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The effect of controlled expression of VEGF by transduced myoblasts in a cardiac patch on vascularization in a mouse model of myocardial infarction

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Abstract

Key requirements for cardiac tissue engineering include the maintenance of cell viability and function and the establishment of a perfusable vascular network in millimeters thick and compact cardiac constructs upon implantation. We investigated if these requirements can be met by providing an intrinsic vascularization stimulus (via sustained action of VEGF secreted at a controlled rate by transduced myoblasts) to a cardiac patch engineered under conditions of effective oxygen supply (via medium flow through channeled elastomeric scaffolds seeded with neonatal cardiomyocytes). We demonstrate that this combined approach resulted in increased viability, vascularization and functionality of the cardiac patch. After implantation in a mouse model of myocardial infarction, VEGF-expressing patches displayed significantly improved engraftment, survival and differentiation of cardiomyocytes, leading to greatly enhanced contractility as compared to controls not expressing VEGF. Controlled VEGF expression also mediated the formation of mature vascular networks, both within the engineered patches and in the underlying ischemic myocardium. We propose that this combined cell-biomaterial approach can be a promising strategy to engineer cardiac patches with intrinsic and extrinsic vascularization potential.

Keywords

cardiac tissue engineering; elastomer; scaffold; bioreactor; cardiac ischemia; angiogenesis; gene therapy

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INTRODUCTION

The adult heart has minimal capacity to regenerate itself after injury, as the turnover rate of adult cardiomyocytes is only 0.1–0.4% per year [1]. Massive cell death associated with myocardial infarction results in the formation of a fibrous non-contractile scar that often leads to congestive heart failure, a condition associated with high mortality. In recent years, cell-based heart repair is emerging as an option for revascularization and regeneration of the infarct bed [2–7]. Cell-based strategies have shown potential to decrease scar formation and enhance heart contractility [8–11]. However, as therapeutic cells are mostly delivered by direct injection, extremely low cell retention and survival immediately after injection and during the first week remain a major limitation of this approach [12].

Engineering a functional cardiac patch could provide retention and conditioning of the implanted cells, leading to improved survival and integration, and rescuing the myocardial function [7]. However, engineering of a viable cardiac patch that is several millimeters thick and contains high density of electromechanically functional cells is still elusive [13]. Several technologies can yield a patch with a large enough surface area [4, 14–16], but generating a thick and compact cardiac construct is challenging due to the limited diffusional depth of oxygen in tissue, which is only ~100 μm at physiologically high cell densities [14].

We previously developed a cell-scaffold-bioreactor approach to perfuse culture medium through thick cell-loaded constructs, which effectively improved cell survival and assembly *in vitro* [17, 18]. Key features of this approach are the combination of an elastomeric scaffold, with advantageous mechanical properties for cardiac tissue engineering [19], and an array of channels providing efficient oxygen delivery while protecting cells from hydrodynamic shear [18]. Upon implantation, the engineered graft also requires rapid vascular invasion to survive and continue to function [13]. Vascular Endothelial Growth Factor (VEGF) is the fundamental regulator of vascular development [20] and its therapeutic overexpression by the cells loaded into the construct is an attractive strategy to guide blood vessel in-growth to the core of the tissue-engineered graft.

A major challenge in vascularizing cardiac grafts lies in the potential for VEGF overexpression to cause significant side effects, such as the growth of vascular tumors (angiomas) in the myocardium and other tissues [21–24]. We have previously found that the therapeutic window of VEGF does not depend on the total dose administered, but rather on its concentration in the microenvironment around each producing cell [25, 26]. In fact, VEGF remains localized in the extracellular matrix and even rare areas of high expression are sufficient to cause the formation of angiomas. When the distribution of VEGF dose was controlled by delivering monoclonal populations of transduced myoblasts, in which every cell produced the same amount of VEGF, stable and functional angiogenesis could be induced over a wide range of VEGF expression levels [25, 26].

Our goal was to combine cell-based gene therapy and cardiac tissue engineering towards generating a fully viable cardiac patch with intrinsic vascularisation potential. To this end, we took advantage of a FACS-based technique we recently developed to rapidly purify homogeneous populations of transduced cells producing a defined level of VEGF, to reliably induce robust and normal angiogenesis [27, 28]. This strategy is not directly applicable to cardiomyocytes, because they cannot be sufficiently expanded *in vitro* to allow efficient transduction and FACS-purification. Therefore, we generated a cardiac patch with intrinsic vascularization potential by seeding channeled elastomeric scaffolds in perfusion bioreactors with a combination of (i) FACS-purified skeletal myoblasts (SM) releasing controlled levels of VEGF and (ii) neonatal rat cardiomyocytes (RCM).

MATERIALS AND METHODS

Cardiomyocytes

Neonatal rat cardiomyocytes (RCM) were isolated from 2 day-old Sprague Dawley rats (Harlan, South Easton, MA) as previously described [18] according to an institutionally approved IACUC protocol. Briefly, heart ventricles were quartered, incubated at 4°C in a 0.06% (w/v) trypsin in Hank's Balanced Salt Solution (HBSS, Gibco, Carlsbad, CA), and subjected to a series of digestions (3–4 min, 37°C, 150 rpm) in a 0.1% (w/v) solution of collagenase type II (Worthington Biochemical Corp., Lakewood, NJ) in HBSS. Cell suspensions from the first 4–5 digestions were collected, and preplated in polystyrene culture flasks for 75 min to remove the readily adhering cardiac fibroblasts. The non-adherent cells, that are enriched for cardiac myocytes, were counted using a hemocytometer and suspended in cardiac medium (CM) consisting of high glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% heat inactivated fetal bovine serum, as previously described [18].

Transduced skeletal myoblasts

Primary skeletal myoblasts (SM) were isolated from neonatal (2–5 day old) C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) as previously described [29]. Briefly, both forelimb and hind limb muscles were collected, minced to a slurry and digested with 1.5 U/ml of collagenase D (Roche Molecular Biochemicals, Indianapolis, IN), 2.4 U/ml of dispase II (Roche Molecular Biochemicals) and 2.5 mM CaCl₂ at 37°C. After filtration through an 80-µm nylon mesh (Becton Dickinson, Franklin Lakes, NJ) cells were plated on collagen-coated dishes, fibroblasts were allowed to attach for 45 minutes and non-adhering myoblasts were removed to a fresh dish.

Myoblast stocks were transduced with a retrovirus expressing the β-gal marker gene [29], expanded and frozen until use. Aliquots were then infected at high efficiency as previously described [27,30] with retroviral vectors expressing either VEGF linked to truncated CD8a as a FACS-quantifiable cell surface marker (pAMFG-VICD8), or CD8a alone as a control (pAMFG-ICD8). Briefly, high-titer retroviral supernatants were obtained from the helper-free, ecotropic φNX packaging cell line after transient transfection with the retroviral construct plasmids [31]. Skeletal myoblasts (4×10^5 per one 60-mm dish) were plated, incubated in 4 ml of fresh viral supernatant supplemented with 8 µg/ml polybrene (Sigma-Aldrich, St. Louis, MO) for 15 minutes at 37°C, and centrifuged in a microplate carrier at 1,100g for 30 minutes at room temperature.

Myoblast populations were expanded at 37°C in 5% CO₂ on collagen-coated dishes, in growth medium consisting of 40% F10, 40% Dulbecco's modified Eagle's medium, and 20% fetal bovine serum, supplemented with 2.5 ng/ml FGF-2, as previously described [32]. FACS-purification of transduced myoblast populations expressing a controlled VEGF level was performed as previously described [27]. Briefly, transduced myoblasts were stained with an antibody specific to mouse CD8a and directly conjugated to allophycocyanin (clone 53–6.7 from BD Biosciences, Basel, Switzerland). Optimized conditions included the use of 0.8 µg of antibody/ 10^6 cells in 200 µl of PBS with 5% BSA for 20 minutes on ice. Cell sorting was performed with a FACS Vantage SE cell sorter (BD Biosciences). Both VEGF-expressing and control transduced myoblasts were expanded for 2 passages after FACS-purification, frozen in 10% DMSO in calf serum and stored in liquid nitrogen.

Channeled elastomer scaffolds

Porous poly(glycerol sebacate) (PGS) scaffolds were prepared as previously described [18], with open interconnected pores ranging from 75 – 150 µm, a void volume of 89%, an elastic

modulus in tension of 282 ± 25 kPa and a compressive modulus of 2.35 ± 0.03 kPa [17]. An array of cubically packed channels (250 μ m in diameter, 500 μ m wall-to-wall spacing) was created using a computerized 40W carbon dioxide laser (Epilog, Pololu Robotics & Electronics, Las Vegas, NV). Scaffolds were cored into disks (8 mm diameter, 1 mm thick), sterilized by autoclave and ethanol gradient (75, 50, 25, and 0% EtOH), and soaked overnight in DMEM containing 10% FBS.

Cell preparation and scaffold seeding

Engineered constructs were prepared using either (i) RCM alone or (ii) a mixture of RCM with control SMs (CD8-SM), or (iii) VEGF-expressing SM (VEGF-SM). One day prior to cell seeding, the scaffolds were coated with laminin (10 μ g/ml, BD Biosciences) [19, 33] and seeded by perfusing a suspension of cells as in our previous studies [17]. The inner 5-mm cores of laminin-coated scaffolds were directly perfused with the suspensions of RCM, CD8-SM or VEGF-SM as specified above, at a velocity of 1 mm/s for 2 hours. In all groups, the seeding density of RCM has been maintained at 1.48×10^8 RCM/cm³ (corresponding to 3.3×10^6 cells per scaffold). The ratio of SM/RCM in the mixture was 0, 1:2 or 1:6, corresponding to 0, 1.65×10^6 or 0.55×10^6 SM/scaffold, respectively.

The cell-seeded constructs were then cultured at unidirectional perfusion flow rate of 0.1 μ L/s for an additional 7 days. Depending on the experiment, cells were cultured on the scaffolds in either of the following media: (a) cardiac medium (Cm) consisting of DMEM containing 4.5 g/L glucose supplemented with 10% FBS, (b) skeletal muscle differentiation medium (Diff) DMEM containing 1 g/L glucose supplemented with 2% horse serum (HyClone, South Logan, UT), and (c) a 1:1 mix of the Cm and Diff (Mix). All media were supplemented with 10mM HEPES, 2mM L-glutamine, and 100 units/mL penicillin (Gibco).

Animal model of acute cardiac ischemia

After 8 days of cultivation, the cardiac patches were implanted into a nude mouse (Harlan) model of cardiac infarction according to an institutionally approved IACUC protocol [6]. Briefly, the left anterior descending coronary artery (LAD) was exposed through a left thoracotomy and tied with a prolene suture. One week after ischemia, the patch was sutured onto the infarct bed (n=6/group). The perioperative mortality was around 50%. Four weeks later, mice were sacrificed and the hearts were harvested.

Cardiac function

Cardiac function was assessed by high- frame-rate, high-resolution echocardiography before myocardial infarction (normal baseline), 1 week after inducing infarcts by LAD ligation (infarct baseline), and 4 weeks after patch implantation. The high-resolution myocardial elastography technique that was previously developed [34, 35] was used. Briefly, a 30-MHz ultrasound transducer (RMV-707B, VisualSonics Inc., Toronto, Canada) was placed on the mouse chest in the parasternal position to obtain a long-axis view and a short-axis view of the left ventricle (LV) of the heart. The field of view was 12mm $\times$ 12mm, the axial resolution was 55 μ m, and the lateral resolution was 115 μ m. The transducer operated on a line-by-line basis. A two-channel, 14-bit waveform digitizer (CompuScope 14200, Gage Applied Technologies Inc., Lachine, Canada) was used to simultaneously acquire the ultrasound radiofrequency (RF) signals and associated ECG at 200 MHz and 8 kHz, respectively. The acquisition involved 180 to 192 RF signals over the entire field of view and lasted approximately 7 min. Using retrospective ECG gating the acquired RF signals were registered off-line to reconstruct the RF image sequence for a complete cardiac cycle at the extremely high frame rate of 8 kHz.

The incremental axial displacements of the myocardium were estimated between consecutive RF frames using a time-efficient, 1-D normalized cross-correlation technique previously described [36]. A window size of 480 μ m and a window overlap of 95% were used. By automatically tracking the same regions of the LV and accumulating the incremental displacements over time [35], the cumulative displacements were obtained representing the total motion from the first frame to any other frame of the cine-loop. A linear Savitzky-Golay digital differentiator with a length of seven samples (140 μ m) [37] was applied to the cumulative displacements to estimate the cumulative strains. The cumulative strains over one cardiac cycle were extracted from a region-of-interest (ROI) that was manually segmented to correspond to the infarcted area that received the cardiac patch. The peak cumulative strains representing the total deformation of the myocardium during systole were determined. In the short-axis view, only the strains in the regions where the radial direction of the myocardial deformation aligned with the axial direction of the ultrasound beam were shown, since only 1-D, i.e. axial, displacements and strains were estimated [34, 35].

Histological evaluation

Whole hearts were harvested, bisected along the short axis, embedded in OCT compound (Sakura Finetek, Torrance, CA), frozen in freezing isopentane, and sectioned for histological evaluation. For general histomorphological evaluation, sections were stained with hematoxylin and eosin (H&E) and Masson trichrome, according to standard protocols. Color images were acquired on a Q-imaging camera mounted onto a light microscope (Olympus, Center Valley, PA).

Immunofluorescence

Tissue sections were fixed in acetone, incubated for 40 minutes in 10% goat serum (at room temperature), and then for 1 hr (at room temperature) in the following primary antibodies: (1) rat monoclonal anti-mouse CD31 (clone MEC 13.3; BD Biosciences) at 1:100, (2) rabbit polyclonal anti-NG2 (Chemicon, Temecula, CA, US) at 1:200, (3) mouse anti-cardiac troponin I (TnI; Millipore, Billerica, MA) at 1:150. Subsequently, tissue sections were incubated for 1 hr (at room temperature) in fluorescently labeled Alexa546 anti-rat, Alexa647 anti-mouse and Alexa488 anti-rabbit secondary antibodies (Invitrogen, Carlsbad, CA) at 1:200. Antibodies were diluted to 1.5% in horse serum and 0.5% in Tween 20 PBS.

Tissue morphometry

The thickness of the different regions in the myocardium (infarct bed, implanted tissue constructs and adjacent native myocardium) was evaluated using tissue sections stained with Masson trichrome. The infarct region could be clearly identified by the blue stained collagenous scar tissue. The implanted constructs were measured as the region right above the infarction. The underlying myocardium was identified as the region around the infarction (beneath or immediately adjacent) and under the patch. Vascular density was measured in tissue sections immunostained with fluorescent antibodies for endothelium (CD31, red) and pericytes (NG2, green). The vessel density was quantified with Image J software (NIH, Bethesda, MD) as the percent area that positively stained for CD31 separately in the three regions: fibrous scar tissue, tissue constructs, and the underlying myocardium for all experimental groups.

Statistics

Data were analyzed using the Prism 4.0a software (GraphPad, La Jolla, CA) and results were presented as means $\pm$ standard error. The significance of differences was evaluated by

analysis of variance (ANOVA) followed by a Bonferroni test. $P < 0.05$ was considered statistically significant.

RESULTS

In vitro co-culture of cardiomyocytes and myoblasts

Since skeletal myoblasts (SM) are highly proliferative *in vitro*, co-culture conditions were optimized to avoid overgrowth of the rat cardiomyocytes (RCM) by myoblast populations during the 8 days of perfusion culture on PGS scaffolds. Control CD8 myoblasts were used for these experiments. Three different media compositions (cardiac medium, Cm; myoblast differentiation medium, Diff; 1:1 mixture of the two, Mix) and three different SM/RCM ratios were investigated. In all groups, the seeding density of RCM has been maintained at 1.48×10^8 RCM/cm³ (corresponding to 3.3×10^6 cells per scaffold).

The ratio of SM/RCM in the mixture was 0, 1:6 or 1:2, corresponding to 0, 0.55×10^6 or 1.65×10^6 SM/scaffold, respectively. As shown in Figure 1A, cardiac medium Cm and Mix medium supported the cultivation of RCM significantly better than Diff medium, and allowed the maintenance of SM without excessive growth. Co-cultures with a SM/RCM ratio of 1:6 in cardiac medium yielded the most reproducible maintenance of both cell types and this condition was adopted for subsequent studies. In all experimental groups, all cell types exhibited spatially uniform distributions throughout the scaffold volume, in agreement with our previous studies using the same method for scaffold seeding [17].

Four experimental groups were formed for animal implantation studies: (1) RCM alone (RCM), (2) 1:6 CD8-SM/RCM (CD8), (3) 1:6 VEGF-SM/RCM (VEGF), and (4) cell-free scaffold (PGS). VEGF-SM were purified by a flow cytometry-based technology we recently developed [27] to generate a population expressing a homogeneous level of VEGF that we have previously shown to be safe and effective (20.2 ± 1.0 ng of VEGF/day/ 10^6 cells). After 7 days in culture, the DNA content of the constructs from the three cell-loaded groups (RCM, CD8 and VEGF) was comparable (Figure 1B).

In vivo cardiac function

High-resolution myocardial elastography allowed myocardium strain to be measured in the region of the infarction, both in the short and the long axes. Strain measures the deformation of cardiac muscle and was quantified at baseline before LAD ligation, 1 week after LAD ligation right before patch implantation, and 4 weeks after patch implantation (Figure 2A). Before surgery, systolic strain values were within the normal range in all mice and very similar between short and long axis (0.50 ± 0.10 and 0.52 ± 0.08 , respectively). One week after infarction, the heart function was significantly compromised in all mice while short- and long-axis strain values were in good agreement (0.16 ± 0.06 and 0.09 ± 0.08 , respectively; at $p < 0.001$ and $p < 0.0001$ compared to the respective pre-infarction values).

To assess the functional effect of treatment, the ratio between the strain values 4 weeks after treatment and right before patch implantation (1 week after infarction) was calculated for each animal. These values therefore represent the fold-change in cardiac function caused by implantation of the different patches. As shown in Figure 2B–C, patches made of scaffold alone or seeded only with RCM did not improve cardiac function (strain ratio of about 1 both in the short and long axes). Addition of control SM caused a slight, but not significant improvement in the strain ratio to approximately 2. In contrast, treatment with patches generated by co-culturing RCM with the SM expressing a specific VEGF level significantly improved cardiac function, by 6- to 10-fold compared to all other groups (short- and long-axis, $p < 0.05$ and $p < 0.01$ respectively).

In vivo patch engraftment

In order to determine the mechanism by which VEGF-expressing patches significantly improved cardiac function, we analyzed the effects of the different conditions on patch engraftment, cell survival and differentiation, infarct size and tissue vascularization. At four weeks after implantation, all PGS scaffolds were completely degraded. Acellular patches made of acellular PGS contained loose fibrous tissue surrounded by a fibrous capsule (Figure 3A–B, Figure 4A). The patches formed from RCM alone or from RCM and control SM (not expressing VEGF) generated fibrous tissues with a high cellular content, but without organized muscle structure (Figure 3C–F, Figure 4B–C). In contrast, implantation of patches containing RCM and VEGF-expressing SM led to the robust generation of dense tissue with compact cellular structure (Figure 3G–H), and without fibrosis (Figure 4D). These tissues also showed best integration with the underlying myocardium, as evidenced by seamless transitions and connections between the tissue layers (Figure 3G).

The thicknesses of the infarct, the patch-derived tissue and the underlying myocardium were quantified on sections stained with Masson trichrome. The thickness of the infarcted region underneath cardiac patches was similar in all groups, except for the RCM alone group, in which the infarct was slightly but significantly thinner (Figure 4E, $p < 0.01$). The improvement in cardiac function following implantation of VEGF-expressing patches was therefore not due to the change in the ventricle geometry in the infarct region.

The implantation of patches based on RCM, control SM and co-culture of RCM and VEGF-expressing SMs respectively decreased, maintained and increased the thickness of the tissue in the patch region, defined as the area above the infarction including the associated capsule, compared with the acellular elastomer patch alone (Figure 4F). Notably, the thickness of the VEGF-expressing patches was significantly higher as compared to all other conditions ($p < 0.01$ vs CD8, $p < 0.001$ vs PGS, $p < 0.001$ vs RCM). Consistently, VEGF expression had a major effect on the survival of the underlying myocardium, leading to about 3-fold greater thickness compared to all other groups ($p < 0.001$; Figure 4G).

Cell survival and differentiation in vivo

In order to determine the effects of controlled VEGF expression on the survival and differentiation of implanted cells, RCM and SM were tracked by expression of cardiac-specific Troponin-I and of the retrovirally encoded β -galactosidase enzyme, respectively. In the absence of VEGF expression, neither RCM nor SM could be found 4 weeks after implantation (Figure 5A–B and Figure 5D, respectively). However, in the VEGF-expressing patches not only both RCM and SM survived, but successfully differentiated into morphologically interconnected cardiomyofibers (Figure 5C) and mature skeletal myotubes (Figure 5E–F), respectively.

Vascularization in vivo

Vessel density was quantified in different areas of the implantation sites: the fibrous capsule, the patch-derived tissue and the underlying myocardium. As shown in Figure 6, the vascularization of the fibrous capsule was similar in all groups (Figure 6A). Since the patches made of PGS alone were completely resorbed and left no recognizable tissue except for the fibrous capsule, vessel density of the patch area could not be measured in this condition. The disorganized tissue generated by patches made of RCM alone or together with control SM had similar vessel densities, whereas VEGF-expressing patches had significantly increased vascular in-growth into the patch area (Figure 6B, $p < 0.01$).

Interestingly, the VEGF-expressing patches induced angiogenesis in the underlying myocardium and caused a greater than 3-fold increase in its vessel density compared to the

condition implanted with a PGS scaffold alone (Figure 6C, $p < 0.001$). In contrast, RCM alone or RCM with control SM, did not affect vascularization of the underlying myocardium (Figure 6C). Controlled VEGF expression induced the formation of vessels resembling mature capillaries, of regular size and surrounded by NG2-positive pericytes (Figure 6D).

DISCUSSION

We demonstrate that a cardiac patch with vascularization potential can be engineered by combining effective oxygen supply during *in vitro* cultivation (by interstitial flow of culture medium through channeled elastomeric scaffolds seeded with cells), with controlled VEGF expression by the component SM cells. In a mouse model of cardiac ischemia, the implantation of such cardiac patch significantly improved cardiac function, through mechanisms involving the formation of mature vascular networks both within the engineered patch and the underlying myocardium. Because these effects were not achieved with either of the three control groups: acellular scaffold, patch based on cardiomyocytes alone or co-cultured with control cells that did not express VEGF, they could be attributed to the sustained release of VEGF. Notably, the *in vivo* vascularization induced by the VEGF-releasing cells was efficient enough to allow survival and differentiation of the cardiac myocytes inside a millimeter-thick cardiac patch.

Vascularization is essential to maintain their viability and function of cardiac patches upon implantation, and to enable integration of the patch tissue to the surrounding myocardium [38, 39]. In previous studies, vascularization of engineered cardiac patches was investigated by either implantation into the omentum [2, 40, 41], which requires a supplementary surgical procedure, and by co-culturing cardiac progenitors and endothelial cells [2, 42]. In the present study, significant improvements of heart function following patch implantation critically depended on the use of VEGF-expressing cells, as RCM alone and RCM co-cultured with control SMs could not rescue cardiac contractility.

Our results suggest that the VEGF-dependent improvement of heart function was due to two related effects. First, the vascularization of the patch enabled the survival and differentiation of the seeded cells, leading to the formation of contractile muscle fibers, and the formation of thick and compact tissue in the patch region. Second, the increased vascular density in the underlying myocardium contributed to the functionality of the native myocardium underlying the patch through improved perfusion and nutrient supply (Figures 4 and 6). The sustained release of VEGF provided the engineered cardiac patch with both intrinsic and extrinsic vascularization potential, supporting the generation of mature cardiac and skeletal myofibers in the patch and of new capillary networks in the surrounding myocardium, respectively. At this point, we cannot distinguish between the relative contributions of vascularization of the patch and the underlying myocardium.

VEGF expression at controlled levels induced the formation of normal capillary networks that were stable and surrounded by NG2-positive pericytes, while angioma growth was not observed. Critical for this result was the application of a technique we recently developed [27, 28], by which we could rapidly FACS-purify a population of transduced cells that homogeneously produced a narrow range of VEGF concentrations that was both safe and effective [25–27]. Our previous studies showed the uniformity of VEGF expression, with each cell producing the same amount of VEGF, was even more important than the overall dose of VEGF. We showed that reducing the total dose of VEGF expression to even much lower levels than those described here, i.e. as low as about 4 ng/10⁶ cells/day, but without eliminating their heterogeneity by FACS-purification, invariably caused lethal angioma growth [25]. Such angiomas were induced by uncontrolled VEGF expression by a variety of

gene delivery systems, such as transduced myoblasts [21, 22, 24, 25], plasmid DNA [23] or adenoviral gene therapy vectors [44], both in skeletal muscle and in the myocardium.

Although implanted cells express homogeneous VEGF levels, the total amount of VEGF production is known to decrease over the first 2–4 days after implantation [44]. We showed in our previous studies that this change was due to the death of myoblasts that were not successfully differentiated, and lower expression of the retroviral promoter *in vivo* as compared to the high-serum culture conditions *in vitro* [25, 27]. Importantly, the expression of VEGF in this model system stabilizes following the initial changes [25, 27].

Homogeneous levels of VEGF expression in myocardium were also achieved by incorporating recombinant factors in biomaterials (reviewed in [45]). However, the use of FACS-purified transduced progenitors has some comparative advantages. Constant release of bioactive recombinant VEGF for at least 4 weeks, a time necessary for stabilization of newly induced vessels [25, 46], can be readily achieved by VEGF secretion from the cells. Providing the whole dose of factor in the biomaterial depot may require implantation of larger volumes of the material, and difficulties in maintaining constant rate of release.

Interestingly, effective vascularization of the whole patch thickness, support of tissue differentiation and improved cardiac function required only ~ 15% VEGF-expressing skeletal myoblasts within the cell population in the patch. It is entirely possible that an even lower fraction of transduced cells may be sufficient, as the minimum necessary amount of VEGF needs to be determined experimentally and may depend on the cell type used to express VEGF. Also, it would be worth exploring the effects of controlled release of VEGF on populations of resident cardiac progenitors, embryonic stem cells or induced pluripotent stem cells [13] and their potential to (re)generate contractile cardiac muscle.

The model system used in this study also has some limitations. Retrovirally transduced myoblasts were used as the VEGF-expressing cells in this work because they provide a well-characterized and highly controlled model of VEGF dose-dependent angiogenesis that we have developed over the last decade [25–28, 32, 47]. However, retroviral vectors sometimes cause neoplastic transformation of transduced stem cells by insertional mutagenesis and activation of endogenous oncogenes by their strong LTR promoter [48]. Also, skeletal myoblasts cannot electrically couple with the resident cardiomyocytes and thus can interfere with cardiac conduction and give rise to arrhythmias [49, 50]. Our results provide proof-of-principle for generating a cardiac patch with both intrinsic and extrinsic vascularization potential, using an approach that could be translated using clinically compliant tools, such as lentiviral vectors and mesenchymal progenitor cells. Self-inactivating lentiviral vectors could ensure sustained stable expression of a transgene while carrying much lower oncogenic potential than retroviral vectors [51, 52]. Mesenchymal progenitor cells from adipose tissue do not cause arrhythmias [53] and are currently undergoing clinical evaluation for the treatment of cardiac ischemia [54,55]. We have recently adapted the high-throughput FACS-based technology that we employed here to rapidly purify transduced mesenchymal progenitors expressing specific VEGF levels [56].

CONCLUSIONS

We show that the combination of effective oxygen supply during *in vitro* cultivation (by interstitial flow of culture medium through channeled elastomeric scaffolds) with controlled VEGF expression *in vivo* (by cell-based gene therapy) can generate a thick cardiac patch with vascularization potential. After implantation in a mouse model of myocardial infarction, normal angiogenesis was induced both within the patch and in the underlying myocardium, leading to survival and differentiation of the seeded cardiomyocytes and

significant improvement of cardiac contractility. This approach could thus provide cardiac patches with both intrinsic and extrinsic vascularization potential, for studies of cardiac regeneration in animal models.

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Figure 1. Cardiomyocyte - myoblast co-culture

(A) Total amount of DNA was measured after 7 days of culture in the constructs generated by cardiomyocytes alone (RCM) or co-cultured with control skeletal myoblasts (SM) in two ratios 6:1 (SM:RCM 1:6) or 2:1 (SM:RCM 1:2). The resulting constructs were then cultured in either cardiac medium (Cm) or myoblast differentiation medium (Diffm) or 1:1 mixture of the two (Mixm). (B) Total DNA content in cardiac constructs generated with cardiomyocytes alone (RCM) or co-cultured for 7 days with either control (CD8) or VEGF-expressing SM (VEGF) in a ratio 6:1 in cardiac medium right before implantation.

Figure 2. Cardiac function

Time line of the in vivo study (**A**): cardiac patches were produced in the laboratory at the same time of the acquisition of the baseline echocardiography (echo) followed by the induction of the infarction. After one week an echo was performed to evaluate the quality of the infarction and followed by the implantation of the cardiac patch. Four weeks after the treatment with the patch a last echo was performed to assess the cardiac function. Strains were measured on high-resolution echo in the region of the infarction, both in the short (**A**) and long (**B**) axes. The graphs represent the ratio between the strain values at the time of patch implantation (1 week after infarction) and 4 weeks later for each animal. VEGF-

expressing patches resulted to be significantly different as compared to all other groups, * = $p < 0.05$ in (A) and $p < 0.01$ in (B).

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Figure 3. In vivo patch engraftment

Hematoxylin and Eosin staining was performed on cross-sections of myocardium treated with PGS scaffold alone (**A, B**), or patches generated by RCM alone (**C, D**) or in co-culture with either control (**E, F**) or VEGF-expressing (**G, H**) SM. Images in panels **B, D, F** and **H** represent high-magnification views of the areas in the black squares in panels **A, C, E** and **G**, respectively. The arrow in panel **G** indicates the seamless integration between the underlying myocardium and the VEGF-expressing patch. Black dashed lines mark the boundary between the patch and the infarcted tissue. Scale bars = 200 μm .

Figure 4. In vivo patch engraftment and thickness quantification

Masson trichrome staining was performed on cross-sections of myocardium treated with PGS scaffold alone (A), or patches generated by RCM alone (B) or in co-culture with either control (C) or VEGF-expressing (D) SM. Thickness of infarction (E), patch plus capsule (F) and underlying myocardium (G) were measured on Masson trichrome sections for all the groups. Black dashed lines mark the boundary between the patch and the infarcted tissue. * denotes $p < 0.01$. Scale bars = 200 μm .

Figure 5. In vivo patch survival and differentiation

Immunofluorescence staining for cardiac Troponin I (red) and nuclei (DAPI) on cross-sections of mouse left ventricles implanted with patches generated with cardiomyocytes (RCM) alone (A), or RCM and control skeletal myoblasts (CD8)(B), or RCM and VEGF-expressing SM (C). (D–F) X-Gal histochemistry is used to detect β -galactosidase activity in SM transduced to express β -gal of patches generated by either RCM and control skeletal myoblasts (CD8) (D), or RCM and VEGF-expressing SM at low and high magnification (E and F, respectively). White lines in the images mark the boundary between the patch and the underlying myocardium (*m*). Scale bars = 200 μ m.

Figure 6. In vivo vascularization

(A–C) Vessel density was quantified in cross-sections of patches made of scaffold alone (PGS), or seeded with cardiomyocytes alone (RCM) or together with control myoblasts (CD8) or VEGF-expressing myoblasts (VEGF). The percentage area occupied by CD31-positive structures (%) was measured in 3 different areas: the surrounding fibrous capsule (A), inside the patch (B) and in the underlying myocardium (C). (D–E) Immunofluorescence staining for endothelium (CD31, red) and pericytes (NG2, green) on cross-sections of mouse left ventricles implanted with patches seeded with RCM and VEGF-

expressing myoblasts (**D**) or with RCM and control skeletal myoblasts (**E**). The patch area is delimited by the white dotted line and indicated by the asterisk (*). Scale bar = 200 μm .