

# Genetic engineering of human stem cells for enhanced angiogenesis using biodegradable polymeric nanoparticles

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**Stem cells hold great potential as cell-based therapies to promote vascularization and tissue regeneration. However, the use of stem cells alone to promote angiogenesis remains limited because of insufficient expression of angiogenic factors and low cell viability after transplantation. Here, we have developed vascular endothelial growth factor (VEGF) high-expressing, transiently modified stem cells for the purposes of promoting angiogenesis. Nonviral, biodegradable polymeric nanoparticles were developed to deliver hVEGF gene to human mesenchymal stem cells (hMSCs) and human embryonic stem cell-derived cells (hESdCs). Treated stem cells demonstrated markedly enhanced hVEGF production, cell viability, and engraftment into target tissues. S.c. implantation of scaffolds seeded with VEGF-expressing stem cells (hMSCs and hESdCs) led to 2- to 4-fold-higher vessel densities 2 weeks after implantation, compared with control cells or cells transfected with VEGF by using Lipofectamine 2000, a leading commercial reagent. Four weeks after intramuscular injection into mouse ischemic hindlimbs, genetically modified hMSCs substantially enhanced angiogenesis and limb salvage while reducing muscle degeneration and tissue fibrosis. These results indicate that stem cells engineered with biodegradable polymer nanoparticles may be therapeutic tools for vascularizing tissue constructs and treating ischemic disease.**

biodegradable polymers | ischemia | nonviral gene delivery

Controlled angiogenesis is an important component of successful tissue regeneration (1) as well as the treatment of ischemic diseases. Differentiated cells such as hematopoietic cells (2) and myoblasts (3) have been shown to induce vessel formation in limb or myocardial ischemic model by expressing angiogenic factors. However, the clinical application of differentiated cells is hindered by the difficulty in obtaining a large cell number, their lack of ability to expand in vitro, and poor engraftment efficiency to target tissue sites. Stem cells are promising therapeutics for revascularization because of their capability of self-renewal, relative ease of isolation, and ability to migrate toward the ischemic tissues (4). Stem cells can contribute to angiogenesis directly, by participating in new vessel formation (5, 6), or indirectly by secreting a broad spectrum of angiogenic and antiapoptotic factors (7, 8). Furthermore, stem cells possess a homing capacity that allows them to migrate toward and engraft into the sites of ischemia or injury. Several factors such as stromal-derived factor 1 $\alpha$  (SDF-1 $\alpha$ ) and CXCR4 play a key role during the stem cell homing process, and overexpression of these chemokines contributes to enhanced homing to the target tissues (9).

Genetic modification of stem cells to express angiogenic factors is a promising approach to further enhance the efficacy of stem cells for therapeutic angiogenesis. Virally modified, VEGF-overexpressing mesenchymal stem cells (MSCs) were reported to enhance angiogenesis (1) in vivo and improve

myocardial function (10). Genetic modification of MSCs with Akt or Bcl-2 gene also improved the therapeutic efficacy of cell transplantation in treating myocardium (11, 12). However, previous studies have largely relied on viral vectors to deliver these therapeutic genes to stem cells, which are associated with safety concerns. Nonviral delivery systems, such as polyethylenimine and Lipofectamine, offer an alternative (3, 13) but are often associated with toxicity and typically provide significantly lower transfection efficiency than a viral-based approach.

Here, we developed biodegradable polymer-DNA nanoparticles to engineer stem cells to efficiently express angiogenic factors for the purpose of promoting angiogenesis in vivo. Compared with the methods of using nonviral gene therapy alone, this combined polymer-stem cells approach takes advantage of the stem cell's ability to target to the ischemic sites. Bone marrow-derived human mesenchymal stem cells (hMSCs) and human embryonic stem cell-derived cells (hESdCs) were modified with angiogenic factor (VEGF) DNA by using poly( $\beta$ -amino esters), a family of hydrolytically biodegradable polymers that can condense DNA to form nanoparticles (14). We show that scaffolds seeded with VEGF-expressing stem cells led to 2- to 4-fold higher vessel densities in the s.c. model. Four weeks after intramuscular injection into mouse ischemic hindlimbs, hMSCs transfected with VEGF markedly enhanced angiogenesis and limb salvage while reducing muscle degeneration and tissue fibrosis. This study is a description of a VEGF high-expressing stem cell therapy for angiogenesis using biodegradable polymer-DNA nanoparticles. The technology described herein may have utility as a tool for promoting therapeutic angiogenesis and treating ischemic disease.

## Results

**Polymer Synthesis.** Poly( $\beta$ -amino esters) (PBAE) were synthesized after a two-step procedure, in which C32-Ac was first prepared by polymerization by using excess diacrylate over amine monomer (Fig. 1A), and C32-Ac was then reacted with various amine reagents to generate amine-capped polymer chains (Fig. 1B). Here, we chose three leading end-modified C32 polymers (C32-103, C32-117, and C32-122), which demonstrated high transfection efficiency in stem cells (15).

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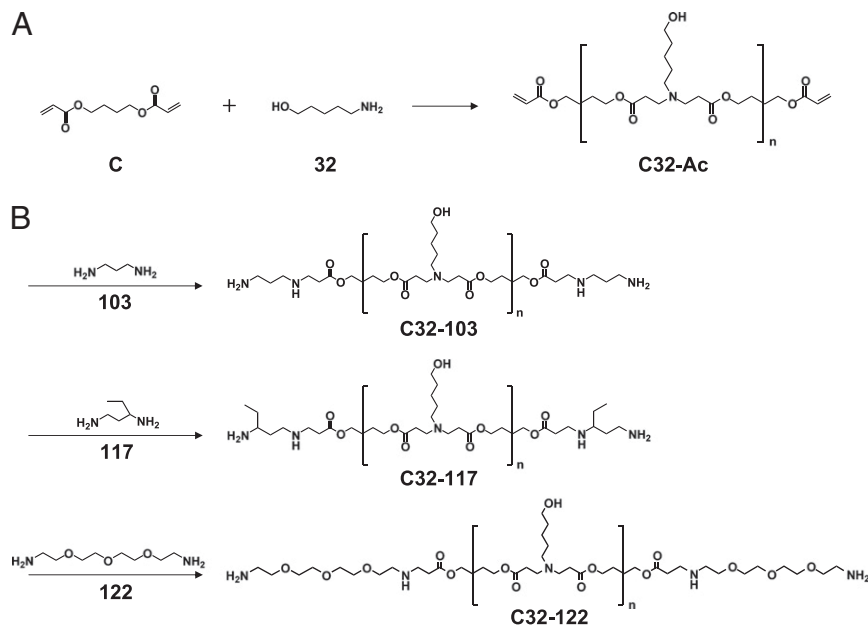
The authors declare no conflict of interest.

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**Fig. 1.** Synthesis of biodegradable poly( $\beta$ -amino esters). (A) Synthesis of acrylate-terminated C32 polymer (C32-Ac). (B) End modification of acrylate-terminated C32 (C32-Ac) with three different amine groups (103, 117, and 122).

**In Vitro VEGF Production.** VEGF production by transfected stem cells was examined by measuring the VEGF concentration in the supernatant of transfected cells by using ELISA. Four days after transfection, VEGF secretion from PBAE-transfected hMSCs or hESdCs was  $\approx$ 1- to 3-fold higher than their respective untransfected controls, and  $\approx$ 1- to 2-fold higher compared with Lipo-fectamine 2000 ( $P < 0.05$ ) (supporting information (SI) Fig. S1). VEGF secretion from day 4 to day 9 slightly decreased and was still significantly higher in PBAE-transfected groups than the control groups (Fig. S1). Cell viability after the PBAE-mediated transfection was 80–90% in both stem cell types (15).

**Enhancement of Angiogenesis in the S.C. Space.** Angiogenesis in s.c. space was examined 2 or 3 weeks after implantation. Compared with acellular scaffold controls, scaffolds seeded with VEGF-transfected hMSCs using three poly( $\beta$ -amino esters) (PBAE) led to markedly increased blood vessel migration into the constructs from adjacent tissues (Fig. 2A), whereas the control groups (hMSCs transfected with C32-103/Luc or Lipo/VEGF) did not appear to be much different from the acellular control. H&E and mouse endothelial cell antigen (MECA) staining of the harvested tissue sections demonstrated 3- to 4-fold-higher vessel density in the hMSC-PBAE/VEGF groups compared with the controls (Fig. 2A and B), and a similar trend was observed with hESdC groups (Fig. 2C).

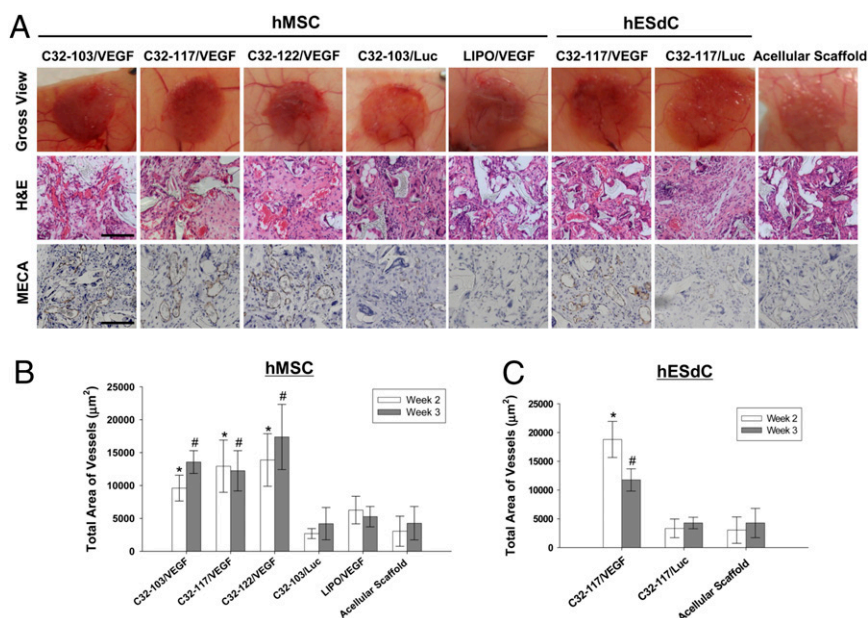
**Enhanced VEGF Production and Homing Factor Expressions in Ischemic Limbs.** VEGF production by the transplanted stem cells in vivo was examined by hVEGF ELISA. Two days after transplantation, C32-122/VEGF-transfected hMSCs produced 6-fold-higher VEGF than did untransfected cells or cells transfected with EGFP and 1-fold higher than the hMSC-Lipo-VEGF group (Fig. 3A). Compared with the normal limb tissues, the ischemic mouse limbs also demonstrated >20-fold increase in *SDF-1 $\alpha$*  expression ( $P = 0.001$ ) (Fig. S24), a chemokine that has been previously shown to stimulate the recruitment of progenitor cells to the ischemic tissues (23). Meanwhile, expression of *SDF-1* receptor *CXCR4* by the transplanted C32-122/VEGF modified

hMSCs was  $\approx$ 3-fold higher than the Lipo-VEGF modified hMSCs ( $P < 0.05$ ) (Fig. S2B).

**Enhanced Cell Survival and Localization of Transplanted Stem Cells.** RT-PCR for human-specific chromosome 17a satellite region confirmed the presence and engraftment of transplanted hMSCs in ischemic tissues, and cell survival was markedly increased in the C32-122/VEGF treatment group (Fig. 3B). Immunofluorescent staining of HNA showed significantly higher localization and retention of transplanted hMSCs in the C32-122/VEGF-treated group compared with the untransfected cells alone or Lipo/VEGF-modified group (Fig. 3C), with  $P < 0.05$  (Fig. S3).

**Enhanced Angiogenesis in Ischemic Hindlimbs.** Four weeks after cell transplantation, immunohistochemical staining for MECA (Fig. 3D) and smooth muscle  $\alpha$ -actin (SMA) (Fig. S4A) demonstrated more extensive microvessel formation in the C32-122/VEGF-modified hMSC group than the controls (PBS, no transfection, or hMSC-C32-122/EGFP). MECA-positive microvessels in the hMSC-C32-122/VEGF group was  $\approx$ 3-fold higher ( $P < 0.05$ ) compared with the PBS-treated group and 50% higher than the Lipo/VEGF-transfected group (Fig. 3E). Similarly, the density of SMA-positive microvessels in the ischemic region was also the highest in C32-122/VEGF-hMSC group (Fig. S4B). Double immunofluorescent staining of HNA and vascular markers [SMA and von Willebrand Factor (vWF)] in the C32-122/VEGF-hMSC group showed high density of HNA-positive cells in the vicinity of blood vessels at day 28 after injection (Fig. 3F).

**Improved Ischemic Limb Salvage.** The therapeutic efficacy of genetically engineered hMSCs in limb salvage was examined by evaluating physiological status of ischemic limbs 4 weeks after surgery. The outcome was rated in three levels: limb salvage (similar limb integrity and morphology as normal limb control of the same animal), foot necrosis, or limb loss. Overall, control groups demonstrated extensive limb loss and foot necrosis and C32-122/VEGF-transfected hMSCs greatly improved limb salvage (Fig. 4A). Triphenyltetrazolium chloride (TTC) staining of muscle samples harvested from the ischemic limbs also showed



**Fig. 2.** Enhanced angiogenesis by genetically engineered stem cells (hMSCs and hESdCs) in s.c. space. (A) Gross morphology of stem cell-seeded PLGA/PLLA scaffolds in s.c. space 3 weeks after implantation and histological (H&E) and immunohistochemical staining (MECA) of tissue sections from constructs harvested at 3 weeks after implantation. (B) Total area of microvessels in the harvested constructs (hMSC implants) at 2 and 3 weeks (\*,  $P < 0.05$ , compared with the control groups (C32-103/Luc, Lipo/VEGF, and acellular scaffold) at 2 weeks and #,  $P < 0.05$ , compared with the control groups at 3 weeks). (C) Total area of microvessels in the harvested constructs (hESdC implants) at 2 and 3 weeks (\*,  $P < 0.05$ , compared with the control groups (C32-117/Luc and acellular scaffold) at 2 weeks and #,  $P < 0.05$ , compared with the control groups at 3 weeks).

more viable tissues in C32-122/VEGF-treated group, which resembled the appearance of normal muscle control (Fig. 4B). Compared with the untransfected hMSCs, cells transfected with VEGF by using our polymer increased the percentage of limb salvage from 12.5% to 50% and decreased the percentage of limb loss from 60% to 20%. In contrast, groups treated with untransfected hMSCs alone, hMSCs modified with EGFP, or Lipo/VEGF-transfected hMSCs still showed substantial limb loss ( $\approx 50\%$ ) and varying degree of foot necrosis (25% to 40%) (Fig. 4C).

#### Reduced Muscle Degeneration and Fibrosis in Ischemic Hindlimbs.

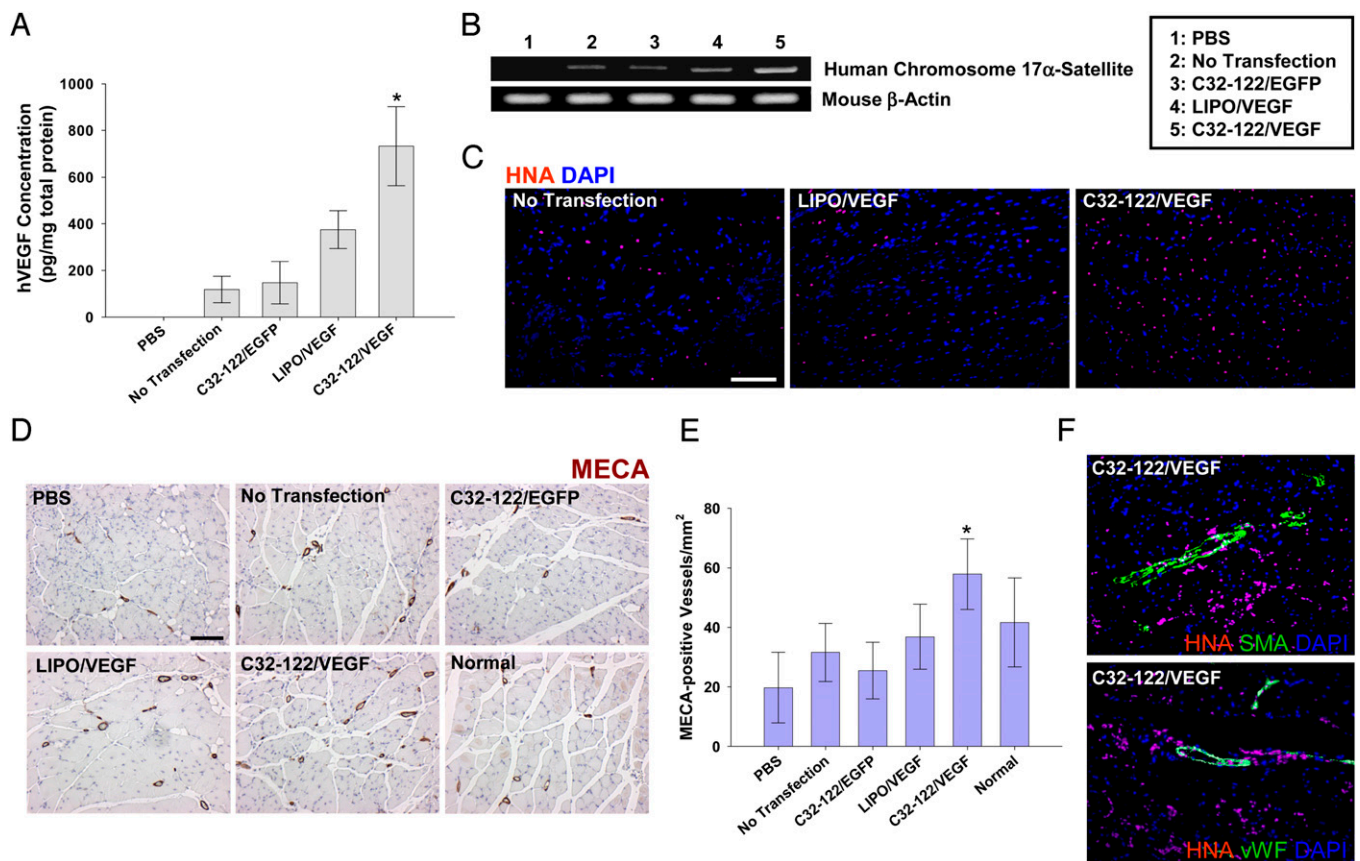
Ischemic limbs harvested at 4 weeks after cell transplantation were used for histological analyses. H&E and Masson's Trichrome staining of the control group (PBS injection) showed muscle degeneration and fibrosis in the ischemic regions (Fig. 4D and E). Transplantation of untreated hMSCs alone attenuated tissue degeneration to some degree but failed to maintain the large muscle fibrils seen in the normal tissue. In contrast, ischemic limbs treated with C32-122/VEGF-transfected hMSCs display substantially reduced tissue degeneration (Fig. 4D) and minimal fibrosis (Fig. 4E and Fig. S5).

#### Discussion

Several strategies have been developed to promote vascular growth, including growth factor delivery (16), cell-based therapy, and gene therapy. Direct delivery of angiogenic growth factors has the potential to stimulate new blood vessel growth in vivo (17) but is often associated with an initial burst of growth factors and a short half-life in vivo (18). The uncontrolled diffusion of angiogenic factors may also cause undesirable side effects. Stem cell therapy holds potential as an alternative approach that may offer advantages by promoting therapeutic angiogenesis through paracrine factor signaling (11, 12) as well as their ability to migrate toward the ischemic tissues (4). However, the efficacy of using stem cells alone to promote angiogenesis remains limited

(19). Combined stem cells and gene therapy may further stimulate angiogenesis by producing desired angiogenic and antiapoptotic factors, but safe and efficient gene delivery to stem cells has been challenging (20, 21). To overcome this hurdle, combinatorial polymer synthesis and high-throughput screening have been used to facilitate the development of nonviral gene delivery systems (22). We have developed biodegradable nanoparticulate polymeric vectors that can deliver DNA into human stem cells with high efficiency and minimal toxicity (15). End modification of the polymers were found to have dramatic effects on multiple steps of gene delivery, including the DNA binding affinity, nanoparticle size, intracellular DNA uptake, and final protein expression (22, 23). To our knowledge, gene transfection efficiency ( $\approx 35\%$ ) using these end-modified polymer nanoparticles (15) was the highest for MSCs in serum-containing transfection conditions compared with previously reported methods using electroporation (16%), poly(L-lysine)-palmitic acid (17%) (24), or commercially available transfection reagents such as FuGene (3%) and DOTAP (5%) (25).

Using optimized poly( $\beta$ -amino esters)-DNA nanoparticles, here, we modified human stem cells to express an angiogenic gene encoding VEGF. Transplantation of PBAE/VEGF-modified stem cells significantly enhanced angiogenesis in a mouse s.c. model and in a hindlimb ischemia model. In contrast, vessel density in the control groups (untransfected hMSCs or hMSCs transfected with C32-122/EGFP) were  $\approx 50\%$  lower compared with the experimental group (C32-122/VEGF) (Fig. 3E and Fig. S4B). This indicates that cells transfected with polymer/control plasmid do not have significant effects on angiogenesis. Furthermore, cells transfected with VEGF by using Lipofectamine 2000 showed only modest efficacy in angiogenesis in both models. ELISA data (Fig. S1) showed that Lipo-VEGF only slightly increased VEGF protein production (40%), whereas our leading polymers led to 3-fold-higher VEGF secretion compared with the untransfected controls. These results suggest that a critical threshold of VEGF dose may be



**Fig. 3.** Promoted angiogenesis in ischemic hindlimbs after transplantation of genetically engineered hMSCs. (A) ELISA results of hVEGF level in mouse ischemic muscles retrieved 2 days after hMSC injection (\*,  $P < 0.05$ , compared with the control groups). (B) Detection of human chromosome 17 $\alpha$  satellite gene expression by RT-PCR in mouse ischemic muscles 2 days after hMSC injection. (C) HNA staining of mouse ischemic muscle 2 days after hMSC injection. (D) Immunohistochemical staining of ischemic muscle tissue sections 4 weeks after cell transplantation for mouse endothelial cell antigen (MECA). (E) Quantification of MECA-positive microvessel density in ischemic regions (\*,  $P < 0.05$ , compared with the control groups of PBS, no transfection, C32-122/EGFP, and Lipo/VEGF). (F) Double immunofluorescent staining of HNA and vascular markers (SMA or vWF) to demonstrate the fate of transplanted hMSCs. Most of transplanted hMSCs (HNA-positive cells) were found in the vicinity of microvessels.

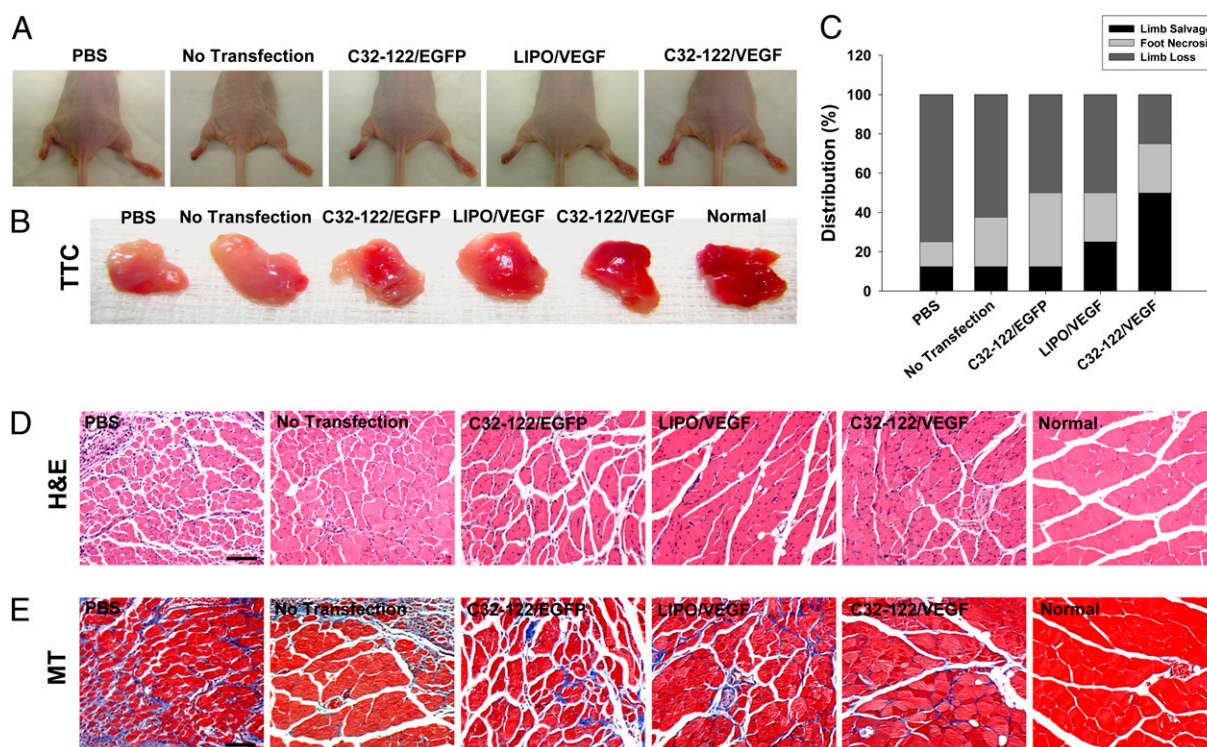
required to achieve significant angiogenesis. Limb ischemia not only led to impaired angiogenesis, but also caused abnormal tissue fibrosis, as shown in Fig. 4E. Imaging analysis of tissue sections stained for collagen (Fig. S5) showed that fibrotic area in ischemic region was markedly reduced by injection of hMSCs transfected with C32-122/VEGF nanoparticles, compared with all of the controls.

Previous work described the use of adenovirus to transduce hMSCs or hESCs for VEGF overexpression and showed at least one order of magnitude higher level of VEGF production in vitro compared with the untransfected controls, which lasted for  $\approx 30$  days (26, 27). Although the amounts of VEGF produced by our polymers are not as high and lasted up to 2 weeks, our results suggest that the level and duration of VEGF production induced by these polymers are sufficient to achieve therapeutic angiogenesis. Virally modified, VEGF-expressing endothelial progenitor cells have been reported to improve neovascularization in a mouse model of hindlimb ischemia and increased limb salvage to  $\approx 60\%$  in comparison with control animals (28). In our study, C32-122/VEGF-modified hMSCs significantly enhanced angiogenesis and increased the percentage of limb salvage to 50% while decreasing limb loss down to 20% (Fig. 4C). Our data indicate that transient, nonviral delivery with PBAE materials may provide therapeutic efficacy comparable with that provided by viral strategies. Furthermore, we hypothesize that the tran-

sient, nonviral degradable nature of these delivery systems may allow for improved safety, relative to adenovirus.

The observed enhanced angiogenesis and reduced tissue necrosis is likely a result of enhanced paracrine signaling from stem cells. Previous work has shown that untransfected stem cells themselves may secrete a broad spectrum of cytokines (7, 10) (e.g., FGF2 and Sfrp2) that can mediate ischemic tissue survival and repair. Together with the up-regulated production of VEGF by PBAE/VEGF transfection (Fig. S1), these paracrine factors secreted by the stem cells may lead to enhanced angiogenesis, decreased cell apoptosis, and better tissue survival, relative to VEGF protein alone. This hypothesis is supported by our in vitro conditioned medium study. We found that conditioned medium from PBAE/VEGF-transfected stem cells led to increased viability of human endothelial cells under hypoxic (1% oxygen) and serum-free conditions, an in vitro model mimicking ischemia (Fig. S6).

Efficient cell engraftment and retention is critical for successful cell-based therapy for promoting angiogenesis. To assess the engraftment and survival of transplanted human stem cells in ischemic mouse limbs, we measured human-specific gene expression level (chromosome 17 $\alpha$  satellite region) in target mouse tissues, which should be directly in proportion to the engraftment and survival of transplanted human cells. We observed significantly enhanced human 17 $\alpha$  expression and human nuclear antigen (HNA) staining in hMSC-C32-122/VEGF group (Fig. 3 B and C), which suggests



**Fig. 4.** Improved ischemic limb salvage by genetically engineered hMSCs. (A) Representative photographs of treated ischemic hindlimbs and controls at 4 weeks. (B) TTC staining of muscles retrieved from treated and control limbs at 4 weeks. (C) Physiological status of ischemic limbs was evaluated 4 weeks after surgery and rated in three levels; limb salvage (similar limb integrity and morphology as normal limb control of the same animal), foot necrosis, or limb loss. (D) H&E staining showed massive muscle degeneration in the ischemic regions of control limbs (PBS-injection group), and such muscle degeneration was markedly reduced in group receiving C32-122/VEGF-transfected hMSCs. (E) Masson's trichrome staining demonstrated significant fibrosis in the control groups, which was greatly attenuated by transplantation of C32-122/VEGF-transfected hMSCs.

enhanced localization and engraftment of genetically engineered stem cells into ischemic sites. This is also supported by the significantly up-regulated gene expression of two stem cell homing factors: SDF-1 $\alpha$  and its receptor CXCR4. The observed enhanced CXCR4 expression is probably due to VEGF-mediated angiogenic signaling (29) and enhanced cell survival. Previous work reported the use of 3D matrices to facilitate localization of transplanted cells and more sustained delivery of angiogenic factors for revascularization. Injection of alginate microparticles with VEGF protein was shown to enhance *in vivo* survival of transplanted cells and the subsequent angiogenesis in hindlimb ischemic tissue (30). However, alginate microparticles are nondegradable, and may not be cleared. In contrast, *ex vivo* genetic modification allows for a transient, matrices-free approach. Our results suggest that PBAE/VEGF-modified stem cells alone without matrices are sufficient to achieve the satisfactory cell engraftment and retention.

In summary, this study suggests that stem cells transiently modified with biodegradable polymeric nanoparticles can promote therapeutic angiogenesis. This technology may facilitate engineering and regeneration of large masses of various tissues such as bone and muscle, as well as complex structures that encompass multiple tissue types. We further hypothesize that this approach could be useful in treating other types of ischemic diseases such as myocardial infarction and cerebral ischemia.

## Materials and Methods

An expanded *Materials and Methods* is provided in the *SI Materials and Methods*.

**Transfection.** Bone marrow-derived hMSCs and hESdCs were obtained and cultured as previously described (31). Cells were transfected with VEGF plas-

mid or control plasmid (EGFP or Luciferase) by using optimized poly( $\beta$ -amino esters) transfection conditions (15). Lipofectamine 2000 (Invitrogen), a commercially available transfection reagent, was used for control transfection (for more detail, see *SI Materials and Methods*).

**S.C. Implantation of Stem Cell-Seeded Scaffolds.** All procedures for surgery were approved by the Committee on Animal Care of Massachusetts Institute of Technology. All constructs ( $1.0 \times 10^6$  cells per scaffold) were implanted into *s.c.* space in the dorsal region of athymic mice. Three experimental groups were studied for hMSCs transfected with: (i) C32-103/VEGF, (ii) C32-117/VEGF, (iii) C32-122/VEGF. Three control groups include (i) hMSC-C32-103/Luc, (ii) hMSC-Lipo/VEGF, and (iii) acellular scaffold alone. For hESdCs, cells were transfected by using either C32-117/VEGF or C32-117/Luc, and the acellular scaffold group was examined as blank control ( $n = 3$ ). All tissue constructs were harvested at 2 or 3 weeks after implantation for analyses (for more detail, see *SI Materials and Methods*).

**Transplantation of Stem Cells into a Mouse Ischemic Hindlimb Model.** Hindlimb ischemia was induced in a mouse model as previously described (6). Immediately after arterial dissection, cells ( $1.0 \times 10^6$  cells per injection) were suspended in 100  $\mu$ L of hMSC growth medium and injected intramuscularly into two sites of the gracilis muscle in the medial thigh. Five experimental groups ( $n = 8$  per group) were examined as following: (i) PBS, (ii) no transfection, (iii) hMSC-C32-122/EGFP, (iv) hMSC-Lipo/VEGF, and (v) hMSC-C32-122/VEGF. All of the animals were killed at the 4-week time point for analyses. All animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (for more detail, see *SI Materials and Methods*).

**Statistical Analysis.** Quantitative data are expressed as mean  $\pm$  standard deviation. Statistical analysis was performed by the ANOVA by using a Bonferroni test. A value of  $P < 0.05$  was considered statistically significant.

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