LANDMARK PROJECT – 2010 Annual Report

Project number: 98I006
Project title: The NCKU-UW Cardiac Stem Cell Bioengineering Consortium
Submitting organization: Institute of Clinical Medicine, NCKU
Period covered: From 08-01-2008 to 07-31-2009
Grant amount: NT$ 7,996,000
Grant utilized: NT$ 7,956,830
Date of report: AUGUST 31, 2010

Principal investigator and Co-PI's names, titles and organizations:

PI: Patrick C.H. Hsieh, M.D., Ph.D. Assistant Professor & Attending Surgeon Institute of Clinical Medicine & Research Center of Clinical Medicine, NCKU & Hospital

Co-PI: Fong-Chin Su, Ph.D. Distinguished Professor & Associate Dean Institute of Biomedical Engineering, NCKU

Charles E. Murry, M.D., Ph.D. Professor & Director Dept. of Bioengineering and Pathology and Cardiovascular Research Center, University of Washington in Seattle
1. **Itemized goals of the project:**
   
   A. To identify novel cardiac stem cell population(s) for therapy.
   
   B. To develop novel nanomaterials for cardiomyocyte-targeted drug delivery.
   
   C. To conduct large animal studies & clinical trials of cardiac delivery of nano-drugs and stem cells for cardiac regeneration.

2. **Achieved “Landmark” accomplishments directly related to this project and how it is compared to the state-of-the-art (no more than 750 words in total):**

   Although the field of cardiac stem cell therapy has significantly advanced over the past years, the premise of regenerating or replacing diseased human myocardium with functional tissue remains unsatisfied. The overall objective of the present international multi-disciplinary program project is to promote myocardial stem cell therapy through nano-/micro-environmental engineering the intramyocardial stem cell niche for *endogenous* cardiac stem cells to reactivate, proliferate, differentiate and repopulate the diseased myocardium. During the past year, the most distinguished accomplishments of this project are—

   1. Demonstrating an early endogenous stem cell replenishment of cardiomyocytes after infarction using cardiac specific inducible Cre-Lox transgenic mice, and discovering that prostaglandin E2 may promote this endogenous cardiac stem cell signaling for regeneration. (Top of the world)
   
   2. Accomplishing a pig model of cardiac regeneration using injectable self-assembling peptide nanofibers and autologous bone marrow stem cells. (Accepted by *Circulation* for publication; top of the world)
   
   3. Achieving the maturation of embryonic stem cell-derived cardiomyocyte progenitors (ES-CP) which is translated into approaches for cardiac repair using co-transplantation of ES-CP and endothelial cells. (Manuscript will be submitted soon; top of the world).
   
   4. Develop novel heart-targeting nano-drugs to improve cardiac regeneration. (Manuscript will be submitted soon; top of the world).
   
   5. Demonstrating that injection of hyaluronan along with endothelial cells promotes angiogenesis after hindlimb ischemia. (Accepted by *Biomaterials* for publication; top in Taiwan).

   Although this research field has been very competitive, these results are the best in the world and we expect to publish 3-4 high impact papers soon. This is based on our results, both at NCKU and at UW-Seattle, showing that endogenous stem cells may regenerate the injured heart, albeit not sufficient to restore the normal heart function (Hsieh et al. published in *Nature Medicine* in 2007, a top journal in experimental medicine) and a combined tissue engineering and stem cell therapy may improve cardiac repair (published in *Nature Biotechnology* in 2007 from Murry’s group, a top journal in biotechnology & bioengineering). And now, we further extend this excitement and pursue to engineer the cardiac nano-/micro-environments for cardiac stem cell therapy using biologically functionalized nanomaterials combined with controlled release of therapeutic molecules. We have employed a team approach which brings together investigators from a broad variety of fields at NCKU and UW, including biochemistry and molecular biology, stem cell biology, biophysics and...
biomechanics, synthetic and biochemical engineering, and clinical medicine. This partnership has allowed us to collaborate and interact in a way that traditionally an individual laboratory or program does not provide.

3. Which items stated above are the best in the world, Asia, or Taiwan?
The first 4 items are the best in the world and the item 5 in Taiwan.

4. Extramural funding currently active, applied or to be applied for supporting the work or related subjects stated above.

- NSC 97IR082 (Patrick Hsieh, PI) 05/01/08 - 04/30/11 (NT$10,756,000)
  Functional Genomics of Regenerative Cardiomyocytes Derived from Stem Cells.
- NHRI EX97-9722SI (Patrick Hsieh, PI) 01/01/08 - 12/31/11 (NT$11,930,000)
  Mechanisms of Cardiomyocyte Regeneration by Stem Cells Using Inducible Cre-Lox Transgenic Mice.

5. Utilization of grant for this year: (Units: Thousand NT Dollars; %)

<table>
<thead>
<tr>
<th>Category</th>
<th>Amount Spent</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consumables</td>
<td>6,145,375</td>
<td>77.23%</td>
</tr>
<tr>
<td>Equipments</td>
<td>1,300,000</td>
<td>16.34%</td>
</tr>
<tr>
<td>International travel</td>
<td>511,455</td>
<td>6.43%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>7,956,830</strong></td>
<td><strong>100%</strong></td>
</tr>
</tbody>
</table>

6. List names of faculty and researchers funded by this project and amount of support.

Patrick Hsieh ($2,756,830)
Jyh-Hong Chen ($600,000)
Yu-Jen Yang ($600,000)
Chwan-Yau Luo ($2,00,000)
Ming-Long Yeh ($2,000,000)

7. Publications by faculty and researchers list above based on work supported by this grant (indicate impact factor, journal ranking and citation number):


• Chiu CT, Wang SP, Shieh DB, Ho CC, Cheng FY, Su CH, Chiu HC, Yeh CS*, Hsieh PC*. “Functional nanoparticles in biomedical applications”. In the *Encyclopedia of Nanoscience and Nanotechnology, J Nanosci Nanotechno*. 2010; in press. (IF=1.435, 86/212)


8. List of invited presentations by faculty and researchers shown in item 6:

**Conference invited speech and chairman (selected)**


9. List all currently funded contracts and grants for PI and each faculty and researchers funded by the project shown in item 8.

1. NSC 97IR082 (Patrick Hsieh, PI), period--05/01/08-04/30/11, title—“Functional Genomics of Regenerative Cardiomyocytes Derived from Stem Cells”.

2. Industrial Technology Research Institute FY97A4 (Corporate Grant; Patrick Hsieh, PI), period--01/01/07-12/31/11, title—“Chemotaxic Control of Stem and Cancer Cells Using Nano-/Micro-Systems”.

3. NHRI EX97-9722SI (Patrick Hsieh, PI), period--01/01/08-12/31/11, title—“Mechanisms of Cardiomyocyte Regeneration by Stem Cells Using Inducible Cre-Lox Transgenic Mice”.

4. NSC 97-3111-B-006-005 (Patrick Hsieh, PI; PPG Project #4), period--12/01/08-11/30/11, title—“Therapeutic Study of Stem Cells in Hyaluronan-Based Tissue Gels for Neovascularization in Ischemic Myocardium”.

5. NSC 99-2323-B-006-003 (Hua-Lin Wu, PI; Patrick Hsieh, Co-PI), period--08/01/09–07/31/11, title—“A Method for the Treatment of Congestive Heart Failure”.

6. NSC 99-2627-M-006-009 (Yu-Jen Yang, PI; Patrick Hsieh, Co-PI), period--08/01/09–07/31/12, title—“Novel nanovectors for gene/drug delivery in cardiovascular regeneration”.

7. NSC 99-2628-B-006-029-MY3 (Jyh-Hong Chen, PI; Patrick Hsieh, Co-PI), period--08/01/10–07/31/13, title—“Embryonic stem cell therapy for myocardial regeneration”.

10. Appendices (reprints of most important recent papers related to this project published by PI & Co-Pls)


Dear Dr. Hsieh,

I am pleased to tell you that your revised manuscript has now been accepted for publication in Biomaterials. Elsevier will be in touch with you shortly regarding proofs and production.

Thank you for submitting your work to Biomaterials.

With regards,

Prof. D.F. Williams, F.R.Eng
Editor-in-Chief
Biomaterials

Biomaterials is once again the leading journal in its field. Impact factors released by ISI in June 2010 showed Biomaterials with an impact factor of 7.365
The Enhancement of Endothelial Cell Therapy for Angiogenesis in Hindlimb Ischemia Using Hyaluronan

Zack C.W. Tang a, Wei-Yin Liao a, Alan C.L. Tang a,
Shih-Jung Tsai b, Patrick C.H. Hsieh a.c.*, 

a Graduate Institute of Clinical Medicine, Research Center of Clinical Medicine and
Department of Surgery, National Cheng Kung University & Hospital, Tainan 70428, Taiwan, R.O.C.;
b Nano-Powder & Thin Film Technology Center, Industrial Technology Research Institute, Tainan, Taiwan, R.O.C.;
c Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, R.O.C.

Correspondence to Patrick C.H. Hsieh, MD, PhD.
Graduate Institute of Clinical Medicine, National Cheng Kung University & Hospital,
138 Sheng-Li Road, Room 7063, Tainan 70428, Taiwan, R.O.C.

E-mail address: phsieh@mail.ncku.edu.tw

Short title: HA-endothelial cell therapy in ischemia.

Word count: 5183

Display: 8 figures

Supplemental materials: 5 supplemental figures
Abstract

Growing evidence shows that injection of hyaluronan (HA) benefits ischemic injury in animals. On the other hand, cell therapy is an emerging approach to treat occlusive arterial diseases, although the low retention rate of cells after direct injection remains a major concern. Here, we tested whether injection of HA along with endothelial cells promotes the retention and growth of transplanted cells, thus improving therapeutic angiogenesis in a mouse model of hindlimb ischemia (HI). In culture, HA improved human umbilical vein endothelial cell (HUVEC) proliferation proportional to HA concentration and protected HUVECs from apoptosis. Subsequently, in immunocompromised mice HI was induced by femoral artery ligation and treatments were given 24 h later. At 4 weeks, injection of HA along with HUVECs had a greater effect for restoring blood perfusion and salvaging the ischemic limb compared to injection of HA or HUVECs alone. In addition, angiogenesis and arteriogenesis were significantly increased by HA + HUVECs injection. Lastly, HA + HUVECs injection resulted in the retention of more cells than HUVECs alone, and allowed their engraftment into the vasculature of the ischemic limb. These results suggest that this combined approach can be translated into a clinical therapy for peripheral artery occlusive disease.

Key words: angiogenesis, endothelial cell, hyaluronan
1. Introduction

Peripheral artery occlusive disease (PAOD), the narrowing or blockage of distal limb arteries, affects ~10 million people in the US. Without effective treatment, PAOD may progress to critical limb ischemia and result in limb loss. Hence, the goal of PAOD treatment is to improve blood supply in the ischemic region, and thus relieve pain, heal ulcers, prevent limb loss, and prolong survival [1].

Previous studies have shown that administering cells is a promising approach for therapeutic angiogenesis as they may produce angiogenic cytokines and participate in vascular regeneration [2-4]. Recent clinical trials have also demonstrated the feasibility and safety of autologous stem cell therapy for PAOD [5-8]. However, the long-term results of these clinical studies remain undetermined and the potential for tumor formation is a concern [9,10]. Another major obstacle is the limited retention of viable cells following transplantation. Evidence indicates that the vast majority of transplanted cells neither survive for long after injection nor remains within a specific location [11-13]. To solve this problem, material-based deployment using hydrogels has been investigated, especially in promoting the efficacy of endothelial progenitor cells [14].

Hydrogels are advantageous serving as cell and drug carriers as well as tissue matrices due to their ability to absorb water and permeate solutes within the swollen matrices. Concerns regarding synthetic polymers inducing non-physiologic cellular responses have caused a shift of interest to using more natural materials, such as stand-alone products, chemically derived, physically derived, or extracellular matrix-derived materials. Prominent examples include collagen [15], fibrin [16], alginate [17], gelatin [18], and hyaluronan (HA), of which HA may be the most biocompatible and biodegradable natural material.
HA is a component of the extracellular matrix, composed of repeating units of disaccharide and \([-\beta\text{-glucuronic acid-}1\rightarrow3-\beta\text{-N-acetylglucosamine-}1\rightarrow4-\]_n, making up a glycosaminoglycan polymer straight chain. It is found primarily in the synovial fluid, cartilage, blood vessels, skin, and umbilical cord. Increasing evidence suggests that HA initiates and regulates angiogenesis, and it has been used as a pro-angiogenesis tool for tissue engineering [19,20]. Since it influences tissue hydration and swelling pressure, HA creates a fluidic and malleable matrix in which cells can change shape during mitosis or penetrate tissue as they migrate [21]. In addition, with the ability to imbibe water 1000 times its own volume, the massive free water space that HA provides permits a better microenvironment for nutrient and waste exchange in contrast to synthetic materials [22]. As a structural component that plays a critical role in blood vessel physiology and pathology, HA regulates the behavior and biomechanical properties of blood vessels. It is well known that HA has various molecular sizes and each regulates specific essential cell behaviors. For example, long-chain HA polymers can be degraded into smaller fragments that stimulate endothelial cell proliferation and promote angiogenesis [23].

Therefore, we hypothesized that an optimal concentration of HA increases proliferation of endothelial cells and protects them from apoptosis. We then tested if intramuscular injection of HA along with endothelial cells promotes angiogenesis, and consequently results in the saving of the ischemic limb and restoration of the blood flow in a mouse model of hindlimb ischemia.
2. Materials and methods

2.1. Hyaluronan solution preparation

Hyaluronan powder (1630 kDa; Sigma) was blended in distilled phosphate buffered saline (PBS) at 4°C for 24 h to achieve total hydration. Next, 1% HA was diluted with sterilized PBS into a series of concentrations (0.1, 0.25, 0.5, and 0.75% by volume) and stored at 4°C before use. Gelatin (1%) as the control treatment was prepared with gelatin powder (Sigma) in PBS, followed by immediate sterilization.

2.2. Umbilical vein endothelial cell isolation and culture

Human umbilical vein endothelial cell (HUVEC) isolation was carried out according to a published protocol [24]. Cells were cultured in M199 medium containing 20% fetal bovine serum (Invitrogen), 5% heparin and 1% endothelial cell growth supplement (Biomedical Technologies Inc.). HUVECs were identified by morphology, immunocytochemical staining for von Willebrand factor (Chemicon), and Matrigel assay (Supplemental Figure 1).

2.3. Cell growth, proliferation, apoptosis and viability

HUVECs (1×10⁴) were cultured on 12-well culture dishes pre-coated with a series of concentrations of HA, and maintained at 37°C and 5% CO₂ for 24 or 48 h 1% gelatin was used as the control group. Cell counting was performed under light microscopy. For the proliferation and apoptosis assays, HUVECs were labeled with propidium iodide (PI) and then evaluated by flow cytometry. Cell apoptosis was induced by multiple approaches, including adding 0.5 μg/ml Puromycin (Invitrogen), 2.5 μM Doxorubicin (Sigma), or serum-free M199 medium, for another 24 h. Cell viability was measured with the MTT assay (Sigma).
2.4. Western blot assay

Whole-cell extracts were collected using a lysis buffer containing 1% (w/v) SDS, 50 mM Tris-Cl (pH 7.4), 5 mM EDTA supplemented with 4× sample buffer (Invitrogen) and proteinase inhibitor cocktail (Sigma) at 1:500 dilution. The following antibodies were used: anti-phospho-ERK, anti-phospho-Akt, and anti-cleaved caspase-3 (Cell Signaling).

2.5. Experimental animals

The National Cheng Kung University Animal Care and Use Committee, and the National Laboratory Animal Center approved all animal research procedures. Nude mice of either sex (3 months old, weight 28 ± 0.6 g) from the National Laboratory Animal Center were used. All animals received anesthesia with Zoletil (50 mg/kg; Virbac, France) and Rompun (0.2 ml/kg; Bayer Healthcare, Germany) before surgery and in vivo measurements.

2.6. Hindlimb ischemia, treatment, and blinding

All procedures were carried out blinded and randomized. The overall surgical mortality rate was 0%. A total of 30 nude mice were divided into 5 groups: 1. sham, in which the left limb was opened without femoral artery ligation (sham group); 2. the left femoral artery and iliac artery were ligated and then cut to induce hindlimb ischemia, then 200 μl PBS was injected intramuscularly (PBS group); 3. Same as for the PBS group (2), but with intramuscular injection of 1% HA (HA group); 4. Same as for the HA group (3), but with injection of 10⁶ HUVECs suspended in PBS (PBS/HUVEC group); 5. Same as for the PBS/HUVEC group (4) but with injection of a mixture of HUVECs and 1% HA (HA/HUVEC group). All these procedures were carried out 24 h after surgery and the injections were made into 4 delivery sites in the ischemic area (50 μl for each site).
2.7. Clinical score and laser Doppler blood flowmetry

Treatment effectiveness was evaluated by gross examination and measuring blood flow to the distal thigh and calf muscles. Limb condition was categorized into several levels at different time points (see Supplemental Figure 2). Microvascular blood flow was measured by laser Doppler (O2C flow meter, LEA Medizintechnik, Giessen, Germany). The blood flow before operation, immediately afterwards, and every week for the following 4 weeks were recorded from both limbs. The data were presented as the ratio of the ischemia limb (left) to the normal limb (right).

2.8. EGFP labeling, cell survival and localization

HUVECs were transfected with a lentiviral vector carrying the enhanced green fluorescent protein (EGFP) plasmid (pAS2.EGFP.puro). Cells were then purified with M199 culture medium containing 0.5 μg/ml Puromycin. EGFP+HUVECs were cultured for at least 3 more passages before use. Cell function was tested with a Matrigel assay (see Supplemental Figure 5). A total of 10^6 EGFP+HUVECs were mixed with 200 μl PBS or 1% HA (n = 6 in each group), and then injected into the ischemic muscles. At the end of 4 weeks, immunohistochemistry was performed to examine cell retention, viability and engraftment.

2.9. Immunohistochemistry and immunofluorescent staining

The distal thigh and calf muscles were fixed in 4% paraformaldehyde, dehydrated, and paraffinized. Fixed samples were deparaffinized, rehydrated, and boiled in 10 mM sodium citrate (pH 7.4) for 10 min, followed by incubation with antibodies against von Willebrand Factor (vWF; Chemicon), VE-cadherin (Invitrogen), SM22α (Abcam), tropomyosin (CH1; DSHB), and GFP (MBJ) at 4°C overnight, and then incubated with Alexa Fluor® 488- or 568-conjugated secondary antibodies (Molecular Probes, Invitrogen). After counterstaining with DAPI (Sigma-Aldrich), sections were mounted and observed under fluorescence microscopy. The capillary and small artery densities were measured and images were taken from 10 randomly-selected ischemic areas.
(200× magnification) in each sample, and blinded quantification was performed in triplicate by manually counting within each section; the 10 values were then averaged.

EGFP⁺HUVECs were measured from images taken from 6 randomly-selected areas (200× magnification) and blinded quantification was performed by manually counting the EGFP⁺HUVECs with or without overlapping VE-cadherin, SM22α, or tropomyosin staining within each section.

2.10. Statistical analysis

All data are presented as mean ± standard deviation. Statistical analysis was performed with analysis of variance (ANOVA) with Bonferroni adjustment. A probability value of $P < 0.05$ was considered statistically significant.
3. Results

3.1. Effect of HA on endothelial cell growth

To investigate whether HA supports endothelial cell growth, we performed in vitro tests as described above. Cell numbers increased proportionally to HA concentration after 24- and 48-h incubation (Figure 1A). HA at 1% (162 ± 37% at 24 h; 235 ± 47% at 48 h) had the optimal effect, especially when compared to 1% gelatin (Figure 1B). Similar results were obtained by PI staining with flow cytometry for cell proliferation (Figure 1C), 35 ± 1.4% at 24 h and 34 ± 1.7% at 48 h for 1% HA; 30 ± 1.9% at 24 h and 25.6 ± 3.1% at 48 h for 1% gelatin (Figure 1D). Consistent with these results, we found that HA induced ERK and Akt phosphorylation in HUVECs, both of which regulate cell proliferation (Figure 1E).

3.2. Effect of HA on endothelial cell apoptosis

As anti-apoptosis is another essential issue in ischemia, we stimulated apoptosis in vitro to investigate whether HA protects endothelial cells from apoptosis. Using a serum-free medium to induce apoptosis, we found a much higher cell density in 1% HA, serum-free medium than in 1% gelatin, serum-free medium (Figure 2A). The MTT assay showed higher viability in 1% HA, serum-free than that in 1% gelatin, serum-free (Figure 2B). HA had a remarkable effect on protecting HUVECs from apoptosis induced by serum-free medium as evaluated by flow cytometry (Figure 2C, 2D). Furthermore, Western blotting (Figure 2E) showed that HUVECs cultured with HA showed attenuated cleavage of caspase-3, thus helping to prevent apoptosis. The anti-apoptotic effect was assessed by two other approaches, which both showed similar results (Supplemental Figures 3 and 4).
3.3. In vivo effects of HA/HUVEC injection

Since HA enhanced endothelial cell proliferation and decreased apoptosis in vitro, we investigated the in vivo effects of HA injection along with HUVECs in hindlimb ischemia. Each week after treatment, the control group (PBS injection) with nude mice showed serious gangrene, resulting in amputation of the ischemic hindlimb (Figure 3A). Intramuscular injection of HA along with HUVECs significantly reduced limb gangrene or occurrence of amputation (Figure 3A), and decreased the clinical score (Figure 3B) compared with the control group. At 4 weeks, all mice injected with PBS had the highest scores and no case of limb salvage (Table 1). Mice injected with PBS/HUVEC had the second highest score and no limb salvage (Table 1). HA-injected mice had the second lowest score and some showed limb salvage (Table 1). Remarkably, mice that received HA/HUVEC injection presented the lowest clinical scores and had mild limb atrophy, although some had gangrenous toes (Figure 3A).

3.4. Blood flow recovery by HA/HUVEC injection

Laser Doppler flowmetry revealed that the ratio of blood flow in the ischemic to the normal limb improved beginning at 2 weeks after injection in the HA/HUVEC group compared with the PBS group (58.95 ± 2.02% in HA/HUVEC group; 38.08 ± 2.75% in PBS group; Figure 4). By week 3, blood flow had recovered to around 70% in the HA/HUVEC group (71.57±7.11%) and was higher than the other groups (57.31 ± 2.95% in PBS group; 63.99 ± 6.22% in HA group; 61.38 ± 4.04% in PBS/HUVEC group; Figure 4). After 4 weeks of treatment, the HA/HUVEC group showed a remarkable recovery of blood perfusion (79.92 ± 3.45%) compared to the other groups (57.59 ± 11.29% in PBS group; 58.66 ± 6.35% in HA group; 67.27 ± 7.72% in PBS/HUVEC group; Figure 4).

3.5. Angiogenesis by HA/HUVEC injection
To investigate if transplantation of HUVECs with HA enhances new vessel formation in hindlimb ischemia, we performed immunohistological staining of the endothelial markers VE-cadherin (Figure 5A-E) and vWF (data not shown), and quantified the capillary density in the ischemic regions. We found that HA/HUVEC injection increased capillary density (863 ± 101 capillaries/mm²) compared with the other groups (635 ± 41 capillaries/mm² in PBS; 652 ± 22 capillaries/mm² in PBS/HUVEC; Figure 5F). Interestingly, injection of HA alone increased capillary density modestly (720 ± 131 capillaries/mm²; Figure 5F), but this was not significantly different from the HA/HUVECs group.

3.6. Arteriogenesis by HA/HUVEC injection

In addition, immunohistological staining for smooth muscle 22α (Figure 6A-E) and α-actin (data not presented) and quantification of small artery density showed that arterioles increased in the HA/HUVEC group (58 ± 21 arterioles/mm²; Figure 6F) compared with the other groups (27 ± 5 arterioles/mm² in PBS; 35 ± 8 arterioles/mm² in PBS/HUVEC; Figure 6F). Similar to capillary density, the HA group showed modestly increased arteriole density (40±13 arterioles/mm²; Figure 6F) but this was not significantly different from the HA/HUVEC group.

3.7. Transplanted cell retention by HA/HUVEC injection

To investigate cell retention and to track cells, we transfected HUVECs with EGFP, and then purified the cultures with Puromycin. More than 90% of the HUVECs expressed EGFP, as detected by flow cytometry (Supplemental Figure 5B). Moreover, EGFP⁺HUVECs were identified by their morphology (Supplemental Figure 5A) and tubular formation on Matrigel culture (Supplemental Figure 5C). After EGFP⁺HUVECs were injected into ischemic muscles, the cell retention ratio was measured by immunostaining for EGFP costained with tropomyosin (Figure 7A). Many more EGFP⁺HUVECs were retained in the ischemic muscles 4 weeks after
HA/HUVEC injection (81.83 ± 8.4 cells/mm²), compared with the PBS/HUVEC group (2.1 ± 0.7 cells/mm²; Figure 7B).

**3.8. Vascular integration by HA/HUVEC injection**

To further examine if transplanted HUVECs integrated into host vessels or contributed to new vessel formation, we performed immunostaining of SM22α and VE-cadherin, overlapping with EGFP+ cells (Figure 8A). EGFP+ cells were seen to integrate into the endothelial layer of pre-existing vessels (Figure 8A) and contributed to new vessels formed entirely from the EGFP+HUVECs (Figure 8B). Interestingly, both of these were detected only in sections from the HA/HUVEC group, but not in the PBS/HUVEC group.
4. Discussion

In the present study, we demonstrated that treatment with HUVECs in combination with HA enhanced angiogenesis and arteriogenesis in a mouse model of critical hindlimb ischemia. The addition of HA prolonged the degree of cell retention, assisted mature HUVECs to survive and engraft into endothelium, recruited host smooth muscle cells, and reduced ischemic limb loss 4 weeks after they were transplanted into the ischemic muscles.

Apart from their potential to become functional, HUVECs are easier to obtain than embryonic or induced pluripotent stem cell- or other somatic stem cell-derived endothelial cells for cell therapy. This also eliminates ethical concerns over the usage of embryonic stem cells. Furthermore, embryonic or induced pluripotent stem cells may induce uncontrollable and unpredictable teratomas [24,25]. To protect the cells, biomaterials have been used to achieve a controlled environment that houses endothelial cells [26-28]. Our in vitro data show that HA not only supports HUVEC proliferation, but also provides anti-apoptotic effects. Indeed, studies from Edelman’s group have strong evidence demonstrating matrix-embedded endothelial cells are well protected from the different vigorous environments [26,28]. To increase cell retention and functional ability for angiogenesis treatment, HA was chosen to serve as the matrix in combination with HUVECs because it has superb qualities, and therefore has potential for clinical utilization.

Toole et al reported similar results showing that prolonged culture (weeks) with both HA-1500 and HA-oligo contributes to endothelial cell proliferation and achieves rapid endothelialization [23], similar to our short-term (hours) results. Our data showed that a larger HA (1630 kDa) at a specific concentration (1%) promoted HUVEC proliferation and activated ERK and Akt phosphorylation. Also, cell proliferation was dependent on HA concentration, showing that HA affects the behavior of endothelial cells. Moreover, apoptosis is a major issue, since
insufficiency of oxygen and nutrients induce apoptosis. Therefore, we induced apoptosis to estimate the anti-apoptotic ability of HA. The data showed that HA protected HUVECs from apoptosis. However, the specific mechanisms remain unclear. Studies have suggested that stimulation of several pathways, such as survivin [29] and caspase-3 [30], protect endothelial cells from apoptosis. Our results showed that HA decreased cleaved caspase-3 expression and prevented apoptosis in HUVECs. Considering these results, HA is a candidate for improving endothelial cell therapy in hindlimb ischemia.

In contrast to the findings of Toole et al, who concluded that smaller HAs have better angiogenic effects [23], our data showed that a large HA polymer increased capillary number after ischemic muscles were treated with HA, or HA with HUVECs. The capillary density in muscles receiving HA along with HUVECs was significantly higher than after PBS treatment. While Toole et al. terminated their in vivo measurements after 2 weeks, we found that the blood flow started to increase at week 2 and continued thereafter, suggesting that angiogenesis had reached a critical level by that time. What is more, we found the arterial density in the HA/HUVEC group was markedly enhanced. Subsequent to angiogenesis, newly-formed vessels with endothelial lining require smooth muscle cells to stabilize the nascent vessels [31]. Our results showing increased arteriogenesis suggest that HA may assist in attracting smooth muscle cells or in enhancing endothelial cell secretion of angiogenic factors. These properties of HA combine to help smooth muscle cells migrate much faster and form a stable artery in a shorter period of time, compared to other treatment groups [2].

Accordingly, angiogenesis and arteriogenesis promoted by HA/HUVEC injection might lead to faster and better repair of the ischemic limb and recovery of blood perfusion. Still, there might be a further factor participating in the repair of an ischemic limb. Studies suggested that cell-based therapy is beneficial to ischemic tissue not only because of the involvement of cells in
vascularization but also for their ability to release angiogenic factors [2-4]. More specifically, Edelman’s group have shown matrix embedded endothelial cells act as regulators that provide complex molecules that may result in vascular repair [27]. Our data actually support the hypothesis that the “paracrine effect” may be the main mechanism in the early stage. However, the mechanism is unclear and we intend to continue working on this. HUVECs mixed in HA might have a similar paracrine effect, releasing angiogenic factors. The blood flow and clinical appearance showed different results in the first week, suggesting that the paracrine effect acts in the early stage of ischemia to prevent muscle from rapid atrophy.

The strategy of transplanting HUVECs combined with HA was so successful that blood flow was improved significantly more than the PBS control group (Figure 4); mice that received HA/HUVEC injections had the lowest scores in clinical appraisal and only had mild atrophy (Figure 3). Interestingly, the PBS control group also showed increased blood flow in the first three weeks, which is possibly due to the innate ability of wound repair and revascularization. At the fourth week, the PBS control group did not continue to recover. By contrary, the HA/HUVEC treatment continued to increase blood flow recovery until the fourth week. Although HA/HUVEC treatment did not fully recover the blood flow, we believe this is possibly because of the short term experimental design or the yet-to-be optimized repair ability of the treatment. Therefore, further studies should be conducted to study the longer term effects of the treatment and to optimize the efficacy of therapy.

In addition, HA enhanced HUVEC retention and engraftment. Four weeks after HUVEC transplantation, EGFP*HUVECs remained in the muscles and a few were found to overlap with capillaries, or were engrafted in the artery surrounded by a layer of smooth muscle cells. However, no EGFP* cells were found in the PBS/HVUEC injection group. This suggested that HUVEC transplantation with injectable HA enhances the cell-based therapeutic effect in the treatment of
hindlimb ischemia. Moreover, those EGFP+ cells remaining within the muscle tissue provide an opportunity for extensive vascularization.

5. Conclusion

Our results showed that adding HA improved the therapeutic effect of HUVECs in restoring an ischemic limb by promoting angiogenesis, arteriogenesis, and perhaps also by a paracrine effect. Therefore, combining the beneficial characteristics of HA with cell-based therapy in an optimal ratio is a promising approach to improving the treatment of PAOD. This combination allowed HUVECs to survive for at least 4 weeks after injection. The HUVECs remained functional as endothelial cells, vascularization was enhanced, and blood flow to the ischemic limb recovered. This approach can be translated into a clinical therapy for ischemia, particularly if endothelial cells are available from patients or a cell bank.

Acknowledgements

This work was supported by the National Science Council (98-3111-B-006-005, 96-2314-B-006-021), the NCKU Integrated Landmark Project (98I006), and the NCKU-ITRI Nano-Innovative Project (98FY-A3).


**Reference**


Figure Captions

**Figure 1.** Hyaluronan improves endothelial cell growth in culture. (A) Photomicrographs of human umbilical vein endothelial cells (HUVECs) cultured on different concentrations of hyaluronan (HA)-coated plates with 1% gelatin as control. (B) Cell counts showing HUVEC proliferation at 24 and 48 h in different concentrations of HA and 1% gelatin. (C) Flow cytometric analysis of propidium iodide-stained HUVECs in different concentrations of HA and 1% gelatin. (D) Quantification of results such as those shown in (C) after 24 and 48 h of culture on HA- or gelatin-coated plates. (E) Western blots (upper panel) and statistics (lower panel) of ERK and Akt phosphorylation of HUVECs cultured on 1% HA- or gelatin-coated plates. GAPDH served as control. *P < 0.05, **P < 0.01, ***P < 0.001.

**Figure 2.** Hyaluronan protects endothelial cells from apoptosis in culture. (A) Photomicrographs of human umbilical vein endothelial cells (HUVECs) cultured on 1% hyaluraron (HA) or gelatin in medium supplied with 10% fetal bovine serum for 24 h, and then switched to culture under serum-free medium for another 24 h. (B) Viability of HUVECs after serum-free starvation for 24 h (MTT assay). (C) Flow cytometric analysis of propidium iodide-stained HUVECs showing percentage of apoptosis in 1% HA or gelatin after 24 h starvation. (D) Quantification of results shown in (C). (E) Western blots (upper panel) and statistics (lower panel) of cleaved caspase-3 from HUVECs cultured on 1% HA- or gelatin-coated plates under 10% serum or serum-free medium. GAPDH served as control. *P < 0.05, ***P < 0.001.
**Figure 3.** Injection of hyaluronan along with endothelial cells promotes limb salvage after ischemia. (A) Gross appearance of mouse hindlimbs 1-4 weeks after surgical excision of the left femoral artery. Lowest panels show magnified images of the left hindlimb 4 weeks after surgery. (B) Clinical scores of mice at 1-4 weeks after hindlimb ischemia. N = 6 in each group; *P < 0.05, **P < 0.01, ***P < 0.001 vs. PBS or PBS/HUVEC group; †P < 0.05, ††P < 0.01, †††P < 0.001 vs. HA group; ‡P < 0.05, ‡‡P < 0.01, ‡‡‡P < 0.001 vs. PBS/HUVEC group. HA, hyaluronan; PBS, phosphate buffered saline; HUVEC, human umbilical vein endothelial cell.

**Figure 4.** Injection of hyaluronan along with endothelial cells increases blood flow in the ischemic hindlimb. Blood flow before, 1 day after, and 1-4 weeks after ischemia in sham and experimental groups measured by laser Doppler flowmetry. N = 6 in each group; *P < 0.05, ***P < 0.001. HA, hyaluronan; PBS, phosphate buffered saline; HUVEC, human umbilical vein endothelial cell.

**Figure 5.** Injection of hyaluronan with endothelial cells increases capillary density in the ischemic hindlimb. (A-E) Representative immunostained images of muscles and capillaries at the mid-thigh level; (A) sham, (B) PBS control, (C) HA alone, (D) HUVECs alone, (E) HA along with HUVECs. Capillaries labeled with anti-VE-cadherin (red), skeletal muscle with anti-tropomyosin (green), and nuclei with DAPI (blue). Magnified images in the peri-injury regions are indicated by arrows. (F) Quantification of capillary density in the peri-injury regions. **P < 0.01. HA, hyaluronan; PBS, phosphate buffered saline; HUVEC, human umbilical vein endothelial cell.
**Figure 6.** Injection of hyaluronan with endothelial cells increases arteriogenesis in the ischemic hindlimb. (A-E) Representative immunostained images of muscles and arterioles at the mid-thigh level; (A) sham, (B) PBS control, (C) HA alone, (D) HUVECs alone, (E) HA along with HUVECs. Arterioles labeled with anti-smooth muscle 22α (red), skeletal muscles with anti-tropomyosin (green), and nuclei with DAPI (blue). (F) Quantification of arteriole density in the peri-injury regions. *P < 0.05, **P < 0.01. HA, hyaluronan; PBS, phosphate buffered saline; HUVEC, human umbilical vein endothelial cell.

**Figure 7.** Injection of hyaluronan with endothelial cells improves transplanted cell retention after hindlimb ischemia. (A) Representative photomicrographs of EGFP⁺HUVECs (green) in the area injected with cells alone (left panels) or with cells and HA (right panels). Nuclei stained with DAPI (blue), and muscles with tropomyosin (red). (B) Cell retention rates as reflected by EGFP⁺ cell counts. ***P < 0.001 vs. PBS/HUVEC. HA, hyaluronan; HUVEC, human umbilical vein endothelial cell.

**Figure 8.** In the presence of hyaluronan, transplanted endothelial cells integrate into pre-existing vessels and form entirely new vessels in the ischemic hindlimb. Three representative sections stained for SM22α (A) and VE-cadherin (B), and then for EGFP⁺HUVECs (green) and DAPI (blue); overlapped images in bottom panels showing co-stained cells (indicated by arrows). HA, hyaluronan; HUVEC, human umbilical vein endothelial cell.
Supplemental Figure Captions

**Supplemental Figure 1.** Characterization of human umbilical vein endothelial cells (HUVECs). (A) Morphology of HUVECs cultured on a gelatin-coated plate. (B) Immunofluorescence staining of von Willebrand Factor in HUVECs. (C) Tube formation of HUVECs cultured on Matrigel assay.

**Supplemental Figure 2.** Clinical score of ambulatory impairment after hindlimb ischemia. Shown is a schematic drawing of clinical scoring of hindlimb ischemia.

**Supplemental Figure 3.** Hyaluronan protects endothelial cells from apoptosis in culture. (A) Photomicrographs of human umbilical vein endothelial cells (HUVECs) cultured on 1% hyaluronan (HA) or gelatin in medium supplied with 10% fetal bovine serum for 24 h, and then adding puromycin (left panel) and doxorubicin (right panel) for another 24 h. (B) Viability of HUVECs after adding puromycin (left panel) and doxorubicin (right panel) for 24 h (MTT assay). ***P < 0.001.

**Supplemental Figure 4.** Hyaluronan protects endothelial cells from apoptosis in culture. (A) Flow cytometric analysis of propidium iodide-stained HUVECs showing percentage apoptosis in 1% HA or gelatin 24 h after adding puromycin and doxorubicin. (B) Quantification of results for those shown in (A). ***P < 0.001. HA, hyaluronan; HUVEC, human umbilical vein endothelial cell.

**Supplemental Figure 5.** Establishment of endothelial cells expressing green fluorescence protein. (A) Morphology of human umbilical vein endothelial cells
(HUVECs) infected with a lentivirus encoding enhanced green fluorescence protein (EGFP). (B) Percentage of EGFP*HUVECs determined by flow cytometry. (C) Representative image of EGFP*HUVECs cultured on Matrigel for 24 h.
Figure 1.

C

1% Gelatin

0.1% HA

0.25% HA

0.5% HA

0.75% HA

1% HA

D

24hr

48hr

Cell proliferation (%)

1% Gelatin

0.1% HA

0.25% HA

0.5% HA

0.75% HA

1% HA

Cell proliferation (%)

1% Gelatin

0.1% HA

0.25% HA

0.5% HA

0.75% HA

1% HA

***

**

*

***

***

***

***
Figure 1.

E  1% Gelatin  1% HA
pERK
GAPDH

1% Gelatin  1% HA

pAkt
GAPDH

Relative percentage
0  100  200  300
1% Gelatin  1% HA

***

Relative percentage
0  100  200  300
1% Gelatin  1% HA

***
Figure 2.

C

6.7%

1% Gelatin

35.2%

1% Gelatin/serum free

7.0%

1% HA

26.1%

1% HA/serum free

D

Apoptosis (%)

1% Gelatin

1% Gelatin/serum free

1% HA

1% HA/serum free
Figure 2.

Cleaved caspase-3

GAPDH

Relative percentage

1% Gelatin  1% HA  1% Gelatin/serum free  1% HA/serum free

***  ***
Figure 3.

A

<table>
<thead>
<tr>
<th>Sham</th>
<th>PBS</th>
<th>HA</th>
<th>PBS/HUVEC</th>
<th>HA/HUVEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>W1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Click here to download high resolution image
Figure 3.

B

Clinical score

Time (weeks)

* compare to PBS
† compare to HA
‡ compare to PBS/HUVEC
Figure 4.

![Bar chart showing blood flow (% of control) over time points D0, D1, W1, W2, W3, W4 for different groups: Sham, PBS, HA, PBS/HUVEC, HA/HUVEC.](Click here to download high resolution image)
Figure 5.

A. Sham

B. PBS

C. HA

D. PBS/HUVEC

E. HA/HUVEC

F. Bar chart showing capillary density (vessels/mm²) for sham, PBS, HA, PBS/HUVEC, and HA/HUVEC treatments. The chart indicates significant differences between groups with ** symbols.
Supplemental Figure 1.
Supplemental Figure 2.

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>Mild claudication with muscle dystrophy</td>
</tr>
<tr>
<td>2</td>
<td>Moderate claudication with muscle atrophy</td>
</tr>
<tr>
<td>3</td>
<td>Severe claudication with muscle atrophy</td>
</tr>
<tr>
<td>4</td>
<td>Toe gangrene</td>
</tr>
<tr>
<td>5</td>
<td>Foot gangrene</td>
</tr>
<tr>
<td>6</td>
<td>Limb amputation</td>
</tr>
</tbody>
</table>

4 + 0.1* (No. of missing toes)
Supplemental Figure 3.

A
1% Gelatin/Puromycin
1% Gelatin/Doxorubicin
1% HA/Puromycin
1% HA/Doxorubicin

B
Viability (%)

**1% Gelatin**
**1% Gelatin/Puro.**
**1% HA/Puro.**

**1% Gelatin**
**1% Gelatin/Dox.**
**1% HA**
**1% HA/Dox.**
Supplemental Figure 4.

A

1% Gelatin/Puromycin

31.9%

1% Gelatin/Doxorubicin

36.4%

1% HA/Puromycin

15.7%

1% HA/Doxorubicin

17.6%

FL2-H

Counts

0 200 400 600 800 1000

B

Apoptosis (%)

1% Gelatin

1% Gelatin/Puro.

1% HA/Puro.

1% HA/Dox.

1% Gelatin/Dox.

***

***
Supplemental Figure 5.
29 June 2010

Patrick C.H. Hsieh, MD, PhD
138 Sheng-Li Road, Room 9B-110
Tainan City 70428
Taiwan

MS ID#: CIRCULATIONAHA/2010/939512
MS TITLE: Intramyocardial Peptide Nanofiber Injection Improves Post-Infarction Ventricular Remodeling and Efficacy of Bone Marrow Cell Therapy in Pigs

Dear Dr. Hsieh,

Thank you for returning your revised manuscript promptly. We are pleased to inform you that it is now acceptable for publication in Circulation’s Cardiovascular Surgery Supplement. Please submit the following items required for publication as soon as possible.

1. Title Page: Please include phone and fax numbers for the corresponding author
2. Abstract: Please reduce to 250 words or fewer (not including key words)
   a. Please use the following headings: Background; Methods and Results; Conclusions
   b. When applicable, include a fourth heading: "Clinical Trial Registration Information". Please list the URL, as well as the unique identifier, for the publicly accessible website on which the trial is registered.
3. Funding Sources:
   - All grant funding agency abbreviations should be completely spelled out, with the exception of the NIH.
4. Figures: If you cite supplemental figures in the text, please do so in sequential order.
5. Original source files for the text and figures are required. Acceptable formats for your manuscript text and tables are Word and WordPerfect. Acceptable figure formats are TIFF, EPS, and PPT. Please return to your Author Area to upload these source files.
6. Online Supplemental Data: A combined PDF of your supplemental data must be provided. The first page of this PDF should include the heading, "SUPPLEMENTAL MATERIAL." Please note that this single PDF should include all of the supplemental material related to your manuscript except for the Video or Movie files. The supplemental material to be included in this PDF is as follows: 1) Supplemental Methods, Supplemental Tables, Supplemental Figures and Figure Legends, and Supplemental References. Lastly, the legends for the Video files should also be included in this PDF. Please upload this PDF to your author area.
7. The Copyright Transfer Agreement form must be completed by each author. Please fax the completed forms to 617-542-6539.
8. Authors should obtain written permission from all individuals who are listed in the “Acknowledgments” section of the manuscript, because readers may infer their endorsement of data and conclusions. The corresponding author must sign the Acknowledgment Section on the Copyright Transfer Agreement. N.B., Signature on this line is required, even if no Acknowledgments section is included in the manuscript.

We will proceed with the processing of your manuscript for publication as soon as all required materials are received. During the copyediting phase, there may be some changes in phrasing, but there will be no alteration of scientific content. When you receive your galley proofs, please read, correct, and return them immediately to avoid delay in publication. Please note that it is your responsibility to make yourself available to review the proofs whenever they arrive, and if you are not available, publication of your manuscript will be delayed.
Page charges for the Surgery Supplement are $300 per page for the first 4 pages and $650 per page after the fourth page. Additional charges also apply for the reproduction of any color figures. You will be billed directly by our publisher, Lippincott Williams & Wilkins.

We are very pleased to have the opportunity to publish this interesting work. Thank you very much.

Sincerely yours,

Y. Joseph Woo, MD
Editor, Surgical Supplement

Joseph Loscalzo, MD, PhD
Editor-in-Chief
 Intramyocardial Peptide Nanofiber Injection Improves Postinfarction Ventricular Remodeling and Efficacy of Bone Marrow Cell Therapy in Pigs

Yi-Dong Lin, MS; Ming-Long Yeh, PhD; Yu-Jen Yang, MD, PhD; Da-Ching Tsai, MS; Ting-Yu Chu, BA; Ya-Yun Shih, MS; Min-Yao Chang, MS; Yen-Wen Liu, MD; Alan C.L. Tang, MS; Tsai-Yun Chen, MD; Chwan-Yau Luo, MD, MS; Kung-Chao Chang, MD, PhD; Jyh-Hong Chen, MD, PhD; Hua-Lin Wu, PhD; Tin-Kan Hung, PhD; Patrick C.H. Hsieh, MD, PhD

Background and Purpose—Growing evidence suggests that intramyocardial biomaterial injection improves cardiac functions after myocardial infarction (MI) in rodents. Cell therapy is another promising approach to treat MI, although poor retention of transplanted cells is a major challenge. In this study, we hypothesized that intramyocardial injection of self-assembling peptide nanofibers (NFs) thickens the infarcted myocardium and increases transplanted autologous bone marrow mononuclear cell (MNC) retention to attenuate cardiac remodeling and dysfunction in a pig MI model.

Methods—A total of 40 mature minipigs were divided into 5 groups: sham, MI + normal saline, MI + NFs, MI + MNCs, and MI + MNCs/NFs. MI was induced by coronary occlusion followed by intramyocardial injection of 2 mL normal saline or 1% NFs with or without 1 × 10^8 isolated autologous MNCs.

Results—NF injection significantly improved diastolic function and reduced ventricular remodeling 28 days after treatment. Injection of MNCs alone ameliorated systolic function only, whereas injection of MNCs with NFs significantly improved both systolic and diastolic functions as indicated by +dP/dt and −dP/dt (1214.5±91.9 and −1109.7±91.2 mm Hg/s in MI+NS, 1693.7±84.7 and −1809.6±264.3 mm Hg/s in MI+MNCs/NFs, respectively), increased transplanted cell retention (29.3±4.5 cells/mm² in MI+MNCs and 229.4±41.4 cells/mm² in MI+MNCs/NFs), and increased capillary density in the peri-infarct area.

Conclusions—We demonstrated that NF injection alone prevents ventricular remodeling, whereas cell implantation with NFs improves cell retention and cardiac functions after MI in pigs. This unprecedented combined treatment in a large animal model has therapeutic effects, which can be translated to clinical applications in the foreseeable future. (Circulation. 2010;122:00-00.)

Key Words: biomaterials ■ bone marrow mononuclear cells ■ cardiac tissue engineering ■ myocardial infarction

Congestive heart failure is a leading cause of death in the United States and other developed countries. The dominant cause of heart failure is loss of myocardium due to coronary artery disease and the limited regeneration potential of cardiomyocytes. Cardiac tissue engineering is a promising and actively developing area of research aiming to repair, replace, and regenerate the myocardium. Several studies have demonstrated the feasibility of this approach and indicated that direct injection of biomaterials into the infarcted myocardium may be beneficial in preventing deleterious remodeling and reducing cardiac dysfunction. Previous studies using intramyocardial injection of self-assembling peptide nanofibers (NFs), a highly biocompatible and biodegradable material, have also revealed their therapeutic potentials for angiogenesis, controlled drug/growth factor release, cell delivery, and stem cell recruitment. These results indicate that NFs may impact a broad spectrum of applications in myocardial tissue engineering.

Cell therapy is another promising approach to heart disease treatments; however, there are many challenges that call for attention such as the extremely low retention and survival rates of implanted cells. This issue is especially apparent in cardiac therapy due to the forbidding microenvironment of a high blood flow rate in the heart and a high degree of ventricular remodeling after myocardial infarction (MI). Thus, it may be necessary to inject cells contained within...
vehicles or biomaterials coupled with nutritional factors to increase cell retention and cell survival rates. Lastly, the lack of angiogenesis in the MI zone is also a critical concern regarding support, survival, and grafting of implanted cells into the host tissue.

Small animal models have been used to attain exciting and promising achievements by different combinations of the mentioned approaches. In contrast, large animal model studies have not been widely performed but are crucial to further investigation of clinical applications. Therefore, we carried out experiments to test the hypothesis that intramyocardial injection of NFs thickens the infarcted myocardium and increases cell retention such that attenuation of post-MI cardiac remodeling and dysfunction can be achieved in a pig model of experimental MI.

**Methods**

A total of 40 sexually mature minipigs (approximately 5 months old) were divided into 5 groups: sham operation, which was performed by opening the chest without coronary artery ligation (sham), MI+normal saline (NS), MI+NFs, MI+autologous bone marrow mononuclear cells (MNCs), and MI+MNCs along with NFs (MNCs/NFs; n=8 in each group). MI was induced by permanent occlusion of the midleft anterior descending coronary artery immediately followed by injection of a total of 2 mL NS or 1% NFs divided among approximately 40 injections into the infarcted area. Freshly isolated 1 x 10^8 autologous MNCs were mixed in 2 mL NS or NFs for injection. Cardiac functions were assessed by echocardiography before and immediately after MI and together with hemodynamic measurements through catheterization 4 weeks later. For additional information, see online Supplemental Material (available at http://circ.ahajournals.org).

**Results**

Injection of Peptide NFs Alone Increases Interventricular Septum Thickness and Prevents Ventricular Remodeling After Infarction

The successful induction of MI was confirmed by a comparably decreased left ventricular ejection fraction (LVEF) immediately postinfarction among groups (LVEF: 62.1±1.0% in sham, 45.1±1.7% in MI+NS, 46.3±3.7% in MI+NFs, 44.0±5.0% in...
MI+MNCs, 44.5 ± 1.6% in MI+MNCs/NFs; all \(P<0.001\) versus sham; Figure 1A).

At 28 days after MI, 68.5 ± 2.3% NFs were retained in the injected regions as determined by high-performance liquid chromatography (Supplemental Figure III). Injection of NFs alone significantly increased both systolic and diastolic interventricular septum thickness (systole: 0.54 ± 0.02 cm and diastole: 0.46 ± 0.03 cm in the MI+NS group; and systole: 0.73 ± 0.05 cm and diastole: 0.57 ± 0.02 cm in the MI+NFs group; Figure 1B). Furthermore, both left ventricular left ventricular end diastolic volume and left ventricular end systolic volume were improved by NF injection (Table), suggesting that this significantly prevented post-MI left ventricular dilatation. NF injection significantly increased the ratio of scar thickness and decreased the ratio of scar length in the infarct (scar thickness ratio: 66.8 ± 2.6% and scar length ratio: 26.0 ± 1.1% in the MI+NS group, and scar thickness ratio: 85.1 ± 5.4% and scar length ratio: 21.0 ± 1.5% in the MI+NFs group; Figure 2). Together these findings imply that the scar expansion and left ventricular remodeling after infarction were prevented by NF injection.

In addition, the post-MI diastolic function was significantly improved after NF injection (\(\times dP/dt\): \(-1109.7\pm 91.2\) mm Hg/s in the MI+NS group, and \(-1751.0\pm 86.9\) mm Hg/s in the MI+NFs group; Table). Consistently, the collagen content at the remote zone (collagen content: 22.5 ± 0.7% in the MI+NS group and 13.0 ± 0.9% in the MI+NFs group; Figure 3) and the global elasticity (as assessed by arterial elastance and maximum chamber elasticity; Table) both significantly revealed improvement of cardiac compliance by NF injection. However, estimation of LVEF by echocardiography and \(+dP/dt\) by catheterization showed no improvement by NF injection (Figure 1A; Table). Combining the finding that there was no significant difference in the ratio of necrotic area between MI+NS and MI+NFs groups (Table 2C), these results suggest that NF implantation alone may not be sufficient for recovery of cardiac performance and cell implantation may be required to increase the contractile component for the improvement of systolic function.

**Injection of Peptide Nanofibers Along With Autologous Bone Marrow MNCs Improves Both Systolic and Diastolic Functions After Infarction**

At 28 days after infarction, injection of autologous bone marrow MNCs alone significantly increased LVEF, systolic interventricular septum thickness, and \(+dP/dt\) (Figure 1; Table), suggesting that MNC injection improved systolic function after MI. However, neither diastolic interventricular septum thickness nor \(-dP/dt\) showed improvement by MNC injection (Figure 1B; Table). Combining the finding that there was no improvement in the maximum chamber elasticity between MI+NS and MI+MNCs groups (Table), these results suggest that MNC implantation alone may not be sufficient for prevention of ventricular remodeling after MI, and NF implantation may be required to increase cardiac compliance and diastolic function.

---

**Table. Hemodynamic Parameters at 1 Month After MI**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham (n=8)</th>
<th>MI+NS (n=8)</th>
<th>MI+NFs (n=8)</th>
<th>MI+MNCs (n=8)</th>
<th>MI+MNCs/NFs (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>102.4±6.3</td>
<td>94.0±4.4</td>
<td>94.0±6.7</td>
<td>95.6±8.1</td>
<td>99.4±6.8</td>
</tr>
<tr>
<td>LVEFP, mm Hg</td>
<td>89.0±4.1</td>
<td>69.6±2.6</td>
<td>77.5±3.4</td>
<td>77.1±5.7</td>
<td>79.1±4.5</td>
</tr>
<tr>
<td>LVESD, mm Hg</td>
<td>8.3±0.7</td>
<td>12.7±0.9</td>
<td>8.6±1.0†</td>
<td>8.6±0.7†</td>
<td>8.5±0.5‡</td>
</tr>
<tr>
<td>LVESV, mL</td>
<td>35.6±3.6</td>
<td>103.0±7.0</td>
<td>73.7±3.6†</td>
<td>58.7±2.4§</td>
<td>51.7±4.6‡</td>
</tr>
<tr>
<td>LVEDV, mL</td>
<td>96.8±6.4</td>
<td>147.5±7.0</td>
<td>120.3±7.0†</td>
<td>115.0±5.7†</td>
<td>113.6±6.1†</td>
</tr>
<tr>
<td>SV, mL</td>
<td>64.9±8.6</td>
<td>44.5±5.8</td>
<td>52.0±5.0</td>
<td>56.2±4.8</td>
<td>61.8±5.0</td>
</tr>
<tr>
<td>CO, mL/min</td>
<td>6636.0±959.1</td>
<td>4133.3±482.2</td>
<td>5084.6±353.2</td>
<td>5247.0±433.3</td>
<td>9879.9±654.8</td>
</tr>
<tr>
<td>SW, mm Hg/mL</td>
<td>4870.5±532.4</td>
<td>2527.0±414.2</td>
<td>3689.1±511.4</td>
<td>4114.5±533.6</td>
<td>4567.0±809.4</td>
</tr>
<tr>
<td>AE, mm Hg/mL</td>
<td>1.52±0.12</td>
<td>0.89±0.07</td>
<td>1.26±0.08*</td>
<td>1.12±0.09*</td>
<td>1.29±0.10*</td>
</tr>
<tr>
<td>+dP/dt/mm Hg/s</td>
<td>1852.7±82.9</td>
<td>1214.5±91.9</td>
<td>1419.4±71.8</td>
<td>1602.1±135.6*</td>
<td>1693.7±84.7†</td>
</tr>
<tr>
<td>−dP/dt/mm Hg/s</td>
<td>−2048.8±293.1</td>
<td>−1109.7±91.2</td>
<td>−1751.0±86.9</td>
<td>−1401.4±127.6</td>
<td>−1800.6±264.3*</td>
</tr>
<tr>
<td>PRSW, mm Hg</td>
<td>65.1±8.4</td>
<td>42.2±5.6</td>
<td>55.8±6.8</td>
<td>60.5±5.9</td>
<td>65.1±13.8</td>
</tr>
<tr>
<td>ESPVR, mm Hg/mL</td>
<td>1.6±2.2</td>
<td>0.7±0.1</td>
<td>1.2±0.2</td>
<td>1.3±0.2</td>
<td>1.5±0.2</td>
</tr>
<tr>
<td>Emax, mm Hg/mL</td>
<td>0.05±0.02</td>
<td>0.06±0.02</td>
<td>0.05±0.02</td>
<td>0.06±0.02</td>
<td>0.05±0.02</td>
</tr>
<tr>
<td>+dP/dt-EDV, mm Hg/s/mL</td>
<td>14.3±4.5</td>
<td>6.5±1.6</td>
<td>9.4±3.0</td>
<td>9.5±2.1</td>
<td>12.0±2.9</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

*\(P<0.05\) versus MI+NS.

†\(P<0.01\) versus MI+NS.

‡\(P<0.001\) versus MI+NS.

§\(P<0.05\) versus MI+NFs.

| \(|P<0.01\) versus MI+MNCs. |

LVEFS indicates left ventricular end systolic pressure; LVEDP, left ventricular end diastolic pressure; LVESV, left ventricular end systolic volume; LVEDV, left ventricular end diastolic volume; SV, stroke volume; CO, cardiac output; SW, stroke work; AE, arterial elastance; \(\tau\), time constant of left ventricular pressure decay; PRSW, preload recruitable stroke work; ESPVR, end diastolic pressure–volume relationship; Emax, end systolic pressure–volume relationship; Emax, maximum chamber elasticity; EDV, end diastolic volume.
Confirming this hypothesis, we found that the LVEF (66.0±1.0% in the sham group, 45.2±1.8% in the MI+NS group, 48.2±2.0% in the MI+NFs group, 52.6±2.1% in the MI+MNCs group, 58.7±1.6% in the MI+MNCs/NFs group; Figure 1A), left ventricular end diastolic pressure, left ventricular end systolic volume, left ventricular end diastolic volume, AE, +dP/dt, −dP/dt, τ, and maximum chamber elasticity were all significantly improved as a result of MNC/NF injection (Table). Although injection of MNCs alone without NFs also improved systolic function after MI, only injection of MNCs combined with NFs improved both systolic and diastolic functions.

Injection of Peptide NFs Along With Autologous Bone Marrow MNCs Decreases Necrotic Tissue and Collagen Content in the Remote Area After Infarction
Consistent with the finding that MNC/NF injection improved both systolic and diastolic functions after MI, the necrotic tissue was significantly reduced after infarction (18.6±1.1%...
in the MI+NS group, 17.5±1.9% in the MI+NFs group, 13.2±2.2% in the MI+MNCs group, and 11.3±1.5% in the MI+MNCs/NFs group; Figure 2C). Furthermore, injection of MNCs/NFs significantly attenuated collagen content in the remote area after infarction, but a similar effect was also detected in the other 2 groups of NFs injection alone and MNCs injection alone (22.5±0.7% in the MI+NS group, 13.0±0.9% in the MI+NFs group, 14.4±3.0% in the MI+MNCs group, and 10.5±2.7% in the MI+MNCs/NFs group; Figure 3).

Injection of Peptide NFs Along With Bone Marrow MNCs Increases Transplanted Cell Retention and Differentiation as Well as Angiogenesis After Infarction

At the border or infarct zone of treated myocardium, our results showed a higher DiI+ cell density in the MI+MNCs/NFs group than in the MI+MNCs group (29.3±4.5 /mm² in the MI+MNCs group and 229.4±41.4/mm² in the MI+MNCs/NFs group, *P<0.001; Figure 4). This result indicates that NFs also serve as a delivery vector to improve the retention of transplanted cells after intramyocardial injection.

To confirm the differentiation fate of the injected MNCs, we used costaining of primary antibodies with DiI signal to trace these cells. To rule out the cytoplasmic autofluorescence, the DiI signal was checked on multiple wavelengths and was detectable only under red wavelength (Supplemental Figure IVA); serial sections with DiI staining only were also used to confirm that the detected signals were dependent on the primary antibodies to these epitopes (Supplemental Figure IVB–C). Surprisingly, we found that 28 days after injection, the surviving MNCs mainly differentiate into endothelial cells as indicated by von Willebrand factor, VE-cadherin, isolectin, and CD31 staining and few into smooth muscle cells (SM myosin heavy chain, SM22α, and
SMo-actin staining), whereas no evidence of differentiation into cardiomyocytes (stained by cardiac troponysin, Nkx2.5, and GATA4; Figure 5; Supplemental Figure VII and data not shown). We also detected MNC differentiation into hematopoietic lineage cells (stained by CD45 and an antibody against macrophage; Supplemental Figure V). Interestingly, when NFs were injected along with MNCs, we found a higher differentiation ratio of MNCs into endothelial cells and smooth muscle cells and still no evidence of cardiomyocytes (MNCs: ratio of endothelial cell differentiation, 56.3 ± 8.8% and ratio of smooth muscle cell differentiation, 3.3 ± 2.1%; MNCs/NFs: ratio of endothelial cell differentiation, 83.0 ± 4.9% and ratio of smooth muscle cell differentiation, 14.5 ± 3.5%; Figure 5). These results indicated that 1 of the benefits of MNC treatment is angiogenesis and this effect is enhanced by injection of MNCs along with NFs. Supporting the results of MNC differentiation into ECs, there was significant additional increase of capillary density in the peri-infarct zone after treatment with NFs along with MNCs (capillary density: 190.4 ± 20.8 number/mm² in the MI+NS group, 352.6 ± 33.5 number/mm² in the MI+NFs group, 533.8 ± 53.9 number/mm² in the MI+MNCs group, and 644.8 ± 64.6 number/mm² in the MI+MNCs/NFs group; Figure 6).

**Injection of NFs Reduces C- Reactive Protein Levels After Infarction**

Although the biocompatibility of NFs is well established,⁵ it has not yet been confirmed in pigs, so we measured C-reactive protein (CRP) levels in the plasma to detect chronic inflammation. There was no CRP level increase at 28 days after NF injection compared with the control. However, CRP is known to rise as a result of MI itself,¹⁷ and our results confirmed this (sham versus MI only). Surprisingly, all treatment groups showed markedly reduced CRP levels, and the combined NF+MNC group showed the greatest decrease (CRP: 19.5 ± 2.2 μg/mL for sham, 50.0 ± 11.2 μg/mL in the MI+NS group, 26.7 ± 4.0 μg/mL in the MI+NFs group, 29.0 ± 6.4 μg/mL in the MI+MNCs group, and 24.7 ± 4.6 μg/mL in the MI+MNCs/NFs group; Figure 7). Interestingly, we also detected significantly decreased leukocyte infiltration in the NF injection area, suggesting that NF injection may not stimulate inflammatory reaction in the infarcted myocardium, which is consistent with a previous study in rats (Supplemental Figure VII).⁵

**Discussion**

The outcome of intramyocardial biomaterial injection has been investigated in only a few studies. In contrast, cell therapy is a more commonly explored approach, but cell retention is an issue and is often briefly addressed or ignored. Although both these treatments have potential uses in therapy for cardiac diseases, they have rarely been tested in large animal models, an essential step before proceeding to clinical trials. In this study, we describe a novel approach that combines biomaterial injections with cell therapy and demonstrate that cardiac functions are regained in a large animal model of MI.

**Local Repair by Biomaterial Injection Preserves Cardiac Geometry and Function**

Jugdutt suggested that although extracellular matrix expression increases in the entire heart after MI, this increase is only beneficial in the infarct zone at the same time as being detrimental in the noninfarct zone.¹⁸ In this study, we showed that NF treatment increased interventricular septum thickness...
after MI, restrained scar extension, and prevented further harmful fibrosis at the remote zone. Moreover, reduction in global cardiac remodeling and diastolic dysfunction after MI was achieved. Interestingly, these results were very similar to a numeric simulation model proposed by Wall et al, which indicated that intramyocardial noncontractile material injection had all of these effects as well as a reduction in elevated myofiber stresses.2

Landa et al reported that intramyocardial biomaterial injection preserves cardiac systolic function 2 months after MI in a rodent model.3 In contrast, we found that NF injection did not improve systolic function at 28 days after MI in pigs. This inconsistency may be due to the different materials, time points, and animal models chosen for the 2 studies. Because the heart failure indices A-type natriuretic peptide and B-type natriuretic peptide showed no difference between the sham and MI+NS groups (data not shown), the heart of a large animal like the pig may still undergo remodeling beyond 28 days after MI, so long-term studies of the effects of NF injection are needed.

Cell Retention Determines Cardiac Functional Improvement by Cell Therapy

Bone marrow MNC transplantation has been reported to benefit the infarcted myocardium.19 We demonstrated similar contributions to cardiac functional improvement after MNC implantation in pigs. Beyond direct MNC injection alone, injection of MNCs along with NFs showed even better amelioration of cardiac function. We believe these beneficial results were mainly due to the ability of NFs to increase cell retention. Furthermore, consistent with our previous studies,6,20 the presence of NFs did not alter the viability of MNCs, which remained approximately 95% viable before injection (Supplemental Methods). NF injection may also increase the cell survival rate due to the increase in capillary density at the border zones 28 days after MI. From this point of view, coinjection of cells with NFs may be highly beneficial for both cell engraftment and angiogenesis effects due to increases in cell retention and survival rates.

Beyond cell retention and survival, evidence from our results also showed an increase in MNC differentiation into endothelial cells and smooth muscle cells after injection of MNCs/NFs compared with injection of MNCs alone. The NFs may act as a scaffold that provides a suitable microenvironment for the MNCs to adhere and perform normal cellular functions.6 Furthermore, differentiation of MNCs into endothelial cells and smooth muscle cells promotes angiogenesis and prevents further apoptosis of cardiomyocytes within the infarct zone, leading to the preservation of cardiac function.21 These results demonstrate the synergistic effect of NF and MNC injection.
Combined MNC/NF Injection Has Complementary Effects for Clinical Application

MNC injection alone significantly increased systolic but not diastolic function after MI, whereas NF injection alone increased diastolic but not systolic function. Importantly, the combined injection of MNCs and NFs improved both systolic and diastolic function. This combination thus acts in a complementary fashion to synergistically benefit cardiac function and is now shown to be feasible in a large animal model with induced acute MI. Because most cardiac patients have chronic heart disease, whether this treatment is applicable for chronic MI cases as well the appropriate time window requires further investigation.

The different conditions of the treatments in this study such as dosage, timing, and location of injections were tuned to be optimal for 25-kg minipigs. Modification is necessary for clinical trial models. However, 1 exception is the requirement for spread-out injections to achieve a well dispersed administration of the cells and NFs. In the future, open-chest intramyocardial injection may be replaced by a catheter transcendocardial approach or minimally invasive thoracotomy for clinical use to minimize the risk and harm of surgery itself.

From a safety perspective, introduction of foreign materials into the body may be seen as hazardous, possibly leading to mechanical failure or arrhythmia caused by change in conductance of the heart. In our study, 2 of 42 animals died with intractable ventricular fibrillation on the operating table before any treatments were administered. There were no negative side effects due to NF treatments.

Mechanisms of MNC Therapy Remain to Be Determined

The mechanisms of cardiac functional improvement by bone marrow cell therapy are still under investigation. Controversies exist concerning myocyte differentiation, paracrine effects, and whether stimulation causes endogenous cardiac repair. Another interesting question is if the transplanted bone marrow cells may integrate into the pre-existing vessels or form entirely new vessels. As shown in Figure 5B and Supplemental Figure VIII, we observed not only integration of MNC-derived smooth muscle cells into pre-existing vessels, but also new vessels formed entirely from the injected MNCs. However, to what extent these effects contribute to the increase of functional improvement after MI remains unknown. Therefore, the specific pathways and mechanisms by which bone marrow cells act require further investigation.

Quevedo et al reported that 14% bone marrow cells differentiate into cardiomyocytes, but our data did not con-
The major difference is that the cells they used were allogeneic bone marrow mesenchymal stem cells, which were conditioned by in vitro culture. On the other hand, the cells we used were autologous bone marrow MNCs, which are nonadherent and mainly consist of hematopoietic cells. In dealing with the controversies surrounding the various results and explanations of bone marrow cell therapy effects, a more stringent approach or confirmation by double experimental methods should be carried out for more convincing results. For example, genetic lineage tracking could be considered for tracing the injected cells in future studies. Here, we attempted to simulate the clinical situation of a single procedure, rather than a sequence of steps, so Dil-labeled MNCs were chosen to allow convenient cell isolation and cell labeling concurrently in MI without a complicated and precarious preprocess.

In conclusion, the present study reveals that intramyocardial injection of NFs alone prevents pathological left ventricular remodeling, whereas injection of cells along with NFs helps raise the cell retention rate and improve cardiac performance 28 days after MI in pigs. To our knowledge, this is the first study to use this novel combined treatment approach in a large animal model and demonstrate positive therapeutic effects. We believe these methods and results can be translated to clinical applications in the foreseeable future.

Acknowledgments
We gratefully acknowledge Iain C. Bruce, PhD (Zhejiang University School of Medicine) for manuscript preparation and the Taitung Animal Propagation Station and the NCKU Animal Center for assistance with pig experiments.
Sources of Funding

This work was supported by the National Science Council (97IR082, 96-2314-B-006-021, 94-2314-B-006-021), the National Health Research Institutes (EX97-9722SI), and the National Cheng Kung University Integrated Landmark Project (98I006).

Disclosures

None.

References


