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Targeting the endothelial progenitor cell surface proteome to identify novel mechanisms that mediate angiogenic efficacy in a rodent model of vascular disease

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Kaczorowski CC, Stodola TJ, Hoffmann BR, Prisco AR, Liu PY, Didier DN, Karcher JR, Liang M, Jacob HJ, Greene AS. Targeting the endothelial progenitor cell surface proteome to identify novel mechanisms that mediate angiogenic efficacy in a rodent model of vascular disease. Physiol Genomics 45: 999–1011, 2013. First published September 10, 2013; doi:10.1152/physiolgenomics.00097.2013.—Endothelial progenitor cells (EPCs) promote angiogenesis, and clinical trials suggest autologous EPC-based therapy may be effective in treatment of vascular diseases. Albeit promising, variability in the efficacy of EPCs associated with underlying disease states has hindered the realization of EPC-based therapy. Here we first identify and characterize EPC dysfunction in a rodent model of vascular disease (SS/Mcwi rat) that exhibits impaired angiogenesis. To identify molecular candidates that mediate the angiogenic potential of these cells, we performed a broad analysis of cell surface protein expression using chemical labeling combined with mass spectrometry. Analysis revealed EPCs derived from SS/Mcwi rats express significantly more type 2 low-affinity immunoglobulin Fc-gamma (FCGR2) and natural killer 2B4 (CD244) receptors compared with controls. Genome-wide sequencing (RNA-seq) and qT-PCR confirmed isoforms of CD244 and FCGR2a transcripts were increased in SS/Mcwi EPCs. EPCs with elevated expression of FCGR2a and CD244 receptors are predicted to increase the probability of SS/Mcwi EPCs being targeted for death, providing a mechanistic explanation for their reduced angiogenic efficacy in vivo. Pathway analysis supported this contention, as “key” molecules annotated to cell death paths were differentially expressed in the SS/Mcwi EPCs. We speculate that screening and neutralization of cell surface proteins that “tag” and impair EPC function may provide an alternative approach to utilizing incompetent EPCs in greater numbers, as circulating EPCs are depleted in patients with vascular disease. Overall, novel methods to identify putative targets for repair of EPCs using discovery-based technologies will likely provide a major advance in the field of regenerative medicine.

endothelial progenitor cell; angiogenesis; proteomics; genome-wide transcript profiling; RNA sequencing; rat model of vascular disease

REGENERATIVE THERAPY APPROACHES using autologous bone-marrow mononuclear cells (BMMCs) for the repair of vasculature function in patients with vascular disease have yielded promising outcomes (68). Both basic and preclinical studies have documented the feasibility and safety of BMMC therapy in a variety of disease models and patients (5). Despite some successes, the degree of heterogeneity in the regenerative capabilities of transplanted autologous BMMCs in clinical trials has been perplexing and warrants further investigation.

Recent data using rodent models suggests that the presence of underlying disease states, genetic and/or environmental in origin, may alter the biological efficacy of autologous BMMC. For example, our laboratory has demonstrated that treatment using BMMC from “healthy” donor rats was effective in vascular repair and improved function in rodent models exhibiting impaired angiogenesis and cardiovascular dysfunction (18). However, BMMCs that were harvested from “diseased” models provided little to no benefit on measures of vessel density (18). These data suggest that the regenerative potential of BMMCs differs significantly based on the health status of the donor, which is determined by both genetic and environmental factors. Thus, the identification of the biological mechanisms mediating the regenerative potential of BMMCs to treat various vascular diseases is critical for development of reliable and effective cell-based therapies. Our laboratory and others speculate that failed BMMC-based treatments may result from reduced numbers and/or biological efficacy of endothelial progenitor cells (EPCs) (5, 18, 19, 49, 68).

EPCs are potent angiogenic cells capable of restoring vessels and improving function of ischemic tissue in peripheral vascular disease and following myocardial infarction (6, 31, 33, 36, 52, 68); for review see Ref. 5. The percentage of EPCs in bone marrow of healthy subjects is estimated at <1–2% of cells, which when mobilized into the bloodstream by endogenous stimuli comprise <0.01% of circulating leukocytes (54). Notably, a significant reduction in circulating EPCs has been associated with advancing age, diabetes, cardiovascular disease, and hypertension (5, 27, 35, 55, 58, 65). Additionally, reductions in the function of EPCs measured in vitro correspond to severity of disease in patients (30, 65). Despite a multitude of studies that suggest either the quantity and/or the efficacy of EPCs is reduced in patients with vascular disease,
the molecular mechanism/s that mediate the angiogenic potential of EPCs have gone relatively unexplored.

The objective of the present research was to characterize the angiogenic potential of EPCs from a donor with a known disease phenotype and elucidate molecular mechanism/s associated with EPC dysfunction in a rodent model of vascular disease. The end goal of this research is to utilize novel strategies to identify cell surface proteins that limit the regenerative potential of EPCs and ultimately neutralize them, which would provide a major advance in regenerative medicine.

Recent work from our laboratory suggests that a salt-sensitive rat model of hypertension (SS/Mcwi) recapitulates many features of vascular disease and EPC dysfunction observed in human patients. For example, skeletal muscle angiogenesis induced through electrical stimulation is impaired in the SS/Mcwi rat (4, 48). Moreover, the number of EPCs contained within the bone marrow fraction is significantly lower in the SS/Mcwi rat donor relative to the SS-13BN/Mcwi controls (54). Herein we demonstrate for the first time that the angiogenic potential of SS/Mcwi EPCs is significantly impaired relative to EPCs from SS-13BN/Mcwi donors and demonstrate a potential molecular mechanism for EPC dysfunction. These data suggest that the SS/Mcwi rat may be a useful model for examining the mechanisms underlying angiogenic efficacy and EPC dysfunction associated with vascular diseases. We hypothesized that the molecular machinery needed for EPCs to interact, adapt, and/or respond to cues in their environment is disrupted in the SS/Mcwi EPCs.

Cell surface proteins are the ideal target for studies of EPC dysfunction, as they reside at the interface between the cell and its surrounding microenvironment and, thus, play a crucial role in cell-cell interactions, signal transduction, and cell fate (11, 28). Prior studies have identified a variety of cell surface proteins that interact with chemokines, hormones, extracellular matrix proteins, and growth factors to regulate angiogenesis under physiological and pathological conditions (11). Therefore, we utilized cell surface capture technology and quantitative mass spectrometry to determine if differences in the expression of cell surface proteins correspond to reduced therapeutic potency of SS/Mcwi EPCs. Outcomes comparing the abundance of cell surface glycoproteins from SS/Mcwi EPCs relative to SS-13BN/Mcwi EPCs revealed several differentially expressed cell surface proteins associated with apoptosis, which correspond to unique variations in the SS/Mcwi rat genome and transcriptome that provide insight into potential mechanisms whereby EPC function can be compromised in cardiovascular disease.

Materials and Methods

Subjects. The Medical College of Wisconsin (MCW) Institutional Animal Care and Use Committee approved all animal protocols. Animals were maintained on water and a low-salt diet (0.4% NaCl) ad libitum. The SS/Mcwi rat strain is an inbred substrain derived from the Dahl-S rat. The SS-13BN/Mcwi consomic rat strain was derived from replacement of chromosome 13 on the SS/Mcwi genetic background with that of the Brown Norway (BN/Mcwi) rat as described previously (15).

Bone marrow cell isolation and endothelial progenitor cell expansion. Isolation of bone marrow from SS/Mcwi and SS-13BN/Mcwi donor rats was performed as previously described (18). Briefly, rats were euthanized with pentobarbital and bone marrow cells were flushed from femurs and tibia using MCD131 media (US Biologicals, E3000-01B) with 5% heat-inactivated fetal bovine serum (FBS). BMMCs were isolated by gradient density centrifugation using Histopaque 1083 (Sigma, 10831). The BMMC fraction was washed and resuspended in MCD131 media containing 10% FBS and the following endothelial cell growth factors (Lonza; EGM-2 cat. CC-316): vascular endothelial growth factor (VEGF), human fibroblast growth factor-2, human epidermal growth factor, insulin-like growth factor-1, ascorbic acid, gentamicin, and amphotericin-B. Cell number and viability were determined with the Countess automated cell counter (Life Technologies).

BMMCs were plated (1–2 × 10⁶ cells/plate) onto 0.1% bovine fibronectin (Sigma, F1141)-coated 100 cm tissue culture dishes and incubated at 5% CO₂ at 37°C. Differential attachment was performed to remove unbound cells on day 3 of incubation, and media changed every 3–4 days. EPCs were identified by typical endothelial cell (EC) morphology and expression of VEGFR2. Approximately 80–90% of cells cultured from SS/Mcwi and SS-13BN/Mcwi donor rats were positive for the EC markers VEGFR2 (flow cytometry) and CD34 (fluorescence microscopy), which is consistent with our prior results using identical isolation and culture procedures showing ~90% of cells are positive for a panel of markers including VEGFR2, CD133, cKIT, CD34, and Dil-ac-LDL and thus classified as EPCs (18, 48). Although the percentage of EPCs-positive cells measured in the bone marrow directly after harvest differs significantly between SS/Mcwi and SS-13BN/Mcwi rat strains, the percentage of cells that were positive for EC markers VEGFR2/CD34 after 10–14 days of expansion/selection in culture was comparable in SS/Mcwi and SS-13BN/Mcwi rat strains.

Electrical stimulation surgery and intramuscular EPC injections. Rats were anesthetized with intramuscular injection of ketamine (70 mg/kg), xylazine (4 mg/kg), and acepromazine (1 mg/kg). Under aseptic conditions, EPCs suspended in 100 μl Dulbecco’s phosphate-buffered saline (DPBS; Life Technologies 14190-136) or vehicle were injected in three or four areas of the tibialis anterior (TA) muscle, and a stimulator implanted (37). After 24 h, the stimulator was activated causing intermittent contractions of the TA and extensor digitorum longus muscles for 8 consecutive hours daily. The contralateral leg was used as a control. All animals were euthanized with an overdose of pentobarbital after 7 days of stimulation.

Skeletal muscle harvest and morphological analysis of vessel density. TA muscles were removed, weighed, and fixed overnight in a 0.25% formalin solution at 4°C and sectioned (~75 μm). Vessels were stained with 30 μg/ml rhodamine-labeled Griffonia simplicifolia I lectin (Vector Laboratories), imaged, and analyzed as previously described (55). Vessel counts from all fields were averaged to a single vessel density, defined as the mean number of vessel-grid intersections per microscope field (0.077 mm²) for each muscle. Within experimental groups mean vessel densities of stimulated muscles were compared with contralateral unstimulated muscles, with all values presented as means ± SE and evaluated by a paired t-test. Differences between groups were evaluated with one-way ANOVA, and significant differences investigated with Tukey’s post hoc test.

Immunocytochemistry and flow cytometry. EPCs were washed, lifted by rocking in 4 ml Enzyme Free Cell Dissociation Solution (Millipore, S-014-B) for 2 min, and resuspended in 2% paraformaldehyde for 10 min at 37°C. EPCs were then permeabilized in ice-cold 90% methanol/10% DPBS solution for 30 min, blocked using 3% goat serum for 15 min, and incubated in anti-Flk-1 rabbit polyclonal antibody for 1 h (1:200, Santa Cruz Biotechnology, sc-315). EPCs were then washed three times and incubated for 60 min in Alexa Fluor 488-conjugated secondary (Invitrogen, A-11034). EPCs were washed three times and resuspended in flow cytometry buffer (0.5% BSA/1 mM EDTA in 10% DPBS). Secondary only labeled cells served as controls. Each analysis included 10,000 events and was run on the LSRII Flow Cytometer (Becton Dickinson).
Sample preparation and labeling of N-linked cell surface glycoproteins. Labeling of N-linked cell surface glycoproteins in EPCs was slightly modified from prior studies (23, 55, 67). Plates of EPCs at 10–14 days of expansion and selection of culture were washed with ice-cold 0.1% FBS (0.1% FBS in PBS, pH 7.3–7.4) and oxidized in 5 ml of 1.5 mM sodium-meta-periodate (Fisher, PI20504) while rocking at 4°C in the dark for 15 min. Plates were washed (0.1% FBS), and EPCs were lifted by rocking in 4 ml Enzyme Free Cell Disassociation Solution (Millipore, S-014-B) for 30–45 min at room temperature, followed by gentle displacement using a transfer pipette. EPCs were pelleted (500 g × 5 min at 4°C) and washed twice with ice-cold 0.1% FBS. EPCs were resuspended in 1 ml of 10 mM biocytin hydrazide (Biotium, 90060) in ice-cold labeling buffer (0.1% FBS, pH adjusted to 6.5) and rocked for 60 min at 4°C in the dark. Labeling buffer was added to wash and pellet EPCs 2× at 800 g × 6 min at 4°C. EPCs were resuspended in 0.1% FBS and 10 µl removed for cell count/viability assay as described above. Cells were pelleted at 800 g × 6 min at 4°C and resuspended in hypotonic lysis buffer (10 mM Tris pH 7.5, 0.5 mM MgCl2) on ice for 10 min. Cells were lysed using a cooled glass dounce homogenizer, pelleted (800 g × 10 min at 4°C), and the supernatant (~4 ml) was collected. We collected 10 µl of supernatant and measured proteins levels measured with a Nanodrop spectrometer. An equal volume of membrane prep buffer (280 mM sucrose, 50 mM MES pH 6.5, 450 mM NaCl, 10 mM MgCl2) was added to the ultracentrifuge tube. Samples were spun at 35,000 g at 4°C for 30 min. The supernatant was removed and 200 µl of fresh membrane wash buffer (25 mM sodium carbonate, adjusted to pH 11) was added to the pellet and set in the thermomixer at 4°C for 30 min. Hypotonic lysis buffer was added to top of ultracentrifuge tube (~12 ml), and the sample was spun at 35,000 g at 4°C for 30 min. The supernatant was discarded, and 300 µl fresh ammonium bicarbonate (100 mM, pH adjusted to 8.0), 40 µl 1% RapiGest (final concentration 0.1%) and 25 µl TCEP (100 mM, 5 mM final) were added to ultracentrifuge tube and placed on the thermomixer at 750 rpm for 30 min at 37°C. The sample was alkylated by addition of 45 µl of 100 mM iodoacetamide (10 mM final) and mixed at 750 rpm for 20 min at 37°C. Trypsin (Promega sequence grade, 20 µg reconstituted in 50 mM acetic acid) was added and mixed at 300 rpm overnight at 37°C (pH adjusted to 8–8.5). Samples were heated to 100°C for 10 min to inactivate the trypsin and spun at 13,000 g for 10 min to remove undigested debris from the supernatant. The supernatant was incubated among 450 µl streptavidin-coated beads (Pierce, 53117) in a MoBiCol spin column and rotated end-over-end at 37°C for 1 h. Beads were then washed with 500 µl of 100 mM ammonium bicarbonate, 10 ml 0.5% Triton X-100 (proteomics grade), 10 mM NaCl, 10 ml of 100 mM ammonium bicarbonate, 10 ml of NaCl, 10 ml of 100 mM sodium carbonate (pH 11), 10 ml of 80% isopropanol, and 10 ml of 100 mM ammonium bicarbonate on a vacuum manifold. For each wash step, beads were transferred to new MoBiCol spin column. After the final wash, beads were resuspended in 450 µl of 100 mM ammonium bicarbonate and 3 µl of PNGaseF (Biolabs, P0705S) and incubated end-over-end at 37°C. Peptide eluents were collected after a quick spin, and 450 µl of 0.1% trifluoroacetic (TFA) was added to the beads and rotated end-over-end for 5 min at 37°C followed by a second quick spin peptide extraction. Eluant from the first and second extraction were combined, and TFA (10%) was added to sample until a pH of 2–4 was reached, and C18 columns (Harvard Apparatus, C-50) were used for desalting. Columns were placed on vacuum manifold and rinsed with 1 ml of 100% acetonitrile, 2 ml of 0.1% TFA, after which the sample was run through the column twice. The column was then washed with 2 ml of 0.1% TFA. Peptides were released and collected using 60 µl of 70% acetonitrile (ACN) in 0.1% formic acid (FA), and followed by 60 µl of 90% ACN in 0.1% FA. Peptides were dried down in a speed-vac and stored at −20°C until analyzed by mass spectrometry.

Relative quantitation of N-linked cell surface glycoproteins by mass spectrometry. Tryptic peptide samples were resuspended in buffer A (98% HPLC H2O, 1.9% ACN, 0.1% FA). For each pair of samples (SS/Mcwi or SS-13HN/Mcwi) that constituted a biological replicate for each rat strain, protein concentrations were measured after cell lysis and diluted appropriately to normalize protein loading (the least concentrated sample was resuspended in 8 µl, and the other diluted to an equal concentration). Tryptic peptide samples (1.9 µl/run) were passed over an in-house C18 resin (particle size 5 µm; Phenomenex, Torrance, CA) packed 10 cm column (inner diameter of 50 µM) coupled to a NanoAccuity UPLC system (Waters, Milford, MA). A 240 min gradient from buffer A to buffer B (98% ACN, 1.9% HPLC H2O, 0.1% FA) was applied to the peptide bound C18 column. Eluted peptides underwent electrospray ionization followed by data acquisition using an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, Waltham, MA). Parent ion scans (MS1) were detected in profile mode in the FTMS portion of the Orbitrap Velos with 10^6 ions collected over a maximum accumulation time of 500 ms. Resolution was set to 30,000 at full width of peak at half-maximum peak height at 400 m/z. Data-dependent setting selected the 10 most abundantparent ions during individual MS1 scans for collision-induced disso- ciation for MS2. For MS2 fragmentation, ion intensity threshold was set to 500, and normalized collision energy of 35% was used for MS2 in the LTQ portion of the instrument with a maximum accumulation time of 25 ms. Dynamic exclusion settings were set to exclude any mass observed more than once in a 30 s time frame for 180 s from selection for fragmentation. Each group had data acquired for four biological replicates (n = 4) comprising two or three technical replicates.

Mass spectrometric RAW data files were uploaded into the MCW Biotechnology and Bioengineering Center Workflow (25) and extracted using Extract MSn 5.0, followed by Sequest and Mascot database search. Parameters for database searching included oxidation of methionine (+16 Da), alkylation of cysteine (+57 Da), and deamidation of asparagine (+1 Da) from removal of N-glycans. Searches were performed against the UniProtKB rodent database including 21,226 proteins. Search parameters also included trypsin cleavage (K, R), three missed cleavages, and precursor tolerance of 1.4 amu. Using in-house Visualize software (26), Sequest and Mascot searches for each individual run were combined via the search combiner matching each spectra to the best match from either search algorithm to avoid redundancy. Each biological replicate then had all Sequest/Mascot combined files for each technical replicate compiled, followed by compilation of all biological replicates. Within Visualize, a relative quantitation comparison of the total values for SS/Mcwi vs. SS-13HN/Mcwi EPCs through spectral counting was performed and normalized for overall scan count.

RNA-Seq and quantitative PCR. For genome-wide transcriptome analysis (14, 38, 43, 45, 66), total RNA is isolated from SS/Mcwi and SS-13HN/Mcwi cells using TRizol according to the manufacturer’s instructions (Invitrogen). Double-stranded cDNA libraries are prepared using the TruSeq RNA Sample Preparation Kit (Illumina). Libraries were quantified by quantitative (q)PCR according to Illumina’s qPCR quantification guide to ensure uniform cluster density. Samples were multiplexed six per lane on a 300 Gb flow cell and paired-end sequenced with an Illumina HiSeq 2000. The obtained samples were multiplexed six per lane on a 300 Gb flow cell and paired-end sequenced with an Illumina HiSeq 2000. The obtained

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qPCR analysis was run as previously described (56). Primers for canonical transcripts CD133 and FCGR2a were IDT PrimeTime qPCR assay #Rn.PT.53a.11119132.g and Rn.PT.53a.10907576, respectively. Custom primers for selected isoforms are described in the Supplementary Material.1

Statistics. Unless otherwise noted, significance was determined by one-way or repeated-measures ANOVA (Sigma Plot; SPSS, Chicago, IL) with post hoc Fisher’s least significant difference where appropriate. All results are reported as means ± SE. Computation of statistical significances of observed ms/ms spectra in SS/Mcwi compared with SS-13BN/Mcwi EPC samples were appropriately adjusted for multiple comparisons (26).

RESULTS

Enrichment and expansion of EPC colonies from BMMCs do not differ on the basis of donor strain. Several assays were performed to assess the confluence, viability, and percentage of EPCs harvested from SS/Mcwi and SS-13BN/Mcwi donor rats. No differences in the confluence [cells per plate; t(1,4) = 0.30, P = 0.78] or viability [t(1,4) = 0.14, P = 0.89] of cells were observed following EPC enrichment and in vitro expansion of BMMCs harvested from each strain. Moreover, no differences in the percentage of presumptive EPCs identified as VEGFR2+/− cells were observed between SS/Mcwi (79.7 ± 6.3%, n = 5) and SS-13BN/Mcwi donor strains (82.7 ± 7.3%, n = 5), [t(1,8) = 0.32, P = 0.76] (Fig. 1). Our data confirmed earlier results from our laboratory that showed the percentage of EPCs do not differ between SS/Mcwi and SS-13BN/Mcwi rats using multiple markers (CD133+, cKit+, CD34+, and VEGFR2+) (49). Specifically, the enrichment strategy used herein (see MATERIALS AND METHODS) yielded ~80% of cells that were VEGFR2−/+, which is comparable to our earlier work using multiple markers (CD133+, cKit+, CD34+, and VEGFR2−/+).

SS/Mcwi EPCs exhibit diminished angiogenic potency that can be overcome by increasing EPC cell number. In the first set of in vivo experiments, SS/Mcwi recipient rats received a direct unilateral intramuscular injection of EPCs from SS/Mcwi or SS-13BN/Mcwi donor rats into the TA muscle prepared for electrical stimulation (8 h/day for 7 days). Note that EPC doses used herein are reported in terms of the total number of cells injected (500–500,000 cells), which correspond to concentrations expressed relative to body weight for ease of comparison to clinical literature (1.5 × 10^3 to 1.5 × 10^6 cells/kg). Microvessel density was measured in the TA muscle of both the stimulated (with cells) and unstimulated (without cells) hindlimb of SS/Mcwi recipient rats. We calculated the mean percent change in vessel density by dividing the total number of vessel grid intersections in the stimulated/EPC injected limb by the total number of vessel grid intersections in the unstimulated limb (see MATERIALS AND METHODS, Ref. 55).

We assessed the effect of EPC treatment on angiogenesis in SS/Mcwi recipient rats by comparing the mean percent change in microvessel density as a function of EPC dose and donor rat strain.

Results from a one-way ANOVA revealed a significant difference in the mean percent change of microvessel density as a function of the dose of EPCs harvested from the SS/Mcwi donors [F(3,33) = 6.004, P = 0.002]. Treatment with the lowest dose of SS/Mcwi donor EPCs resulted in only a modest (9.2 ± 2.2%, n = 15) increase in vessel density in the stimulated TA of SS/Mcwi recipient rats (Fig. 2A, white bars), which in the absence of any EPC treatment exhibits impaired angiogenesis (2.1 ± 3.0%, n = 13) as reported previously (18). However, we found increasing the dose of SS/Mcwi EPCs was positively correlated with an increase in microvessel density (r^2 = 0.58, P < 0.0005). By increasing the dose of SS/Mcwi EPCs to 1.5 × 10^6 cells/kg, we were able to fully restore the angiogenic response to electrical stimulation in the TA muscle of SS/Mcwi recipient rats. Specifically, direct injection of 500,000 SS/Mcwi EPCs (~1.5 × 10^6 cells/kg) into the stimulated limb resulted in a 27.0 ± 3.5% increase in microvessel density (n = 9), which corresponds to levels observed in rodent strains that exhibit intact angiogenesis (17, 50).

In contrast to studies using EPCs from the SS/Mcwi strain, treatment using EPCs from SS-13BN/Mcwi donor rats robustly increased microvessel density in the stimulated TA of SS/Mcwi recipient rats at every dose tested (1.5 × 10^6 to 1.5 × 10^6 cells/kg). Because EPCs from SS-13BN/Mcwi donor rats exhibited potent angiogenic properties, no dose-response effect on microvessel density was observed [Fig. 2A, black bars; F(3,24) = 0.155, P = 0.925]. Notably, as few as 500 SS-13BN/Mcwi EPCs (1.5 × 10^3 cells/kg) were sufficient to fully restore angiogenesis in the TA muscle of SS/Mcwi recipient rats (22.3 ± 3.7%, n = 9) to levels observed in various rats strains that exhibit intact angiogenesis, including the SS-13BN/Mcwi donor strain.
EPC SURFACOME REVEALS VASCULAR DISEASE MECHANISMS

Fig. 2. Angiogenic potency of endothelial progenitor cells is reduced in the SS/Mcwi rat. A: plot illustrates the mean percent change in microvessel density from unstimulated to electrically stimulated tibialis anterior (TA) muscle in SS/Mcwi recipient rats as a function of the number and source of EPCs. SS/Mcwi recipient rats received injection of EPCs harvested from either SS/Mcwi or SS-13BN/Mcwi donors directly into the TA (500–5 × 10^5 cells in volume of 100 μl) followed by 7 days of electrical stimulation, euthanasia, and subsequent morphological assessment of vessel density. Analysis revealed that the efficacy of SS/Mcwi EPC treatment to restore angiogenesis in the TA of SS/Mcwi recipients was dependent on the dose (i.e., cell number), whereby increasing the number of SS/Mcwi EPCs resulted in a significant increase in microvessel density [univariate ANOVA, F(1,33) = 4.253, P = 0.047]. In contrast, SS-13BN/Mcwi EPCs acted as potent stimulators of angiogenesis when injected directly into the TA of SS/Mcwi recipients regardless of the dose [univariate ANOVA, F(3,24) = 0.155, P = 0.925]. B: plot illustrating the significant interaction (*) between the donor rat strain (SS/Mcwi vs. SS-13BN/Mcwi) and dose of EPCs injected (500 vs. 500,000) on microvessel density in the TA of SS/Mcwi recipients [2-way ANOVA, F(1,34) = 4.253, P = 0.047].

Fig. 3. EPC-mediated rescue of angiogenesis in SS/Mcwi recipients requires electrical stimulation. Plot illustrates the mean percent increase in microvessel density from baseline PBS injections in the TA in response to EPC relative to baseline PBS injections (Fig. 3). In the absence of electrical stimulation, the mean percent change in microvessel density following treatment with 500 SS/Mcwi EPCs (−0.62 ± 0.8%, n = 5) compared with SS-13BN/Mcwi EPCs (2.0 ± 1.8%, n = 5) was not significantly different [t(1,8) = 1.3, P = 0.23]. These data suggest that EPCs interact with product/s of electrical stimulation (e.g., proangiogenic factors and/or ischemic environment) to induce angiogenesis in the SS/Mcwi recipient rats.

Differential cell surface protein composition of SS/mcwi vs. SS-13BN/SS EPCs suggest differences in immune signaling. We hypothesized that misregulation of one or more proteins at the cell surface of the SS/Mcwi EPC disrupt integration, signaling, differentiation, and/or survival of EPCs, providing a mechanistic explanation for their reduced angiogenic potency. Therefore, we applied cell surface capture technology and mass spectrometry (23, 53, 67) to qualitatively and quantitatively...
identify proteins at the cell surface of EPCs from SS/Mcwi relative to SS-13BN/Mcwi rats. EPCs were harvested from bone marrow of SS/Mcwi and SS-13BN/Mcwi rats and enriched (see MATERIALS and METHODS). Approximately 1.5–8.0 × 10⁶ EPCs per biological replicate were labeled as described above, streptavidin purified, reduced and alkylated, and digested with trypsin. N-glycan tryptic peptides passing all stringent filters (see MATERIALS and METHODS) were detected for 128 proteins in either SS/Mcwi, SS-13BN/Mcwi, or both rat strains. Of the proteins detected, 91% were canonically annotated to the plasma membrane demonstrating highly specific enrichment with this technique. The SS/Mcwi EPC N-glycan surface proteome included 110 proteins, and the SS-13BN/Mcwi EPC N-glycan surface proteome included 102 proteins. There were 54 proteins detected in both cell types (50 of which were annotated to plasma membrane) that were not significantly different. These included several EPC/EC markers such as VEGFR2, VE-cadherin, ICAM, and integrin beta-1. Abundance differences relative to donor strain were also observed (Fig. 4); 25 proteins were significantly increased in SS/Mcwi EPCs (all annotated to plasma membrane), 5 proteins were significantly increased in SS-13BN/Mcwi (3 annotated to the plasma membrane), 26 proteins were unique to SS/Mcwi EPCs (25 annotated to plasma membrane), and 18 to SS-13BN/Mcwi EPCs (13 annotated to plasma membrane).

To analyze and describe the potential biological relevance of differentially expressed proteins from the cell surface proteome of SS/Mcwi vs. SS-13BN/Mcwi EPCs, we subjected proteins that remained after stringent filter criteria (see MATERIALS and METHODS) to literature search and pathway analysis using ProteinCenter software (see MATERIALS and METHODS). To assess the biological differences between SS/Mcwi and SS-13BN/Mcwi EPCs we focused on those proteins unique or significantly increased in each cell type. After the appropriate accession numbers for proteins unique or significantly increased from the filtered SS/Mcwi and SS-13BN/Mcwi EPC comparisons were uploaded, biological process involvement was annotated. A list of 50 unique or significantly increased proteins was generated for SS/Mcwi EPCS, and a list of 23 unique or significantly increased proteins was generated for SS-13BN/Mcwi EPCs. For each EPC donor rat strain, the top five biological processes were annotated. From these top five biological processes we observed unique biological functions ascribed to SS/Mcwi EPC surface proteins, which were involved in immune system process (Table 1) and cell adhesion (Table 2). Since introgression of chromosome 13 from the BN strain onto the SS/Mcwi background restored the angiogenic efficacy of EPCs, we further narrowed candidate biological functions by filtering for those containing significantly differentially expressed proteins encoded by genes on chromosome 13.

Differential expression of candidate proteins located on chromosome 13 correspond to variations in the SS/Mcwi genome and molecular markers of cell death. Within the cell surface proteome expressed from rat chromosome 13, only two proteins were significantly differentially expressed in SS/Mcwi relative to SS-13BN/Mcwi EPCs (Table 1). FCGR2 was increased by 1.7-fold in SS/Mcwi (normalized P = 2.08 E-5), and the natural killer cell receptor 2B4 (CD244) was increased by sixfold in SS/Mcwi (normalized P = 2.68 E-3). Comparative analysis of the BN rat reference genome to that of the SS/Mcwi rat genome using data retrieved from the Rat Genome Database (20) revealed a high degree of sequence variation (SNVs) in the 1.78 Mb region of chromosome 13 containing the FCGR2a gene, the provisional FCGR2a-like gene LOC498276, and the CD244 gene (RGD, http://rgd.mcw.edu; rn5; 93,968,431–95,748,528; Fig. 5). Moreover, evidence of copy number variation (CNV) for CD244 in the SS/Mcwi rat genome has been reported previously (Guryev et al., Ref. 24) that was positively associated with increased CD244 protein expression in SS/Mcwi EPCs observed herein. Interestingly, the membrane-bound ligand CD48 that targets CD244 was detected on the cell surface of both SS/Mcwi and SS-13BN/Mcwi EPCs but was not differentially expressed relative to rat strain. Although the CD48 gene is located in close proximity of CD244 on rat chromosome 13 (RGD, http://rgd.mcw.edu; rn5; 94,737,447–94,760,975), it is located outside of the CNV region and is highly conserved between SS/Mcwi and SS-13BN/Mcwi rats (Fig. 5). Similar to protein expression data for the gene CD48, no differences in mRNA expression of the CD48 gene using state-of-the-art RNA-seq transcriptome profiling was observed (data not shown).

Analysis of transcript expression in this region of interest (ROI) using genome-wide transcriptome profiling revealed that expression of the canonical candidate genes CD244 and FCGR2a were not different (Fig. 6), but rather specific isoforms of the CD244 (Fig. 6, isoform D/rn5) and FCGR2a (Supplementary data, isoform B-D/rn5) were significantly differentially expressed in the transcriptome of the SS/Mcwi compared with SS-13BN/Mcwi EPCs (Baylor 5.0/rn5; 93,968,431–95,697,538). Real-time quantitative PCR (q-PCR) for canonical transcripts (RefSeq), and a subset of isoforms, were performed based on expression levels and availability of primer sets (Fig. 7A).

Fig. 4. Comparison of total cell surface proteins observed between SS/Mcwi and SS-13BN/Mcwi EPCs. Within Visualize, a relative quantitation of the total values for SS/Mcwi vs. SS-13BN/Mcwi EPCs through spectral counting was performed and normalized for overall scan count. To be considered for additional analysis, a protein with a significant G-score must 1) have been observed in at least 3 biological replicates, 2) have differed by >1.5-fold abundance between the strains, and 3) have an abundance ratio in the same direction (e.g., higher in SS/Mcwi derived samples) for 3 of the 4 biological replicates. Of the 128 proteins that met these stringent criteria, 54 proteins were not significantly different, 5 significantly increased in SS-13BN/Mcwi EPCs, 25 significantly increased in SS/Mcwi EPCs, 18 unique to SS-13BN/Mcwi EPCs, and 26 unique to SS/Mcwi EPCs. Of the total proteins detected ~91% were conically annotated as secreted or plasma membrane (PM) proteins (gray).
confirmed expression of the canonical transcript for CD244 (IDT PrimeTime qPCR assay #Rn.PT.53a.11119132.g) not different in EPCs from SS/Mcwi compared with SS-13BN/Mcwi EPCs \([t(1,6) = 1.18, P = 0.284]\), as predicted by RNA-seq results. Expression of the CD244 isoform D was significantly increased in EPCs from SS/Mcwi compared with SS-13BN/Mcwi EPCs as predicted by RNA-seq results and in line with protein expression data \([t(1,6) = 2.15, P = 0.037; \text{one-tailed}]\). However, despite the result of “no difference” in the expression of the CD244 isoform E by RNA-seq analysis, the expression of the CD244 isoform E was significantly increased in SS/Mcwi compared with SS-13BN/Mcwi EPCs \([t(1,6) = 2.97, P = 0.025]\). Such increases in the expression of a subset of CD244 isoforms measured from SS/Mcwi EPCs were consistent with an increase in CD244 cell surface protein in SS/Mcwi compared with SS-13BN/Mcwi EPCs, but not results from RNA-seq analysis (Fig. 6).

Expression of FCGRa transcripts were significantly increased in expression in EPCs from SS/Mcwi compared with SS-13BN/Mcwi EPCs, as predicted by our cell surface protein expression findings. Specifically, the FCGR2a canonical transcript was significantly increased in SS/Mcwi EPCs compared with SS-13BN/Mcwi EPCs by qPCR \([t(1,6) = 10.91, P = 0.00161]\). All proteins listed were plasma membrane associated or secreted; peptides were found in at least 3 of 4 SS/Mcwi endothelial progenitor cell (EPC) biological replicates and are annotated as rat accessions and mouse when not available. The SS-13BN/Mcwi scan count ratio and \(P\) values are normalized to total scan count. NA, not available; boldface, significantly differentially expressed in SS/Mcwi relative to SS-13BN/Mcwi EPCs.

**Table 1.** Comparison of SS/Mcwi and SS-13BN/Mcwi EPC cell surface proteome implicated in immune response

<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
<th>Chr.</th>
<th>Peptide Count</th>
<th>Scan Count</th>
<th>SS/Mcwi EPCs</th>
<th>SS-13BN/Mcwi EPCs</th>
<th>SS/BN13 Ratio</th>
<th>(P) Value</th>
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<tbody>
<tr>
<td>Q8R5M8</td>
<td>cell adhesion molecule 1 (CADM1)</td>
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<td>1</td>
<td>12/6.8</td>
<td>1</td>
<td>1/6.2</td>
<td>10.91</td>
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<td>1</td>
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**Table 2.** Comparison of SS/Mcwi EPC and SS-13BN/Mcwi EPC cell surface proteome implicated in cell adhesion

<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
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<tr>
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<td>1</td>
<td>12/6.8</td>
<td>1</td>
<td>1/6.2</td>
<td>10.91</td>
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<td>mast cell antigen AD1 (CD63)</td>
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<td>0.00321</td>
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<tr>
<td>P01830</td>
<td>Thy-1 membrane glycoprotein (CD206)</td>
<td>7</td>
<td>1</td>
<td>45/35.1</td>
<td>1</td>
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<td>Q61830</td>
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All proteins listed were plasma membrane associated or secreted; peptides were found in at least 3 of 4 SS/Mcwi endothelial progenitor cell (EPC) biological replicates and are annotated as rat accessions and mouse when not available. The SS-13BN/Mcwi scan count ratio and \(P\) values are normalized to total scan count. NA, not available; boldface, significantly differentially expressed in SS/Mcwi relative to SS-13BN/Mcwi EPCs.
script $t(1,6) - 2.01, P = 0.045$; one-tailed; IDT PrimeTime qPCR assay #Rn.PT.53a.10907576], FCGR2a isoform A $t(1,6) - 3.06, P = 0.011$; one-tailed], and FCGR2a isoform E $t(1,6) - 2.02, P = 0.044$; one-tailed] were significantly increased in expression in EPCs from SS/Mcwi compared with SS-13BN/Mcwi EPCs (Fig. 7B). Note that expression of FCGRa mRNA using quantitative PCR differed from RNA-seq results (Fig. 6). On the basis of observed inconsistencies between RNA-seq expression results and qPCR results, we predict that minor errors inherent in assembling and mapping short reads onto a reference genome may be amplified in samples collected from the SS/Mcwi rat in the ROI because they are guided by the Brown Norway rat reference genome (rn5). Such errors in the ROI located on Chr13 would not be predicted for samples collected for the SS-13BN/Mcwi rat, as Chr13 from the BN background to generate the SS-13BN/Mcwi rat. Moreover, errors associated with assembly and mapping of reads in our ROI may have been further exacerbated by the occurrence of a copy number variant containing CD244 gene/s (Guryev et al., Ref. 24). Regardless, these data provide probable mechanisms whereby differences in the gene sequence of FCGR2a and CD244 and mRNA expression associate with increased expression of these proteins at the SS/Mcwi EPC surfaceome.

Given that activation of FCGRs and/or CD244 receptors at the cell surface have been shown to play role in apoptosis (9, 41, 63) and regulate proliferation (51, 57), it was not surprising that pathway analysis of the transcriptome profiles of EPCs

Fig. 5. Region of rat reference genome (rn5) containing FCGR2 and CD244 genes. Illustration of genes within our region of interest (ROI) (1.78 Mb, http://rgd.mcw.edu; rn5; 93,968,431–95,748,528) and corresponding SNVs generated by comparison of SS/Mcwi genomic sequence to the Brown Norway reference genome. SNV data from 3 separate analyses in 2 independent laboratories were plotted. Genes differentially expressed at the transcript and/or protein levels are colored blue. The CD48 gene, whose protein product interacts with CD244 at the cell surface, was not differentially expressed at the transcript and/or protein levels (black). The remainder of the genes in the ROI were not differentially expressed at the transcript and/or protein levels and are colored gray.

Fig. 6. Transcriptome and pathway analyses identified molecules differentially expressed at the gene level (left) and the isoform level (right) in EPCs from SS-13BN/Mcwi relative to SS/Mcwi rats associated with cell death, proliferation, and cell motility. Note that expression of molecules significantly decreased in SS-13BN/Mcwi relative SS/Mcwi EPCs (red) or significantly increased in 3 biological replicates SS-13BN/Mcwi EPCs (green) illustrates highly reproducible gene expression across all 3 biological replicates (BN13_rat 1–3) analyzed with RNA-seq ($P < 0.05$). Generally speaking, molecules that were identified as significantly differentially expressed using a gene level analysis (left column, *) were those that contained few isoforms that were “changing” in the same direction (e.g., ANPEP and MX1). Because changes in expression of isoforms are summed for analyses at the gene level, the identification of genes with isoforms that are changing in opposite directions or genes with many isoforms require analysis at the isoform level (e.g., CALCRL, GNAQ, and CD244).
EPC SURFACOME REVEALS VASCULAR DISEASE MECHANISMS

Fig. 7. Quantitative PCR analysis of EPCs from SS-13BN/Mcwi relative to SS/Mcwi rats for key genes identified by RNA-seq. Relative fold-change in mRNA expression of CD244 isoforms (A) and FCGR2a isoforms (B). *P < 0.05 by standard t-test relative to SS/Mcwi; NS, not significant.

from SS/Mcwi (n = 3) relative to SS-13BN/Mcwi (n = 3) revealed the top biological function ontologies associated with genes significantly differentially expressed were “cell proliferation” (249 molecules) and “cell death” (229 molecules). Pathways involved in “cell movement” (163 molecules) and “cell morphology” (158 molecules) were also implicated. Generally speaking, the expression pattern of differentially expressed genes/isoforms predicted: 1) the “activation” of cell death pathways in EPCs from SS/Mcwi rats (activation score of 0.541), and 2) the activation of proliferation and motility pathways in EPCs from the SS-13BN/Mcwi rats (activation score -1.681 and -1.005, respectively). Of particular note, 33 genes (or at least one of their isoforms) were differentially expressed in EPCs from SS/Mcwi compared with SS-13BN/Mcwi rats that were assigned to the regulation of all three functions: cell death, Proliferation, and cell movement (Fig. 6). Taken together, these data suggest deficits in the angiogenic potential of SS/Mcwi EPCs are associated with an enhancement of cell death and/or dysfunction of cell migration and proliferation that correspond to differences in CD244 and Fcgr2a at the level of the genome, transcriptome, and cell surface proteome.

DISCUSSION

The goals of the present study were to characterize EPC regenerative angiogenic properties in the context of underlying vascular disease and to identify candidate mechanisms using well-characterized rodent models. Using the salt-sensitive SS/Mcwi rat model of vascular disease and its functionally normal consomic pair (SS-13BN/Mcwi), we identified candidate mechanisms through a combination of in vivo angiogenesis assays, genomics, transcriptomics, and proteomics analyses.

Bone marrow-derived EPCs are superior to bone marrow mononuclear cells for restoration of angiogenesis in a rodent model of vascular disease. Prior work shows a variety of microvascular impairments in the SS/Mcwi rat including vascular rarefaction (8) and impaired angiogenesis (17), suggesting the SS/Mcwi rat is a useful model of impaired vascular disease (18). Although we previously demonstrated that direct delivery of heterogeneous BMMCs from SS/Mcwi rat donors failed to rescue angiogenesis in the angiogenesis impaired SS/Mcwi rat, here we show that EPCs enriched in vitro from BMMCs facilitate angiogenesis in SS/Mcwi recipient rats. These data demonstrate for the first time that cultured EPC-enriched cell injections are superior to freshly isolated BMMCs as a cell-based treatment for revascularization of tissue in a rodent model of human vascular disease.

EPC-mediated angiogenesis is facilitated by electrical stimulation. Here we also demonstrate, for the first time, that the angiogenic efficacy of bone marrow-derived EPCs is dramatically enhanced by direct electrical stimulation of the target muscle. Injection of EPCs in the absence of stimulation, regardless of donor genotype, had essentially no effect on vessel density relative to saline-injected control. When a single injection of 500 EPCs was paired with electrical stimulation, the mean vessel density in SS/Mcwi recipients increased relative to saline-treated contralateral limb, regardless of the donor strain. This finding clearly demonstrates that electrical stimulation modulates EPC-mediated angiogenesis in vivo in skeletal muscle of SS/Mcwi recipient rats even when relatively low numbers of “incompetent” EPCs are used. Given that a low yield of circulating EPCs from patients with vascular disease has been reported in several clinical studies as one of the major hurdles to successful application of EPC-based therapy (5), our data suggest that electrical stimulation of tissue requiring revascularization may enhance the efficacy of EPCs beyond what can be gained with EPCs administered as the “sole therapy” and overcome limitations of reduced yield of EPCs in patients.

Not all EPCs are created equal. The present study is the first to utilize a well-characterized model of human vascular disease and their immune-compatible consomic pair to demonstrate an effect of disease phenotype on EPC function in vivo to narrow in on potential mechanism/s. Herein, we demonstrate that bone marrow-derived EPCs harvested from donors with vascular disease (SS/Mcwi) were significantly less effective (i.e., incompetent) at restoring angiogenesis than EPCs from the normal consomic immune compatible SS-13BN/Mcwi donors. Specifically, treatment of SS/Mcwi recipients with SS-13BN/Mcwi EPCs were ~2.5-fold more effective at restoring angiogenesis than SS/Mcwi EPCs. It is important to note that no differences in the expansion, viability, or percentage of EPCs were measured in vitro between these two strains prior to EPC injections and electrical stimulation. Therefore, the differences reported here reflect differences in the potency of EPCs relative to the donor strain rather than any indirect effects related to growth, viability, or expression of EPC phenotype.
Generally speaking, EPCs from rats with known vascular disease (SS/Mcwi model) exhibited dysfunction that closely mirrored reports from human studies in which autologous EPC treatments were far less effective than expected based on favorable outcomes performed in animal models (5). It has long been speculated that EPC dysfunction with regards to their regenerative/repair potential is a consequence of underlying disease states (e.g., diabetes, cardiovascular disease, peripheral artery disease, lupus), providing a mechanistic explanation for their negligible benefit to patients in some clinical trials (5). Therefore, the utilization of well-characterized models of human vascular disease herein may be a superior approach to identify mechanism/s that determine the efficacy of autologous EPC-based treatment in humans than traditional models of surgically induced ischemia/vascular damage treated with EPCs from a “nondiseased” source (29, 31, 33, 34, 36, 44).

Role of the immune system in regenerative potential of EPCs and angiogenesis. Given the considerable number of possible determinants of EPC dysfunction, ranging from an inability to respond appropriately to electrical stimulation and/or growth factors to expression of adhesion proteins for binding and integration, we focused our attention on the interface between the cell and its environment. To identify candidate mechanisms at the cell surface, we utilized state-of-the-art cell surface capture technology (23, 53, 67) to map and quantitate the CD244 proteins at their cell surface, may be targeted for cytotoxicity and increase cytokine production (41). These data have been shown to stimulate natural killer (NK)-mediated cell triggering cytokine release and global damage to surrounding cells. Along similar lines, CD244 expression on target cells has been identified on endothelial cells (1). However, to our knowledge, this is the first study to identify: 1) CD244 and FCGRs on the cell surface of SS/Mcwi EPC specifically impede their function.

Increased expression of FCGRs on endothelial cells and a wide variety of other cell types has been shown to activate an immune response that results in apoptosis (i.e., antibody-dependent cellular cytotoxicity) or phagocytosis of the target cell (63). Under some conditions, FCGR binding can also trigger cytokine release and global damage to surrounding cells. Along similar lines, CD244 expression on target cells has been shown to stimulate natural killer (NK)-mediated cell cytotoxicity and increase cytokine production (41). These data suggest that SS/Mcwi EPCs, which overexpress FCGR2 and CD244 proteins at their cell surface, may be targeted for destruction in vivo upon exposure to relevant IgGs and/or interaction with immune NK cells. Interestingly, SS/Mcwi EPCs that are immediately harvested and counted from bone marrow mononuclear fraction are significantly reduced compared with SS-13BN/Mcwi EPCs (49), which suggests proliferation and/or survival in vivo is hindered but rescued, in part, in vitro. This idea is consistent with our data showing that there are no differences in the expansion, viability, or percentage of EPCs cultured for 12–14 days from SS/Mcwi and SS-13BN/Mcwi donor rats measured prior to direct injections, which occur in the absence of IgGs and other immune cells. Although this presumption is the most parsimonious explanation based on our data and the literature, results of our genome-wide transcriptome profiling experiments implicate biological processes related to cell death and apoptosis, as well as those related to proliferation and cell migration.

Alternative roles for FCGR and CD244 in progenitor cell function and angiogenesis have gone largely unexplored, although recent evidence suggests ligation of CD244 by CD48 may have proapoptotic (41) and/or proliferative actions (9) that may critically depend on the cell type or “state” of the cell. Although FCGRs have been identified as mediators of graft-versus-host immune response (40), the dysfunctional phenotype of SS/Mcwi EPCs is not likely to result from of graft-versus-host rejection given that these EPCs are isolated from genetically identical inbred rat donors. Future work will deduce the exact effect and mechanisms whereby FCGR and/or CD244 expression on EPCs influence angiogenesis, although these are beyond the scope of the present study.

Linkage of the genome to disease phenotype through interrogation of the transcriptome and proteome. Comparative genomics analysis identified evidence of CNV in the genome of SS/Mcwi rat (24) that provides an explanation for the increase in CD244 transcript and protein expression in EPCs within the SS/Mcwi rat strain shown herein. It is worth noting that the membrane-bound ligand CD48 was also identified in the cell surface proteome of both SS/Mcwi and SS-13BN/Mcwi EPCs but was not significantly differentially expressed at the protein or transcript level. This is particularly interesting because the CD48 gene is located in close proximity to CD244 on rat chromosome 13, but it is located outside of the CNV region and is highly conserved between SS/Mcwi and SS-13BN/Mcwi rats. Therefore, the comparable expression of CD48 mRNA and protein at the cell surface of SS/Mcwi and SS-13BN/Mcwi EPCs corresponds to prediction based upon the common gene sequence in both rat strains.

Additionally, we located multiple strain-specific variations in genomic sequence in regions of chromosome 13 containing FCGR2a gene and the provisional FCGR2a gene (LOC498276). We identified transcripts via RNA-seq profiling that correspond to these genes in samples of peripheral blood (data not shown) that are known to contain circulating EPCs (59), as well as mRNA harvested directly from SS/Mcwi and SS-13BN/Mcwi EPCs reported herein. Therefore, it is reasonable to assume that these gene products are indeed being expressed. Previously, FCGR proteins have been identified on endothelial cells (1). However, to our knowledge, this is the first study to identify: 1) CD244 and FCGRs on the cell surface of EPCs and 2) genomic variation and abundance differences that associate with angiogenic dysfunction of EPCs from SS/Mcwi rats. Taken together, these data identify probable genomic mechanisms that explain the increase in expression of FCGR and CD244 proteins in the SS/Mcwi EPC surfaceome. Interestingly, accumulating evidence suggests FCGR and CD244 associate with disease susceptibility in several rodent models of human disease and likely generalize to human conditions (2, 24).

Remarkably, the identification of genetic variations that correspond to aberrant cell surface protein expression in EPCs of the SS/Mcwi rodent model of vascular disease shown herein have previously been shown to associate with a number of autoimmune and autoinflammatory diseases in humans (2, 3, 10, 13, 29, 40, 42, 46, 47, 64). CNV variations in SS/Mcwi rat gene CD244 have also been reported in other strains including...
the spontaneously hypertensive rat (SHR) (24). This result was intriguing to us given that, like SS/Mcei rats, SHRs exhibit vascular abnormalities including rarefaction (12, 21). Thus, it is possible that elevated expression of CD244 and/or FCGRs may be a general mechanism of EPC dysfunction. To this point, results of our transcriptome profiling analysis suggest that replacement of a single chromosome (Chr 13) harboring CD244 and FCGR genes corresponds to a global change in gene networks known to regulate cell death, cell proliferation, and cell migration.

It remains unknown whether EPC dysfunction observed herein and/or in human vascular diseases is a cause or consequence of vascular damage and ultimately organ damage/failure. Regardless, repair of such mechanisms in endogenous EPCs, or autologous EPCs expanded in vitro, may provide a major advance in enhancing efficacy of these progenitors for use in repairing vascular disease. Future attempts at downregulating FCGR2a and/or CD244 expression on EPCs in vitro prior to treatment may prove to be a novel approach to enhance the efficacy of autologous EPC-based treatments. Through understanding these mechanisms, regenerative therapeutic approaches using EPCs to repair vascular function in diseased patients may be realized.

Since the original theoretical description of the flow of information from DNA to RNA to protein by Francis Crick in 1958 (16), significant advances in technology allowed the present study to collect, analyze, and present “omic” data from all (3) steps of the original process. While each technique itself is unlikely to illuminate mechanism/s underlying complex biological phenomena and disease due to biological complexity and technical limits, when they were used together, we were able to obtain a global picture of processes occurring within a “single cell.” Thus, the present study highlights the tremendous advantage of combining analyses of the genome, transcriptome, and proteome for elucidating mechanisms in rodent models that are relevant to human disease.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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