Antibodies, cytospin hematologic stains, flow cytometry analysis, and FACS purification of hEB cells

Wright-Giemsa stains of cytospin cells, and *in situ* immunofluorescence of fixed and permeabilized hematopoietic colonies, adherent endothelial cells, and mesenchymal cells in MHE clusters were done exactly as previously described[1], following incubation with 10 μg/ml acetylated Dil-LDL (Molecular Bioprobes), and staining with anti-Vimentin (Lab Vision), CD31-FITC (BD Biosciences), and Ulex Europeaus agglutinin-1-FITC (Vector Labs). Nucleii were counter-stained with DAPI.

Surface markers were evaluated by FACS on enzymatically-dissociated single hEB cells, at different time points, following treatment with Accutase (SIGMA), or on individually picked and pooled hematopoietic colonies. Fluorochrome-conjugated monoclonal antibodies (from Becton Dickinson) included BB9-PE, BB9-APC: (anti-ACE), CD36-PE, CD164-PE, CD31-PE, CD34-PE, CD34-APC, CD45-PE, CD45-APC, CD71-CyPE, CD13-PE, glycophorin A (CD235A, GlyA)-PE, VE-cadherin-PE, CD56-PE, CD19-PE, CD43-PE (AbCAM), KDR-PE, and KDR-APC (R&D Systems). Viable cells were gated for analysis, and appropriate isotype and secondary antibody controls compared for each sample. Live cells from dissociated hEB, or picked colonies were analyzed for surface marker expression on a FACSort (BDIS) instrument and analyzed with CellQuest and FloJo softwares. Hemoglobin F (HbF), hemoglobin A (HbA), gamma chain, and epsilon chain expression were evaluated by intracellular FACS staining methods, as previously described.[1] Briefly, Hemoglobin F (HbF), Hemoglobin A (HbA), gamma chain, and epsilon chain expression was evaluated by intracellular FACS staining of fixed, permeabilized colony cells (FIX and PERM, Caltag) using fluorochrome-conjugated monoclonal antibodies. Fluorochrome-conjugated monoclonal antibodies used included gamma chain specific anti-HbF-PE (Caltag), anti-embryonic epsilon chain-FITC (clone 0900-50; Cortex Biochem), or the following unconjugated murine anti-HbA antibodies (which do not cross-react with HbF, or embryonic hemoglobins): anti-beta 6-Glu epitope of HbA (clone: 0300-50; Cortex Biochem), and anti-beta hemoglobin chain antibody (Perkin Elmer Wallac; as previously described.[2] Secondary PE-conjugated anti-mouse antibodies were obtained from Southern Biotech.

FACS purification of hEB cells: day 5-9 hEB cells used for single cell hemangioblastic assays, BL-CFC assays, FACS analysis, or qRT-PCR analyses were FACS-purified after differentiation of hEB in the presence of SF LDM, supplemented with 50 ng/ml each of BVF2H. Post-sort viability was greatly augmented if hEB were enzymatically-disaggregated into clumps 24-48 hours prior to harvesting for FACS-sorts, and recultured into fresh SF LDM with BVF2H that was additionally supplemented with 50 ng/ml each of TPO, IL-6, and 5 μg/ml angiotensin II peptide (SIGMA). hEB suspensions were passaged through 70 μm strainers, and immunostained on ice in SF medium with BB9-APC or BB9-APC and CD34-PE monoclonal antibodies. Single, purified $BB9^+$ hEB cells were incubated in 1 μM Rock inhibitor (Y-27632; Calbiochem) during immunostaining, and during the period of FACS collection. Single post-sorted hEB cells were reanalyzed by FACS, and cell numbers were adjusted for viability and purity. 5,000-150,000 viable hEB cells were recultured in BL-CFC assays or hemangioblastic fibronectin CFC assay conditions as described in Methods.

Quantitative real-time RT-PCR (qRT-PCR) analysis

Total RNA of hEB cells was isolated using RNEasy reagents (Qiagen). RNA samples were treated with RNAse-free DNAse (Qiagen) to remove contaminating genomic DNA. First strand cDNA was reversed-transcribed with oligo-dT priming using the Superscript system (Invitrogen). Quantitative PCR (qPCR) was performed using iQ SYBR Green Supermix reagents and PCR reactions were analyzed with an iCycler thermal cycler instrument (BioRad). Specified human gene-specific PCR primers were as previously described,[1] and as outlined Table S1. Transcripts of target genes and actin controls for each cDNA sample were amplified in duplicate/triplicates. All qPCR reactions were confirmed for specificity of a single PCR product by electrophoresis on 2%-4% agarose gels. Comparative quantification ("fold change expression") of each target gene was performed based on cycle threshold (C_T) normalized to actin using the $2\Delta\Delta C_T$ method.[3]

Clonality Studies

Cell dose and mixing experiments were conducted using H1 (WA01) hESC subclones that have integrated lentiviral constructs expressing either green fluorescent protein (EGFP, Clontech), or dsRed fluorescent protein (RFP, Clontech) from the EF1α promoter, following standard lentiviral transduction methods.[4] 50:50 mixtures of day 5-8 hEB single cell suspensions, pre-differentiated in SF LDM medium + BVF2H, from GFP-H1 hESC, RFP-H1 hESC, or control H1 hESC were cultured in BL-CFC assay conditions in $BVF2H^{+}TPO^{+}$IL-6, as before. Blast colonies developing from 50:50 GFP-RFP hEB cell mixtures were individually plucked from methylcellulose medium and secondarily recultured onto fibronectin-coated plates in SF hemangioblastic methylcellulose CFC assay conditions, as described in Methods. Normalized gene lists are presented in Table S2.

Gene Microarray Hybridization and Analysis of hEB Samples

Undifferentiated H1 hESC cells were highly-purified away from PMEF by FACS with Tra-1-60 immuno-staining from feeder-free cultures that had been passaged onto Matrigel. Embryoid bodies were differentiated as described above in SF + BVF2H conditions, and collected at Days 3, 5, 7, and 10 of hEB differentiation for RNA isolation. Total RNA was isolated from cell pellets using TriZol reagent (Invitrogen) followed by RNeasy mini kit with DNase on-column digestion (Qiagen). RNA was quantified with NanoDrop ND-1000 followed by quality assessment with 2100 Bioanalyzer (Agilent Technologies) according to manufacturer's protocol.

Sample amplification and labeling procedures were carried out with Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) with minor modifications. Briefly, 0.4 μg total RNA was reverse-transcribed into cDNA by MMLV-RT using an oligo dT primer (System Biosciences) that incorporates a T7 promoter sequence. The cDNA is then used as a template for *in vitro* transcription in the presence of T7 RNA polymerase and Cyanine-3 labeled CTP. Labeled cRNA was purified using RNeasy mini kit (Qiagen). RNA spike-in controls (Agilent

Technologies) were added to RNA samples before amplification and labeling according to manufacturer's protocol. 0.825 μg of each Cy3-labeled samples were used for hybridization on Agilent 4× 44K whole human genome microarray (G4112F) at 65ºC for 17 hours in a hybridization oven with rotation. After hybridization, microarrays were washed and dried according to manufacturer's protocols. Microarrays were scanned with an Agilent Scanner using Agilent Scan Control 7.0 software, and data were extracted with Agilent Feature Extraction 9.5.3.1 software. Processed signal intensities were imported into GeneSpring GX 7.3.1 using function of Enhanced Agilent FE Import. Signal intensities of repeat probes were automatically averaged during data import. Each array median was used for inter-array normalization. Background-subtracted signal intensities were further filtered to discard those probes with intensities lower than 30% of the array median (~ 1× background) if that was the case for all five time points before performing data analysis. 26,756 probes were used to determine genes with most significant changes. To choose top up- or down-regulated genes, all probes were first filtered by RefSeq ID, and taken first probe into account if a Refseq ID has repeat probes. The resulting 17,064 probes were then used for array median normalization and filtered by 30% of the array median intensity to discard probes with low signals at all time points before further data analysis.

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