in non-viral vector development has demonstrated increased transfection efficiency [3]. Of these polymers, those that employ lipid modifications to improve transfection or target cardiovascular tissues have proven themselves to be extremely beneficial.

Water-soluble lipopolymer (WSLP) consists of a low molecular weight branched PEI (1800) and cholesterol. The cholesterol moiety adds extra condensation by forming stable micellar complexes and was later employed for myocardial gene therapy to exploit the high expression of lipoprotein lipase found within cardiac tissue. Use of WSLP to deliver hypoxia-responsive driven expression of hVEGF to ischemic rabbit myocardium has proven to provide for even better expression in cardiovascular cells than Terplex and has demonstrated a significant reduction in infarct size ($13 \pm 4\%$, $p=0.001$) over constitutive VEGF expression ($32 \pm 7\%$, $p=0.007$) and sham-injected controls ($48 \pm 7\%$). A significant reduction in apoptotic values and an increase in capillary growth were also seen in surrounding tissue.

Recently, investigations have begun using bioreducible polymers made of poly(amido polyethyleneimines) (SS-PAEI). SS-PAEIs breakdown within the cytoplasm through inherent redox mechanisms and provide for high transfection efficiencies (upwards to 60% in cardiovascular cell types) with little to no demonstrable toxicity. In vivo transfections in normoxic and hypoxic rabbit myocardium have proven to exceed those results of WSLP transfections by 2–5 fold [8]. This new breed of polymer(s) may allow for decreased doses and use of new molecular mechanisms not previously available due to low transfection efficiencies.

However, little development has been seen in the use of new gene agents for treatment of myocardial ischemia and infarction. Current treatment consists of using mitogenic factors, described decades earlier, alone or in combination to spur angiogenesis or modulating intracellular Ca^{2+} homeostasis through SERCA2a but have to date, failed to demonstrate clinical efficacy. Recent data suggests that axonal guidance cues also act on vasculature neo-genesis and provide a new means of investigation for treatment.

Clinical Gene Therapy for Therapeutic Myocardial Angiogenesis

Ischemic heart disease is the leading cause of death in the United States today. Currently, the mainstay of therapy for ischemic heart disease (IHD) is revascularization. Nearly 2,000,000 cardiac catheterizations and 553,000 coronary artery bypass grafting procedures are performed annually [9]. Technological developments in these areas have led to an improved survival and quality of life for patients with IHD. However, increasing evidence suggests that revascularization alone is insufficient for the longer-term management of many patients with IHD. A significant number of patients will develop congestive heart failure (CHF) each year following ischemic events to the myocardium despite having received revascularization therapy. In addition, revascularization of ischemic myocardium can result in reperfusion injury, which is associated with a cascade of events promoting additional myocyte loss and detrimental ventricular remodeling [10]. To address these issues, innovative therapies that extend beyond revascularization and towards protection of myocytes and preservation of ventricular function are required. Recent developments have led to a better understanding of the pathophysiology of the progression from ischemic heart disease to CHF. The emergence of gene therapy and proteomics offers the potential for innovative prophylactic and treatment strategies for IHD.

Clinical gene therapy, after undergoing strict regulatory controls affecting its bench to bedside translation, is rapidly becoming an effective treatment for numerous disease states

[11–13]. Unfortunately, current progress in gene therapy has been guided by prior severe adverse clinical events [14,15]. While there are currently over 1347 clinical gene therapy trials underway worldwide, only 11 are currently active for myocardial gene therapy [16,16]. The majority of these myocardial gene therapy trials are directed towards promoting neovascularization to treat end stage ischemic heart disease or congestive heart failure. Previous trials have demonstrated that the genes and delivery procedures for therapeutic angiogenesis are safe, but efficacy remains unproven [17]. Several criteria must be addressed for improved performance of the genes and delivery procedures for therapeutic angiogenesis: temporal transgene expression, transgene expression levels, the appropriate angiogenic molecule, and proper gene carrier selection.

The selection of a proper gene transfer method is crucial for efficacious myocardial gene therapy. There are two major types of gene delivery carriers in use today: viral and non-viral gene vectors. Virus-mediated gene therapy includes the use of modified, replication defective viruses: retroviruses, adenoviruses, adeno-associated viruses, and herpes viruses. Viral delivery systems are still predominantly used, due to their higher transfection efficiency compared to non-viral systems. Viral gene delivery systems, however, possess serious risks that limit their clinical use: *i*) induction of host immune responses against viral components; *ii*) the risk of potential chromosomal insertion of viral sequences into the host chromosomes, which could lead to undesirable cell transformations, such as the activation of oncogenes and the inactivation of tumor suppressor genes; *iii*) size limitations of DNA for encapsulation; *iv*) high cost of production; and *v*) difficulties in quality control [9,10,18–21]. It is for these reasons that the development of non-viral systems is becoming increasingly attractive. Non-viral gene delivery systems offer numerous advantages over viral vectors including: *a*) excellent safety profiles; *b*) unlimited DNA size for loading; *c*) relatively low cost of production; and *d*) a simple quality control process [22].

Advances within recent years have expanded our understanding of non-viral gene delivery. Non-viral gene carriers must meet the following criteria for efficient transfection: 1) protect DNA by condensation; 2) be able to escape from the endosome; 3) exhibit minimal toxicity within cells and tissues; and 4) allow for reproducible large scale production. While the spectrum of materials and methods has increased in recent years, polyethylenimine (PEI) remains the standard against which most non-viral gene carriers are compared.

Polyethylenimine Conjugates

The molecular weight of PEI plays a significant role in transfection efficiency and toxicity [23]. Lower molecular weights of PEI exhibit poor transfection characteristics with virtually no toxicity. As the molecular weight of PEI increases, transfection efficiency increases along with cytotoxicity. The reduced transfection efficiency of PEI 1.8kDa is attributed to its dissociative properties in physiological salt concentrations. The addition of amine groups provides the charge for condensation and allows for the conjugation of targeting moieties and shielding agents to overcome the extracellular and intracellular obstacles, which can plague polycationic gene delivery. The addition of a cholesterol moiety can increase the transfection efficiency of polymer carriers.

Cholesterol uptake is accomplished through receptor-mediated endocytosis and helps to maintain plasma membrane integrity [24]. Most cholesterol is supplied by circulating lipoproteins, and although the cholesterol demands of the heart and skeletal muscle are low [25], selective uptake may be mediated by lipoprotein lipase (LPL) [26]. A cholesterol moiety enhances transfection efficiency within the myocardium over naked DNA, while reducing the toxicity seen from other non-viral carriers as LPL expression is high within cardiac muscle [27].

Several cholesterol based cationic lipids have been generated as gene carriers and have demonstrated high transfection efficiency in other mammalian cell types [28–30], but most have lacked an effective endosomal escape mechanism such as found in PEI. One polymer, however, water-soluble lipopolymer (WSLP), possesses efficient endosomal release and demonstrates the utility of such modifications in gene delivery [31].

Delivery of WSLP to the aortic smooth muscle cell line, A7R5, demonstrated similar transfection levels compared to PEI25kDa while maintaining low cytotoxicity [32]. This transfection and cell viability was also much greater than commercially available products, Lipofectamine and SuperFect. Increasing amounts of free cholesterol or LDL dramatically affected luciferase expression while PEI1.8kDa was not significantly affected, thus demonstrating cholesterol's effect on enhanced transfection through receptor-ligand interactions. Further evaluation demonstrated the superior transfection efficiency of WSLP as compared to Terplex in both A7R5 and H9c2 cell lines [33]. Interestingly, minute increases in VEGF production were demonstrated to protect these cells under hypoxic conditions. Oxygen levels were not altered with increased VEGF expression, but VEGF is known to stimulate anti-apoptotic factors to enhance survival under hypoxic conditions [34].

Upon in vivo administration of WSLP containing the gene for VEGF (WSLP/hVEGF-165) to rabbit myocardium, VEGF expression was measured at almost 50% more than naked pDNA and measurable expression was evident at 14 days post injection [32]. Bio-distribution studies demonstrated significant hVEGF within the myocardium at four days post-injection but negligible amounts within the lung, liver, or serum compared against controls (see Figure 1). These results indicate that there was little to no spread of the vector beyond the injection site, which is a concern with viral gene delivery vectors.

Efficacy of WSLP/hVEGF-165 delivery in a rabbit infarct model was determined by infarct size, increase in capillary density, decrease in apoptosis and ventricular wall thickness. A 57% decrease in the area at risk of infarct in the treated myocardium compared to ligation controls was noted using a tetrazolium-based assay. Wall thickness was significantly increased as well in the group treated with WSLP/hVEGF-165. The mechanisms underlying these results can be attributed to the increase in new blood vessel formation, as demonstrated by the increase in capillary density. The decrease in apoptotic index of the infarct border of treated myocardium may be in part due to the protective effects of VEGF itself (and its regulation of downstream anti-apoptotic factors) and the increase in neovascularization.

The modification of PEI will continue to play an important role in novel gene delivery for both local and systemic administration. Merely slight modifications based on administration route have shown increases in transfection, targeting, and toxicity. Future modifications will continue to increase our knowledge in the non-viral gene delivery field.

Biodegradable Polymers

Key modifications to increase polymeric gene delivery efficiency will incorporate molecules that have the ability to discriminate between differences in biologic microenvironments, including pH, ionic or redox potentials [35]. One type of modification has taken advantage of changes in pH to assist in targeting through the incorporation of acid-labile linkages to facilitate polymer degradation within the local environment of a tumor [36], or to assist in endosomal release of stabilizing complexes [37–42] or polyplex degradation [43–45]. The latter becomes important as polyplex unpacking is seen as a rate-limiting step for non-viral mediated gene delivery, especially for large polymer constructs [46]. These acid-labile polymers may exhibit poor transfection efficiency due to either rapid hydrolysis or poor endosomal escape, demonstrating that in the design of hydrolytically degradable carriers, a balance needs to exist between extracellular hydrolytic stability and rapid intracellular

degradation. This is further compounded by difficulties in vector handling and post-modifications of these hydrolysis sensitive polymers [45]. A more rational approach is to have the complex escape the endosome and decomplex within the cytoplasm.

Bioreducible polymers use disulfide bonds instead of ester linkages to take advantage of the differences in reductive potential between the extracellular and intracellular microenvironments in normal as well as disease states. Several novel gene carriers incorporate disulfide bonds into their structure for cargo release upon entry into the subcellular space(s) [47–50]. Delivery of nucleotides by means of redox-sensitive gene carriers has included mRNA [51], antisense oligonucleotides [39,42,52], siRNA [53] and plasmid DNA [54–58]. These studies have incorporated numerous cell lines and several disease states, but until recently have not included cardiac tissues [8].

Poly(amido amine)s, (PAA's) are considered a family of peptidomimetic polymers that are generated by Micheal type addition of primary and secondary aliphatic amines to bisacrylamide monomers [59]. As such, poly(amido amine)s (PAAs) are more hydrolytically stable than poly(amino ester)s, and have good water solubility and biodegradability. Such polymer characteristics have garnered interest for biomedical applications, including drug and gene delivery [60]. Modifications of these PAA's with carboxylic acid side groups provide transfection efficiencies similar to that of 25 kDa branched polyethylenimine (pEI) [61]. Upon protonation in the endosome, a conformational change is induced to allow escape of the polyplexes, but amide hydrolysis is too slow to completely free the DNA from the complexes in the cytoplasm [62]. Work using poly(glycoamidoamine)s has demonstrated transgene expression similar to PEI and low cytotoxicity in BHK and H9C2 cell lines, but further investigation exploiting the reductive nature of cardiac cells was never initiated [63–65].

Recently, novel poly(amido amine)s were generated containing disulfide linkages to create a highly efficient degradable gene carrier [66–68]. Differences exist in the polymer backbone, but most of the novel poly(amido amine)s have similar toxicity profiles and degradative profiles in reductive environments. Although it is difficult to directly compare transfection efficiencies amongst the polymers due to the different cell lines used, the difference in transfection between PEI and pABOL in COS-7 cells resembles that of reducible histidine and poly-lysine vectors [69]. Most relevant to this discussion, however, is the use of poly(amido ethylenimine)s to deliver DNA to cardiovascular cells, such as arterial endothelial cells and smooth muscle cells.

Reducible poly(amido ethylenimine)s (SS-PAEI)s utilize multiple disulfide linkages to decrease toxicity while exploiting the characteristics of PEI to protect and condense DNA [66]. This condensation/protection begins at w/w ratios of polymer to DNA of 6:1 and maintains a stable particle size of under 200nm and zeta-potential of +32mV over a broad range of w/w ratios (6/1 to 48/1). Reported results indicate that regardless of the cell type, the SS-PAEI's exhibited similar or higher transfection levels than bPEI 25k, with some as high as 20 times higher, with negligible toxicity. In depth fluorescent studies suggest that there may not be a difference in cellular uptake of the complexes by flow cytometry analysis [53], although the cellular distribution of the fluorescent nucleotides was significantly more diffuse in the reducible poly(amido ethylenimine)s [53,66]. Further experiments were conducted to determine if this reductive potential could be extended to other cardiovascular cell lines such as H9C2.

SS-PAEI with CMV driven eGFP transfected greater than 50% of H9C2 cells as opposed to only 11% of H9C2 cells when bPEI 25k was the polymer carrier [8]. This result correlated well when comparisons of a hypoxia responsive promoter (RTP801) driving luciferase

yielded a significant difference in expression between the two polymers. This effect also translated over to the more highly branched version of SS-PAEI, triethylenetetramine (TETA) (Figure 2a). TETA transfected H9c2 cells had significantly higher luciferase expression ($p < 0.01$) than bPEI25k (0.75:1 w/w) without the associated toxicity (Figure 2b). Interestingly, while the difference of expression among the polymers remained similar when using the RTP801 promoter driving VEGF expression, it was the difference between normoxic and hypoxic environments that was striking. Typically, a 2 to 3 fold increase under hypoxic conditions is seen regardless of the polymer used, but here VEGF expression in H9C2 cells was striking, with a 76-fold increase in expression with the SS-PAEI carrier and 22-fold increase using the bPEI25k carrier. This result was attributed to post-translational modifications by the protein kinase C pathway [72–70]. Direct myocardial injections of SS-PAEI were compared against that of WSLP, and VEGF expression with the SS-PAEI carrier was twice as high as WSLP with only half of the injected dose (100ug SS-PAEI to 200ug WSLP). Confirmation of these findings was carried out using 200ug of naked DNA, WSLP, and TETA in infarcted myocardium expressing luciferase under control of the RTP801 promoter (See Figure 3). The TETA treated myocardium expressed a greater than 5-fold increase in expression compared to WSLP and a ten-fold increase compared to naked DNA. These results demonstrate the importance of both the carrier and the plasmid on expression within the myocardium and that the combination of both should be given careful consideration.

Non-viral Targeting Vectors of Myocardium

Targeting of these reducible polymers has not yet occurred but may be accomplished through the addition of shielding molecules such as polyethylene glycol (PEG) and targeting moieties. Unfortunately, antibodies specific to cardiac surface markers are scarce. However, it was first theorized by Khaw et al that if intracellular myocardial proteins egress upon an ischemic event and can be measured in the serum, then there must also be an entry that may allow for a means of targeting [73].

This hypothesis led to the development of a cardiac myosin-specific antibody, 2G4, which has been used to facilitate the uptake of ATP by several different versions of immunoliposomes containing primarily phosphatidyl choline, cholesterol, and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and PEG [74–77]. The use of these immunoliposomes was demonstrated to efficiently transfect H9C2 cells in vitro, but in vivo experimentation never followed [78]. Others have since investigated the use of targeted immunoliposomes to the ischemic myocardium using antibodies towards P-selectin and intracellular adhesion molecule 1 (ICAM-1) but have only placed their emphasis on drug delivery [79]. With the advent of fast, efficient, and inexpensive genetic testing assays, particularly microarrays, it may be possible to elucidate novel factors that are upregulated during ischemic events that may also be used as targeting molecules.

Molecular Targeting of Myocardium

In lieu of specific targeting ligands, molecular targeting confers tissue or cellular specificity at the transcriptional level. These targeting methods are primarily used to bypass non-specific interactions of the gene vectors with other tissues [80–83]. These issues are of primary concern with all gene therapy applications and so interest remains high. Cell or tissue specific promoters are constitutively active and thus may not provide specific cues for environmental changes within tissues. In response to hypoxia, cells are drawn to the hypoxic environment via VEGF and other factors via a gradient [84]. If myocardium is transfected with a tissue-specific constitutive promoter, the gradient is never turned off and aberrant growth may occur. Temporal expression is deemed to be crucial for myocardial gene

therapy. Whereas myocardial gene therapy has placed importance on temporal expression in regards to onset or length of expression; it seems that for angiogenesis to occur properly one should place more emphasis on promoters and enhancers involved in that particular process. Numerous hypoxia-related cis- and trans- elements have been described over the years. Recent work using the RTP801 promoter to drive hVEGF-165 has demonstrated efficacy with regards to a decrease in infarct size, a decrease in apoptosis, an increase in capillary density and an increase in ventricular wall thickness in an acute myocardial infarct rabbit model, when compared to ligation only controls and hVEGF-165 driven by the constitutive promoter SV40 [33]. More evidence is required to directly correlate the effects of this ischemia driven promoter to its observed effects in vivo; however, it appears to be a promising component towards the goal to guide and promote therapeutic angiogenesis.

Studies elucidating molecular mechanisms of vascular development have been guided by discoveries of key mitogens for cardiovascular cells. Several of these factors have been or are in use in clinical trials today [17] based on gene targeting experiments supporting a simplified paradigm of vascular formation. Evolution of this idea is now focusing on understanding blood vessel formation as an integrated network that defines not only capillaries from arteries and veins by size and location but the molecular mechanisms involved as well. This paradigm has similarities to the neurobiologists' idea of integrative circuitry within the neural system. In fact, it has become increasingly clear that many of the factors involved in neural guidance cues coincide with angiogenesis [85].

Netrin-1 was demonstrated to be a potent mitogen and chemotactic agent for human vascular cells on par with the most widely used agent, VEGF. Vascular sprouting was observed in the chorioallantoic membrane and corneal micropocket assays. Since then, Netrins have been demonstrated to guide developmental and therapeutic angiogenesis in the rat hind-limb ischemia model [86]. The identification of this neural guidance cue provides us with opportunities to advance our knowledge of vascular regeneration in the hopes of ischemic tissue restoration. To this end, we inserted Netrin-1 downstream of the RTP801 promoter and created the pRTP801-Netrin-1 plasmid to investigate its role in reducing infarct size and restoring ventricular wall thickness in comparison to its proven counterpart, pRTP801-hVEGF, in the rabbit acute myocardial infarct model.

WSLP was complexed to 200ug of the pRTP801-Netrin-1 plasmid and 200ug of the pRTP801-hVEGF plasmid and injected into the ischemic border zone following ligation of the circumflex coronary artery. Twenty-eight days following injection, hearts were excised and photographed prior to picric acid staining to determine infarct size. Photographs of the epicardial surface demonstrate the powerful effect that the mitogenic agents have on the vasculature surrounding the injection sites (marked with sutures) (Figure 4). Pale white surfaces demarcate scar tissue as well as loss of muscle fibers within the ligation controls (Figure 4a). The pRTP801-VEGF165 treated hearts (Figure 4b) do not demonstrate either of these findings and overall seem to represent relatively well-preserved myocardium. The pRTP801-Netrin1 treated hearts (Figure 4c) have accumulated large vascular eruptions that have migrated through to the endocardial surface (Figure 4d). The limited diffusive pattern was not completely unexpected as the Netrins have several integrin binding domains that most likely play a part in its role of axonal guidance and vascular patterning [87]. This transmural movement correlated with increased ventricular wall thickness in the Netrin treated hearts compared to the VEGF treated and ligation only controls and comparable wall thickness in the Netrin treated hearts to the uninfarcted controls (Figure 5a). The pRTP801-VEGF165 treatment yielded significant results in terms of preservation of LV wall thickness compared to ligated controls but did not prevent a significant decrease in wall thickness compared to the uninfarcted controls. pRTP801-Netrin1 treatment, however, failed to yield significant decreases in left ventricular infarct size (Figure 5b). The presence of thickness of

the wall due to the increased vascular beds may signify aberrant growth. Netrin has been shown to demonstrate dual functionality regarding angiogenesis by either inducing proliferation or inhibition depending on its concentration [88]. Dose response curves and functional studies are crucial to provide a more complete analysis of Netrin1's ability in restoration of ischemic and infarcted myocardium. By itself, Netrin1 expression under hypoxic control was insufficient to affect a global change as seen with VEGF. One factor alone may not be sufficient to induce therapeutic angiogenesis. Thus, the use of new angiogenic factors with different biological profiles from previously discovered mitogens is important. Netrin1's ability to maintain LV wall thickness signifies that, in combination with VEGF, a synergistic response may be achieved in infarcted myocardium as seen in the CAM assay [85].

Conclusion

The work described in this article demonstrates current advances in the field of non-viral polymers for therapeutic gene delivery to the myocardium. It is essential that continuing innovations in non-viral gene delivery, molecular and cellular biology be combined to increase acceptance of non-viral polymeric gene delivery as a viable option for the clinical treatment of ischemic myocardium.

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Figure 1. Direct Injection of RTP801-VEGF/WSLP Complexes into Infarcting Myocardium Remains at Injection Site

A left thoracotomy was performed on 5 New Zealand White rabbits and the proximal circumflex coronary artery was ligated. Immediately following ligation, rabbits were injected with 200ug of RTP801-hVEGF complexed to WSLP in a 10:1 N:P ratio (5 total injections) surrounding the area at risk. The rabbits were sacrificed four days later and tissues were excised and blood collected. ELISA was performed on homogenized tissues using the Quantikine human VEGF ELISA kit (R&D Systems, Burlingame, CA). The heart was the only organ that demonstrated any significant hVEGF protein above control values. The lung, liver and plasma showed little to no evidence of hVEGF protein.

Figure 2. The bioreducible polymer, TETA, shows superior transfection characteristics over bPEI25k in cardiovascular cells

H9c2 cells were grown to 60% confluency in 24 well plates with complete media (DMEM + 10% FBS). Complete media was replaced with serum-free DMEM and wells were transfected with 450ng pCMV-Luc complexed with either bPEI25k at 0.75:1 (w/w) or TETA at 6 or 12:1 w/w. (a) Cells were lysed and luciferase was analyzed 48 hrs later. Luciferase activity in TETA transfected cells were highly significant ($p < 0.01$, One-way ANOVA) in comparison to bPEI25k and controls. (b) Viability was measured by MTT assay 20 hrs following transfection. Toxicity was not evident in TETA transfected groups while bPEI25k showed 25% toxicity.

Figure 3. TETA exhibits significant reporter gene expression in infarcted rabbit myocardium

A left thoracotomy was performed on 5 New Zealand White rabbits and the proximal circumflex coronary artery was ligated. Immediately following ligation, rabbits were injected with 200ug of RTP801-Luc alone or complexed to either WSLP in a 10:1 N:P ratio or TETA in a 6 or 12:1 w/w ratio (5 total injections) surrounding the area at risk. The left ventricle of the hearts were excised, weighed and homogenized, four days following injection. Luciferase activity was measured and normalized against tissue weight. Differences between the groups were statistically significantly ($p < 0.05$, One-way ANOVA). Luciferase expression was almost 10x greater than naked DNA alone and 4x greater than WSLP.

Figure 4. Ischemia-driven Netrin1 Expression Directs Transmural Capillary Formation in Ischemic Rabbit Myocardium

A left thoracotomy was performed on 5 New Zealand White rabbits and the proximal circumflex coronary artery was ligated. Immediately following ligation, rabbits were injected with 200ug of RTP801-hVEGF or RTP801-Netrin1 complexed to WSLP in a 10:1 N:P ratio (5 total injections) surrounding the area at risk. The rabbits were sacrificed four weeks later and the hearts excised to assess the area at risk of infarct and wall thickness. Representative photographs of the excised hearts demonstrate significant scarring in the left ventricle of ligation only controls (a), minor scarring of the left ventricular wall of the RTP801-hVEGF treated group (b), and significant neo-vascular eruptions around each injection site (blue sutures) in RTP801-Netrin1 treated animals (c). The endocardial surface of the RTP801-Netrin1 treated hearts shows transmural neo-vascular eruptions corresponding to the epicardial surface.

Figure 5. Ischemia-driven Netrin1 Expression Decreases Infarct Size and Increases Wall Thickness

Excised hearts were stained with 10% Formalin and saturated picric acid for 48hrs. All images were captured at a predetermined fixed focal length and focal distance such that a full-width 4368 pixel image captured a focal field width of 86mm. (a) Contrast was enhanced and brightness adjusted with parameters recorded, areas of the image not relevant to the analysis were bounded out and blackened, thresholding was performed to yield a binary image of black scar tissue on white background, outlining of the black (scar) areas was performed, and the “Analyze Particles” function was used to quantitate the pixels inside the outline. This permitted conversion of the resulting scar tissue area in pixels, A_{pxl} to an

affected area in mm^2 , A_{mm^2} , using the conversion $A_{\text{mm}^2} = (86\text{mm})^2 \times A_{\text{pxl}} / (4368\text{pxl})^2$. (b) Wall thickness was measured using a Marathon electronic digital micrometer (Marathon Watch Co., Ontario, Canada) at the base of papillary muscles of the left ventricle. The micrometer was adjusted so that the myocardial wall was immobile between the micrometer poles but negligibly compressed or distorted.