Controlled Angiogenesis in the Heart by Cell-Based Expression of Specific Vascular Endothelial Growth Factor Levels

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Abstract

Vascular endothelial growth factor (VEGF) can induce normal angiogenesis or the growth of angioma-like vascular tumors depending on the amount secreted by each producing cell because it remains localized in the microenvironment. In order to control the distribution of VEGF expression levels *in vivo*, we recently developed a high-throughput fluorescence-activated cell sorting (FACS)-based technique to rapidly purify transduced progenitors that homogeneously express a specific VEGF dose from a heterogeneous primary population. Here we tested the hypothesis that cell-based delivery of a controlled VEGF level could induce normal angiogenesis in the heart, while preventing the development of angiomas. Freshly isolated human adipose tissue-derived stem cells (ASC) were transduced with retroviral vectors expressing either rat VEGF linked to a FACS-quantifiable cell-surface marker (a truncated form of CD8) or CD8 alone as control (CTR). VEGF-expressing cells were FACS-purified to generate populations producing either a specific VEGF level (SPEC) or uncontrolled heterogeneous levels (ALL). Fifteen nude rats underwent intramyocardial injection of 10^7 cells. Histology was performed after 4 weeks. Both the SPEC and ALL cells produced a similar total amount of VEGF, and both cell types induced a 50%–60% increase in both total and perfused vessel density compared to CTR cells, despite very limited stable engraftment. However, homogeneous VEGF expression by SPEC cells induced only normal and stable angiogenesis. Conversely, heterogeneous expression of a similar total amount by the ALL cells caused the growth of numerous angioma-like structures. These results suggest that controlled VEGF delivery by FACS-purified ASC may be a promising strategy to achieve safe therapeutic angiogenesis in the heart.

Introduction

ISCHEMIC HEART DISEASE IS ONE OF the most frequent causes of death worldwide (Roger *et al.*, 2011). Revascularization strategies, such as percutaneous angioplasty or coronary artery bypass grafting, are possible to restore blood flow distal to a vascular occlusion. However, morbidity and mortality remain high, despite optimal medical and surgical treatment, and many patients could benefit from additional angiogenic therapy aimed at stimulating the growth of new blood vessels in the ischemic tissue.

Vascular endothelial growth factor (VEGF) is the most potent and specific angiogenic factor and controls vascular

growth both in physiological and pathological settings (Ylä-Herttuala *et al.*, 2007). However, newly formed vessels require VEGF expression *in vivo* for approximately 4 weeks in order to become stable and persist indefinitely (Dor *et al.*, 2002; Ozawa *et al.*, 2004; Tafuro *et al.*, 2009) making protein delivery an ineffective strategy due to limited half-life. On the other hand, excessive VEGF expression has the potential to induce the growth of angioma-like vascular tumors (Lee *et al.*, 2000; Schwarz *et al.*, 2000). We previously found that, since VEGF binds tightly to extracellular matrix, the induction of normal or aberrant angiogenesis depends strictly on its amount localized in the microenvironment around each producing cell and not on the total dose delivered (Ozawa

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et al., 2004; von Degenfeld *et al.*, 2006). This finding helps to explain the apparently narrow therapeutic window for VEGF gene delivery and its lack of efficacy at safe doses in clinical trials of therapeutic angiogenesis (Ylä-Herttuala *et al.*, 2004), in which only the total dose of vector could be controlled, but not its expression in individual cells, leading to a heterogeneous distribution of microenvironmental levels within the tissue (Banfi *et al.*, 2005). In order to achieve homogeneous delivery of specific VEGF doses *in vivo* as a clinically applicable therapeutic strategy, we have recently developed a fluorescence-activated cell sorting (FACS)-based technology to allow the high-throughput purification of cells that express a desired VEGF level from a heterogeneous population of transduced progenitors, by quantitatively linking VEGF expression to that of a FACS-quantifiable syngenic cell surface marker (truncated CD8a; Misteli *et al.*, 2010). Proof-of-principle was obtained in skeletal muscle, by showing that such a purified population of VEGF-expressing primary myoblasts yielded robust, normal, and stable angiogenesis and completely avoided angioma growth, both in normal (Misteli *et al.*, 2010) and chronically ischemic (Wolff *et al.*, 2012) skeletal muscle.

However, skeletal myoblasts are not an ideal cell source for cardiac delivery, because they can lead to potentially fatal ventricular arrhythmias. In fact, they have been shown to differentiate into contractile skeletal myofibers that remain electrically uncoupled from the surrounding cardiomyocytes (Menasche *et al.*, 2008). On the other hand, mesenchymal stem cells, and particularly adipose tissue-derived stem cells (ASC), are an attractive source for cardiac cell delivery (Choi *et al.*, 2011). Indeed, ASC can be easily procured in large quantities from liposuction material (Zuk *et al.*, 2002), may have the potential to differentiate into cardiomyocytes (Jumabay *et al.*, 2010; Planat-Benard *et al.*, 2004), and have been reported not to increase the risk of arrhythmias (Sanchez *et al.*, 2010). Therefore, here we sought to establish a human ASC-based platform for controlled VEGF expression in the heart. We found that transduced and FACS-purified mesenchymal progenitors, homogeneously expressing a specific VEGF level, were effective in inducing robust normal angiogenesis in the myocardium, while preventing the growth of aberrant angioma-like vascular structures caused by uncontrolled expression.

Methods

Cell culture

Adipose tissue was obtained from two healthy donors undergoing plastic surgery after informed consent and according to a protocol approved by the Ethical Committee of Basel University Hospital. All investigations conformed to the Declaration of Helsinki. Tissue was minced and digested with 0.15% collagenase (Worthington Biochemical Corporation, Lakewood, NJ) in phosphate-buffered saline (PBS) at 37°C under continuous shaking for 60 min. Released cells were strained through a 70- μ m nylon mesh, plated at a density of 10^5 cells/cm², and cultured in high-glucose Dulbecco's modified Eagle's medium with 10% fetal bovine serum, as described (Scherberich *et al.*, 2007). After 4 days, cells were retrovirally transduced twice a day according to a recently optimized high-efficiency protocol (Helmrich *et al.*, 2012) for a total of six rounds. The retroviral vectors expressing rat VEGF₁₆₄ and truncated rat CD8a have been

previously described (Wolff *et al.*, 2012). We have previously found that neither the transduction procedure nor VEGF expression affected the surface marker expression profile, proliferative ability, and multilineage differentiation potential of the transduced ASC (Helmrich *et al.*, 2012). After 12 days, when cells were about 90% confluent, they were FACS-sorted with a BD Influx Cell Sorter (BD Biosciences, Basel, Switzerland) to generate the three different treatment groups, according to a previously published protocol (Misteli *et al.*, 2010; Wolff *et al.*, 2012).

VEGF ELISA measurements

The production of rat VEGF₁₆₄ and human VEGF₁₆₅ was quantified using specific Quantikine VEGF Immunoassay enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Abingdon, UK). One milliliter of medium was harvested from ASC cultured in a 60-mm dish, following 4 hr of incubation, filtered, and analyzed in duplicate. Results were normalized by the number of cells and time of incubation. Four separate dishes of cells were assayed for each cell type ($n=4$).

Flow cytometry

Cells were stained with fluorescently labeled specific antibodies against human CD31, CD34, CD73, CD90 (BD Biosciences), VEGFR2 (R&D Systems, Abingdon, UK), CD105 (AbD Serotec, Düsseldorf, Germany), and troponin I (Millipore, Zug, Switzerland) or with appropriate isotype control antibodies (BD Biosciences) according to manufacturers' instructions and analyzed with a FACS-Calibur Flow Cytometer (BD Biosciences). Retrovirus-encoded CD8a was detected with a specific mouse monoclonal antibody to rat CD8a (clone OX-8; BD Biosciences) as previously described (Wolff *et al.*, 2012). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

In vivo cell implantation

Animals were treated in compliance with Swiss Federal guidelines for animal welfare and all procedures were approved by the Veterinary Office of the Canton Bern (Bern, Switzerland) and conform to the Directive 2010/63/EU of the European Parliament. Male nude rats (Hsd: RH-rnu/rnu, average weight 252 ± 16 g) were anesthetized with 2.5% isoflurane and 10 mg/kg buprenorphine with tracheal ventilation at 80 cycles/min (Small Animal Ventilator 683, Harvard Apparatus, Holliston, MA). The heart was exposed through a left thoracotomy and 10^7 cells were injected into five sites of the anterior wall. Four weeks posttreatment, rats were sacrificed by exsanguination under the same anesthesia conditions as already described, and the hearts were then collected, embedded in OCT compound (Sakura Finetek, Torrance, CA), and frozen in isopentane cooled in liquid nitrogen. In some experiments, fluorescein isothiocyanate (FITC)-labeled *Lycopersicon esculentum* lectin (250 μ g in 250 μ l; Vector Laboratories, Burlingame, CA) was injected into the femoral vein and allowed to circulate for 4 min before sacrifice in order to label perfused vessels as previously described (von Degenfeld *et al.*, 2006).

Tissue staining

Histological analyses were performed on heart cryosections. Hematoxylin and eosin (H&E) staining was performed

according to standard protocols. ALU *in situ* hybridization was performed as previously described (Kasten *et al.*, 2005). Immunofluorescence was performed using the following primary antibodies and dilutions: mouse-anti-rat CD45 (BD Biosciences; 1:200), mouse-anti-rat CD31 (Clone TLD-3A12; AbD Serotec, Düsseldorf, Germany; 1:100), rabbit-anti-mouse NG2 (Millipore, Zug, Switzerland; 1:200) and mouse-anti-human cardiac troponin I (Millipore, Zug, Switzerland; 1:200), mouse anti- α -smooth muscle actin (Clone 1A4; MPBiomedicals, Basel, Switzerland; 1:400), anti-human leukocyte antigen (HLA) (Biolegend, San Diego, CA; 1:200). Fluorescently labeled secondary antibodies (Invitrogen, Basel, Switzerland) were used at 1:200.

Vessel measurements

The amount of blood vessels was quantified on images taken from CD31-stained sections with two methods. Six representative fluorescent images per sample (three samples per group) were acquired with a 20 $\times$ objective on an Olympus BX61 microscope (Münster, Germany) on cross-sections of the anterior wall taken at 300- μ m intervals in the area where injections were performed. Blind analysis was performed with either Image J software (National Institutes of Health, Bethesda, MD) or AnalySIS D software (Soft Imaging System, Münster, Germany). The area fraction occupied by endothelial cells was calculated by measuring the number of CD31-positive pixels in each image with Image J software and normalizing it to the total area. Alternatively, vessel length density (VLD) was quantified as previously described (Banfi *et al.*, 2012) by tracing the total length of CD31-positive vessels in AnalySIS D software (Soft Imaging System) and dividing it by the total area of each field.

In some experiments, the amount of perfused vessels was quantified by similarly tracing the total length of lectin-positive structures and dividing it by the total area of each field (20 $\times$ objective). The fraction of total vessels that was functionally perfused was calculated by dividing this value by the total length of CD31-positive vessels in each field. Measurements were performed with AnalySIS D software (Soft Imaging System) on sections obtained after intravascular perfusion with *L. esculentum* lectin as described above (nine fields per sample and three samples per group).

Statistics

Data were analyzed with the statistical software Prism 4.0a (GraphPad, La Jolla, CA) and SPSS 17.0 (IBM, Armonk, NY). Data are presented as means $\pm$ standard deviation. Comparison between groups was performed using a one-way ANOVA between groups with Bonferroni correction for the VEGF quantification and a mixed-effects model for the angiogenesis quantification; $p < 0.05$ was considered statistically significant.

Results

Generation and FACS purification of VEGF-expressing ASC

In order to minimize *in vitro* expansion, ASC were transduced with retroviral constructs carrying the gene for rat VEGF₁₆₄ linked to the FACS-quantifiable cell-surface marker CD8, or CD8 alone (Fig. 1) at the earliest possible time-point (i.e., 4 days after isolation). Transduction efficiency >90% was routinely achieved before first confluency, which was

consistently reached in 12 days (Fig. 1A). At the first passage, control cells (CTR), which were transduced with the control virus expressing CD8 alone, were FACS purified to eliminate the few nontransduced cells and yield a purely CD8-positive population (Fig. 1B). In parallel, VEGF-expressing ASC were used to generate two distinct populations. Part of the cells were FACS purified through a specific narrow gate, designed according to a technique we recently developed (Misteli *et al.*, 2010), to produce a homogeneous cell population expressing a specific moderate VEGF level (SPEC). The rest of the cells were sorted to purify all CD8-positive cells, in order to

FIG. 1. Generation and fluorescence-activated cell sorting (FACS) purification of ASC. **(A)** Time-line for the generation of genetically modified adipose tissue-derived stem cells (ASC). Structure of the control **(B)** and vascular endothelial growth factor (VEGF)-expressing **(C)** retroviral constructs. Ψ =retroviral packaging signal; LTR=long terminal repeats; IRES=internal ribosome entry site. FACS purification of control ASC **(B)**, expressing only the cell surface marker CD8 (CTR), or VEGF-expressing ASC **(C)** as populations producing either a specific homogeneous level (SPEC) or all heterogeneous levels (ALL). In the FACS plots: black curves=negative control; green open curve=primary transduced control cells; green tinted curve=purified control cells; red open curve=primary transduced VEGF cells; grey tinted curve=purified ALL cells; red tinted curve=purified SPEC cells. Color images available online at www.liebertpub.com/hgtb

generate a population expressing all the heterogeneous VEGF levels present in the primary transduced population (ALL) (Fig. 1C). Therefore, cell expansion prior to injection could be limited to two to three passages, corresponding to 14–20 doublings, including transduction and FACS purification.

Before injection, transduced and FACS-purified cells displayed the classic fibroblast-like morphology, typical of early-passage ASC, without noticeable differences between the groups (Fig. 2A). Immunofluorescence staining confirmed that FACS-purified cells in all three groups were homogeneously CD8 positive (Fig. 2A). *In vitro* VEGF release

by cells from the different groups was quantified at the same time. Both VEGF-expressing populations (SPEC and ALL) produced a similar amount of rat VEGF (about 130 ng/10⁶ cells/d), which instead was not detectable in the supernatants of control cells (Fig. 2B). Since ASC were of human origin, expression of endogenous human VEGF was also assessed. All cell groups produced a similar amount of human VEGF, corresponding to only about 10% of the retrovirus-derived rat VEGF (Fig. 2C), showing that exogenous VEGF overexpression did not influence the basal levels of expression of the endogenous factor.

FIG. 2. Cell morphology and VEGF expression. **(A)** Before *in vivo* injection, cell morphology was assessed by bright field microscopy (left column) and CD8 expression by immunofluorescence (right column; CD8=green and nuclei=blue). **(B,C)** Enzyme-linked immunosorbent assay (ELISA) quantification of rat **(B)** and human **(C)** VEGF in the culture supernatants of transduced cells (expressed in ng/10⁶ cells/d); **p* < 0.01. Color images available online at www.liebertpub.com/hgtb

In order to determine whether VEGF overexpression during *in vitro* expansion may cause a selection of specific ASC subpopulations and consequently differences in the cellular composition of the injected populations, we investigated the expression of a panel of mesenchymal, endothelial, and cardiac markers. No significant differences were found in the composition of the three populations (Table 1). In all cases the large majority of cells expressed at least one of the mesenchymal markers CD73, CD90, or CD105, each of which was detected on 70% to 80% of the cells. Less than 4% of the cells were positive for CD31, which is expressed by endothelial as well as some hemopoietic cells. However, the other endothelial markers CD34 and VEGF-Receptor 2 (VEGFR2) were expressed by <1% of cells in all three groups, suggesting that the frequency of endothelial cells in the injected populations was negligible and not affected by VEGF overexpression. We could also exclude spontaneous differentiation into a cardiac lineage during cell expansion, since the cardiac marker troponin I was not detectable.

Myocardial angiogenesis

To assess the quality and quantity of vascular growth induced in the myocardium by controlled or heterogeneous VEGF expression, immunodeficient nude rats were used to prevent rejection of human ASC. Thirty nude rats, divided into the three treatment groups (CTR, SPEC, ALL), received 10^7 total cells divided in five intramyocardial injections each, with a mortality of 3.3% (1/30). After 4 weeks cardiac angiogenesis was assessed by immunofluorescence staining ($n=5$ per group). Both VEGF-expressing populations induced robust angiogenesis compared to myocardium injected with control cells (Fig. 3A). Vessel density was quantified by measuring the area fraction occupied by CD31+ endothelial cells, which is not affected by the size of lumens, and the results showed a significant two- to three-fold increase by both VEGF-expressing populations compared to controls (Fig. 3B). Since the vessels induced by the ALL population displayed an irregular morphology, with heterogeneous sizes, vascular growth was independently

quantified by measuring VLD, which indicates the total length of vessels in a given tissue area, irrespective of their diameter or endothelial cell content (Ozawa *et al.*, 2004). As shown in Fig. 3C, both VEGF-expressing populations caused a net increase in VLD by about 60%.

However, vessels induced by controlled VEGF expression (SPEC) displayed the morphology of normal and stable capillaries, were homogeneous in diameter, and associated with the slender processes of typical pericytes positive for the chondroitin sulfate proteoglycan NG2, similar in quality to vessels visible in the control group, while occasional regular-shaped arterioles were the only smooth muscle-covered vessels present (arrows in Fig. 3A). By contrast, heterogeneous VEGF expression by ALL cells caused the widespread growth of enlarged aberrant vessels (arrowheads in Fig. 3A), characterized by heterogeneous sizes, the presence of multiple lumens, and a lack of normal pericytes, substituted by a thick coat of smooth muscle cells, positive for both NG2 and smooth muscle actin (SMA; Fig. 3D), similar to the progressively growing angioma-like vascular structures previously described by us and others (Lee *et al.*, 2000; Ozawa *et al.*, 2004). Therefore, only controlled specific VEGF expression induced angiogenesis that was both robust and normal.

Vessel perfusion

We further investigated whether the new vessels induced by the VEGF-expressing populations were functionally connected to the circulation and perfused *in vivo*. Fluorescent *L. esculentum* lectin was injected intravenously and allowed to circulate in order to label all vascular structures in which blood flow was present ($n=5$ per group). Co-staining of sections with CD31 (Fig. 4A) showed that both normal and aberrant vessels, induced by SPEC and ALL cells, respectively, were labeled with the lectin. The percentage of perfused vessels out of the total amount of the CD31-positive structures (Fig. 4B) was higher than 90% in all the experimental groups, revealing that most of the vessels were functionally connected to the host vasculature. However, quantification of the amount of perfused vessels showed that, similarly to the simple measurement of histologically visible vessels in Fig. 3C, both VEGF-expressing populations induced a net increase in the density of physiologically functional vessels by about 50% compared to the control cells (Fig. 4C).

Cell engraftment

In order to determine the degree of stable engraftment of the injected cells, human ASC were tracked in harvested tissue by immunofluorescence staining for the human-specific histocompatibility antigens of class I (HLA-class I), which are expressed on all cells of human origin. Four weeks after injection, only rare human ASC were still detectable in the rat myocardium (Fig. 5). ASC were not integrated with the cardiomyocytes, but rather remained as isolated single cells in between troponin I-positive fibers (white arrows in Fig. 5). No differences were observed between the three treatment groups. Although unlikely, it is possible that some injected cells may have down-regulated HLA-class I expression *in vivo*. Therefore, we also determined the degree of engraftment by *in situ* hybridization to the human-specific Alu sequence in the genomic DNA of ASC. This unrelated technique confirmed that ASC engraftment 4 weeks after

TABLE 1. PHENOTYPE OF TRANSDUCED ADIPOSE-TISSUE STEM CELL POPULATIONS

	CTR	SPEC	ALL
Mesenchymal			
CD 73	74.2±0.1%	79.9±1.1%	75.8±0.4%
CD 90	69.7±4.4%	78.7±3.0%	70.9±2.3%
CD 105	69.5±10.1%	77.5±6.5%	74.9±0.2%
Endothelial			
CD 31	3.6±4.3%	3.8±2.2%	2.8±0.2%
CD 34	0.7±0.7%	0.6±0.2%	0.9±0.2%
VEGFR2	0.2±0.2%	0.5±0.1%	0.1±0.1%
Cardiac			
Trop I	0.0±0.0%	0.4±0.6%	0.1±0.1%

Expression of the indicated mesenchymal, endothelial, and cardiac markers by the different populations of transduced and purified ASC at the time of *in vivo* injection, measured by flow cytometry. Data represent the percentage of cells positive for the indicated marker in each population and are expressed as the mean±standard deviation ($n=2$ independent donors); p was not significant for each marker expression between all groups.

FIG. 3. Myocardial angiogenesis. **(A)** Immunofluorescent staining for endothelium (CD31, in red), pericytes (NG2, in green), smooth muscle cells (SMA, in cyan), and nuclei (DAPI, in blue) in sections of myocardium injected with control cells (CTR) or ASC expressing a specific homogeneous VEGF level (SPEC) or uncontrolled heterogeneous levels (ALL). White arrows and arrowheads indicate normal, regular-shaped arterioles and aberrant angioma-like structures, respectively. Angiogenesis was quantified either as the area fraction occupied by CD31-positive pixels **(B)** or as vessel length density **(C)**. Results are expressed as the mean $\pm$ SEM; $*p < 0.01$. **(D)** Immunofluorescent staining showing in greater detail an aberrant vascular structure in the ALL group, displaying irregular, multiple lumens and a thick smooth muscle coat.

injection was very limited, without noticeable differences between the groups (data not shown).

Inflammatory reaction

Inflammation can stimulate vascular growth (Arroyo and Iruela-Arispe, 2010). Therefore, we investigated whether the induction of normal or aberrant angiogenesis by the different VEGF-expressing populations might correlate with a different recruitment of inflammatory cells. In fact, although nude rats

specifically lack CD8⁺ cytotoxic T lymphocytes and are therefore tolerant to xenogenic cell transplantation, they possess a full complement of all other lymphoid and myeloid populations and, therefore, are capable of mounting a completely effective inflammatory reaction (Vos *et al.*, 1980). As shown in Fig. 6, H&E staining showed a moderate mononuclear cell infiltrate at the site of cell injections in all experimental groups. CD45 immunostaining confirmed that the infiltrate was composed of mature hematopoietic cells, whose frequency was similar in the control and both VEGF-expressing groups.

FIG. 4. Vessel perfusion. **(A)** Immunofluorescent staining for endothelium (CD31, in red), smooth muscle cells (SMA, in cyan) and perfused vessels (*L. esculentum* lectin, in green) in sections of myocardium injected with control cells (CTR) or ASC expressing a specific homogeneous VEGF level (SPEC) or uncontrolled heterogeneous levels (ALL). White arrowheads indicate a large aberrant angioma-like structure. **(B)** Quantification of perfused vascular fraction, measured as the percentage of vessels that were labeled by circulating lectin. **(C)** Quantification of the functionally perfused vessel length density (VLD), measured as the total length (in mm) of lectin-positive structures per section area (in mm²). Results are expressed as the mean $\pm$ SEM; * $p < 0.001$.

FIG. 5. Cell engraftment. Survival of human ASC was assessed by immunostaining for HLA-Class I (red), while cardiomyocytes were detected by troponin I expression (green) and nuclei by DAPI staining (blue) 4 weeks after injection of control cells (CTR) or the two VEGF-expressing populations (ALL and SPEC). White arrows indicate single engrafted human cells.

FIG. 6. Inflammatory reaction. Inflammation was assessed by hematoxylin and eosin (H&E) staining and by immunofluorescent staining for CD45 (green) with nuclei detected by DAPI staining (blue) 4 weeks after injection of control cells (CTR) or the two VEGF-expressing populations (SPEC and ALL). Color images available online at www.liebertpub.com/hgthb

Discussion

In the current study, we showed that cell-based expression of a specific moderate VEGF level in the myocardium is effective in inducing robust growth of normal and mature vessels, while completely avoiding the formation of aberrant angioma-like vascular structures associated with uncontrolled VEGF production.

Chronic myocardial ischemia causes progressive deterioration of cardiac function and can lead to end-stage heart failure. Therapeutic angiogenesis, aimed at growing new blood vessels by the delivery of appropriate growth factors, is an attractive complementary strategy to increase blood flow in the ischemic myocardium for many patients treated by coronary revascularization (Roger *et al.*, 2011). VEGF is the master regulator of angiogenesis in both development and disease (Carmeliet, 2003). However, despite encouraging initial clinical results, clear evidence of therapeutic efficacy has not been achieved in clinical trials of VEGF gene delivery for coronary or peripheral artery disease (Gupta *et al.*, 2009). Increasing evidence suggests that the apparent paradox between the crucial biological function of VEGF and its lack of a therapeutic window may be due to the difficulty of ap-

propriately controlling its dosage distribution *in vivo* (Banfi *et al.*, 2005; Ozawa *et al.*, 2004). In fact, VEGF has the potential to induce significant adverse effects, such as increased blood vessel permeability with edema (Thurston *et al.*, 2000) and aberrant vascular proliferation (Schwarz *et al.*, 2000; Springer *et al.*, 1998) if even rare “hotspots” of excessive expression are not avoided (Ozawa *et al.*, 2004). In particular, VEGF overexpression has been shown to induce angioma growth in the heart both with cell-based expression (Lee *et al.*, 2000) and gene therapy vectors (Schwarz *et al.*, 2000). Furthermore, such aberrant vessels failed to contribute to blood flow recovery in ischemic tissue (von Degenfeld *et al.*, 2006) and even generated functional arteriovenous shunts that excluded the downstream microcirculation (Choi *et al.*, 2011). Clinical angioma formation has been reported only once, after intra-arterial delivery of a plasmid DNA vector (Isner *et al.*, 1996). The safety profile of therapeutic angiogenesis approaches in clinical trials has been very good, but the overall efficacy has been disappointing (Gupta *et al.*, 2009). Indeed, retrospective analyses have shown that when VEGF was delivered at a safe dose, it did not generate sufficient angiogenesis to correct cardiac or skeletal muscle ischemia (Karvinen and Ylä-Herttuala, 2010; Ylä-Herttuala

et al., 2004). The current conundrum, therefore, is that, on the one hand, increased efficacy is required, but on the other, strong expression easily leads to a loss of safety.

Our results show that the use of transduced and FACS-purified ASC can overcome this limitation, allowing efficient and sustained VEGF expression without compromising safety. In fact, only controlled VEGF expression by the SPEC cells could induce angiogenesis that was both robust, leading to a 60% increase in vessel quantity, and safe, by completely preventing angioma appearance. This amount of vessel growth is similar to that induced by controlled VEGF expression in both normal and ischemic skeletal muscle (Ozawa *et al.*, 2004; von Degenfeld *et al.*, 2006), where it was effective in causing collateral arteriogenesis and restoring blood flow after femoral artery ligation (von Degenfeld *et al.*, 2006).

It should be noted that the ALL and SPEC populations expressed equal total amounts of VEGF, but led to profoundly different outcomes. In fact, despite being a secreted factor, VEGF binds tightly to the extracellular matrix (Park *et al.*, 1993) and its effects remain localized to the microenvironment around each producing cell *in vivo* (Springer *et al.*, 2007). As a consequence, areas of excessive concentration do not diffuse *in vivo* and lead to aberrant angiogenesis, whereas similar total tissue VEGF amounts can induce exclusively normal vascular growth if all cells express homogeneous levels and “hotspots” are avoided (Ozawa *et al.*, 2004; von Degenfeld *et al.*, 2006).

These results underscore the principle that the outcome of VEGF gene delivery does not depend just on the global amount of factor achieved in the tissue, but rather that controlling the distribution of VEGF expression levels in the microenvironment *in vivo* is necessary and sufficient to reliably induce only normal angiogenesis. This paradigm was initially determined in skeletal muscle, but had not been investigated before in the heart. The ASC-based platform described here also provides the means to achieve controlled VEGF expression in the myocardium.

Naive ASC have been described to have an intrinsic cardioprotective and pro-angiogenic potential, due to the spontaneous production of paracrine factors (Meirelles Lda *et al.*, 2009), and at least two clinical trials (APOLLO and PRECISE; Perin *et al.*, 2010; Houtgraaf *et al.*, 2012) are currently investigating the potential of unmodified ASC to ameliorate cardiac ischemia. Whether the delivery of naïve ASC may be sufficient or whether expression of specific genes may be necessary to improve their therapeutic potential is a currently debated issue, for which no definitive data are available (Mushtaq *et al.*, 2011). In our conditions, VEGF overexpression significantly extended the angiogenic potential of naïve ASC, since SPEC cells increased myocardial vessel density by 60% compared to control cells. Indeed the amount of endogenous human VEGF produced by ASC was only about 10% of the amount of transgenic rat VEGF secreted by the SPEC cells, suggesting that this low level is insufficient to initiate angiogenesis.

In agreement with previous reports (Bai and Alt, 2010), we observed very limited human ASC engraftment 4 weeks after intramyocardial injection by two independent methods. Results in other tissues have shown that approximately 4 weeks of sustained VEGF expression are required before new vessels become independent (Dor *et al.*, 2002; Ozawa *et al.*, 2004). Interestingly, newly induced vessels persisted in the injection

areas despite the disappearance of most implanted cells, suggesting either of two possible explanations: (1) tissue-specific differences allow vessels in the myocardium to stabilize after VEGF expression shorter than 4 weeks, or 2) the production of other paracrine factors by ASC may accelerate the stabilization of VEGF-induced vessels. Conditional switching of VEGF overexpression specifically in the heart of transgenic mice showed that normal induced vessels were stable after 4 weeks, but not after 2 weeks, whereas aberrant vascular structures always depended on continued VEGF production for their survival (Dor *et al.*, 2002). On the other hand, among the many paracrine factors produced by ASC there are some involved in vascular stabilization, such as transforming growth factor- β and basic fibroblast growth factor (Meirelles Lda *et al.*, 2009). Further experiments, beyond the scope of the present work, are required to address the relative role of these possible mechanisms.

It should be noted that the experiments reported here were performed in normal, nonischemic myocardium in order to rigorously investigate the specific effects of homogeneous or heterogeneous expression of similar total amounts of VEGF, in the absence of the confounding effects of the multiple endogenous angiogenic factors upregulated by ischemia. Also, the angiogenic response has been analyzed only 4 weeks after implantation because it has been consistently found before that the switch between normal or aberrant VEGF-induced angiogenesis takes place before this time-point. In fact, vessels induced by moderate VEGF levels that are normal by 4 weeks have been found to persist unmodified for more than a year (von Degenfeld *et al.*, 2006), whereas morphologically aberrant structures evolve over the course of several more weeks into angiomas that eventually bleed and kill the animal (Lee *et al.*, 2000; Misteli *et al.*, 2010; Ozawa *et al.*, 2004).

In conclusion we obtained proof-of-principle that controlled VEGF expression by transduced and FACS-purified ASC is safe in inducing robust growth of uniformly normal blood vessels in nonischemic myocardium. These results suggest that this cell-based gene delivery approach, by affording precise control over the distribution of expression levels *in vivo*, could help overcome a significant obstacle in the development of safe and efficacious strategies for therapeutic angiogenesis in the heart. Based on these results, further investigations in models of chronic myocardial ischemia are warranted in order to assess both the safety of this approach in the presence of endogenous factors and its potential to ameliorate cardiac function.

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Author disclosure statement

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