SUPPLEMENTARY INFORMATION

In vivo growth-restricted and reversible malignancy induced by Human Herpesvirus-8/ KSHV: a cell and animal model of virally induced Kaposi's sarcoma

Agata D'Agostino Mutlu[1,7] Lucas E. Cavallin[1,2,7], Loïc Vincent[3], Chiara Chiozzini[1], Pilar Eroles[1], Elda M. Duran[2], Zahra Asgari[1], Andrea T. Hooper[3], Krista M. D. La Perle[4], Chelsey Hilsher[5], Shou-Jiang Gao[6], Dirk P. Dittmer[5], Shahin Rafii[3] and Enrique A. Mesri[1,2*#]

Supplementary Materials and methods

LANA, K8, and Kaposin immunofluorescense

The cell monolayers and frozen sections or paraffin embedded sections from tumors were fixed in 4% paraformaldehyde for 10 min, washed with PBS and permeabilized with 0.2% Triton-X in PBS 30 min. After blocking in PBS containing 10% FBS for 20 min, samples were incubated 1 h at 37°C with anti-LANA 1:1000, or anti-K8.1 1: 3000 (Advanced Biotechnologies, Columbia, MD), and anti-Kaposin 1:500 (Chemicon, Temecula, CA). After PBS washing, samples were incubated another hour with secondary antibodies, anti-rat IgG or anti-mouse IgG, respectively, conjugated to Cy3 or Cy5 1: 100 (Calbiochem, San Diego, CA), washed again and incubated with DAPI 0.5 ug/ml in water for 5 min. Samples were mounted in glycerol 50% in PBS. Pictures were

taken using an Olympus fluorescent microscope equipped with a digital camera and a Zeiss LSM-510 Confocal Laser Scanning Microscope.

RNA isolation and reverse transcription.

Total RNA from tissue culture cells was isolated by using the Absolutely RNA® Microprep™ kit (Stratagene, La Jolla, CA). Solid tumor pieces were resuspended in 750 μl TRI Reagent™ (Sigma-Aldrich Corp. St Louis, MO) and disrupted using an Ultra-Turrax T8 (IKA Labortechnik, Germany). RNA was isolated according to the supplier's protocol, precipitated and subsequently suspended in 50 μl DEPC-treated water at 56°C for 10 minutes. Residual DNA was removed by using the DNA-free RNA kit™ (Zymo Research, Orange, CA). The RNA was reverse transcribed as per our previously described procedures (Fakhari and Dittmer, 2002).

Real-time quantitative PCR

PCR primers and procedures were previously described (Dittmer, 2003; Fakhari and Dittmer, 2002). Cycle-threshold values (CT) were determined by automated analysis. The threshold was set to five times the standard deviation (SD) of the non-template control (NTC). Dissociation curves were recorded after each run to verify that the same, primer-specific, single reaction product was generated every time. Hierarchical clustering was performed using SPSS v11.0 (SPSS science, Chicago, IL) as previously described (Dittmer, 2003). Samples were normalized to TBP or KSHV LANA (primer lat234) mRNA (Dittmer et al., 1999), centered by median of gene, normalized to +/- 1

and ordered by hierarchical clustering using ArrayMiner™ (Optimal Design Inc., Brussels, Belgium) software and standard correlation metric. VEGF-A, B, D; VEGF-R1, R2; Angpt1, 2; Tie-1 and Tie-2 mRNA levels were measured using Taqman One Step RT-PCR Kit and Gene Expression Assays (Applied Biosystems, PA, California) normalized to β2-microglobulin using ΔΔCT methodology as per ABI provided protocols (Duran et al., 2005).

VEGF ELISA

Cell supernatants were assayed after clearing by centrifugation at 2,000 x g RT for 5 min. ELISA for mouse-VEGF was performed using an R&D Quantikine kit (R&D Systems, Inc, Minneapolis, MN).

CD-31, CD-34 and flk-1 and VEGF-R1 Immunohistochemistry of tumors

Tumors were removed from animals, cryoprotected by O.N. incubation in 30% sucrose/PBS and 1 h incubation in OCT/30% sucrose PBS (1:1), then embedded in Tissue-Tek OCT compound, and immediately frozen in liquid nitrogen. Frozen sections 8 μm were fixed in acetone and immunoperoxidase staining was performed by standard IHC methods. Briefly after blocking, samples were incubated ON with anti-CD31, -CD34 or -KDR (BD Pharmingen, San Diego, California) or isotype control. After 30 min of incubation with goat anti-rat IgG (BD Pharmingen, San Diego, California), they were developed using Elite-Vectastain ABC-peroxidase (Vector, Burlingame, California) for 30 min and DAB substrate (Vector, Burlingame, California). Slides were counterstained with hematoxylin and mounted. Pictures were taken using an Olympus microscope

equipped with a digital camera. For VEGF-R1 staining, frozen sections were fixed with acetone for 5 min, and endogenous peroxidase was quenched using glucose oxidase method and non-specific protein block. Primary anti-VEGFR-1 (3 µg/ml; ImClone, New York, NY) and anti-rabbit IgG as control (3 µg/ml; Jackson ImmunoReasearch Laboratories, West Grove, PA) were incubated for 1 h at RT in protein block. After washing, secondary anti-rabbit antibodies (1 µg/ml, Jackson ImmunoReasearch Laboratories, West Grove, PA) were added for 30 min. Slides were then washed and incubated in streptavidin-HRP (1 µg/ml, Jackson ImmunoReasearch Laboratories, West Grove, PA) for 30 min at RT. Positive immunoreactivity was developed using DAB (Dako, Carpinteria, CA).

Flow cytometry on tumors

Tumor bearing animals were sacrificed. Tumors were removed and dissociated in collagenase I (Gibco-BRL, Gaithersburg, MD) at 37^{O}C for 2 h. Tumors were then meshed in a cell strainer, and the single cell suspension was stained with rat anti-mouse VEGFR-1, VEGFR-2 and VEGFR-3 (clones MF1, DC101 and 31C1, respectively; ImClone, New York, NY) or an unspecific isotype-matched rat antibody as a control for 30 min at 4^{O} C. After washes in PBS/1% BSA, cells were subsequently incubated with secondary Cy5-conjugated goat antibodies (Jackson ImmunoReasearch Laboratories, West Grove, PA) for 30 min at 4^{O} C. Cells were then washed, fixed in 1% PFA and analyzed using a Coulter Elite Flow Cytometer (Coulter; Hialeah, FL).

Microarray Procedure and data analysis

Using the Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA), we examined gene expression levels of three biological samples of mECK36, mEC-V, mECK36-KSHV-Null, and mECK36 tumors. RNA isolation (Trizol; Invitrogen, Carlsbad, California), Turbo DNase treatment (Ambion, Austin, TX), and RNA purification (RNAeasy; Qiagen, Chatsworth, CA) were done following the manufacturer's recommendations. The integrity of RNA was confirmed with a bioanalyzer (model 2100; Agilent Technologies, Palo Alto, CA). We synthesized, labeled, and hybridized cRNA onto arrays at Genome Explorations (Memphis, TN) according to standard Affymetrix methods. Raw data intensity profiles were analyzed using the GeneSpring 7 (Agilent, Palo Alto, CA) to perform microarray normalization and statistical analysis. Statistically significant genes were selected by ANOVA test to obtain the largest gene list that gave a false discovery rate of <1% (Reiner et al., 2003). Significantly expressed genes were clustered with GeneSpring 7 (Agilent, Palo Alto, CA) using the Pearson correlation method. The Data sets for Figure 6E were obtained from Gene Expression Omnibus (GEO). Aorta Endothelial Cells: GSM44658, GSM44659, and GSM44663. Erythroid Progenitor: GSM27212 and GSM27214. GMP: GSM27216. Lineage depleted bone marrow (Lin^-BM): GSM72905, GSM72907, and GSM72909. $Lin^-Sca1^-cKit^+$: GSM72887 and GSM72889. $Lin^-Sca1^+cKit^+$: GSM72879, GSM72881, and GSM72883. $Lin^-Sca1^-cKit^-$: GSM72891, GSM72893, and GSM72895. Total BM: GSM72899, GSM72901, and GSM72903. $Lin^-Sca1^+cKit^-$: GSM72897. Liver Endothelial Progenitor samples were kindly given by Drs. David Salomon and Dr. Sunil Mathan Kurian, The Scripps Research Institute (Cherqui et al., 2006). Mouse skin samples were obtained from GEO data sets GSM26945, GSM26946, and GSM26947.

Statistical Analysis

A Student's test was applied for the statistical determinations. $P<0.05$ was considered significant. All the experiments were repeated at least three times.

SUPPLEMENTARY FIGURES

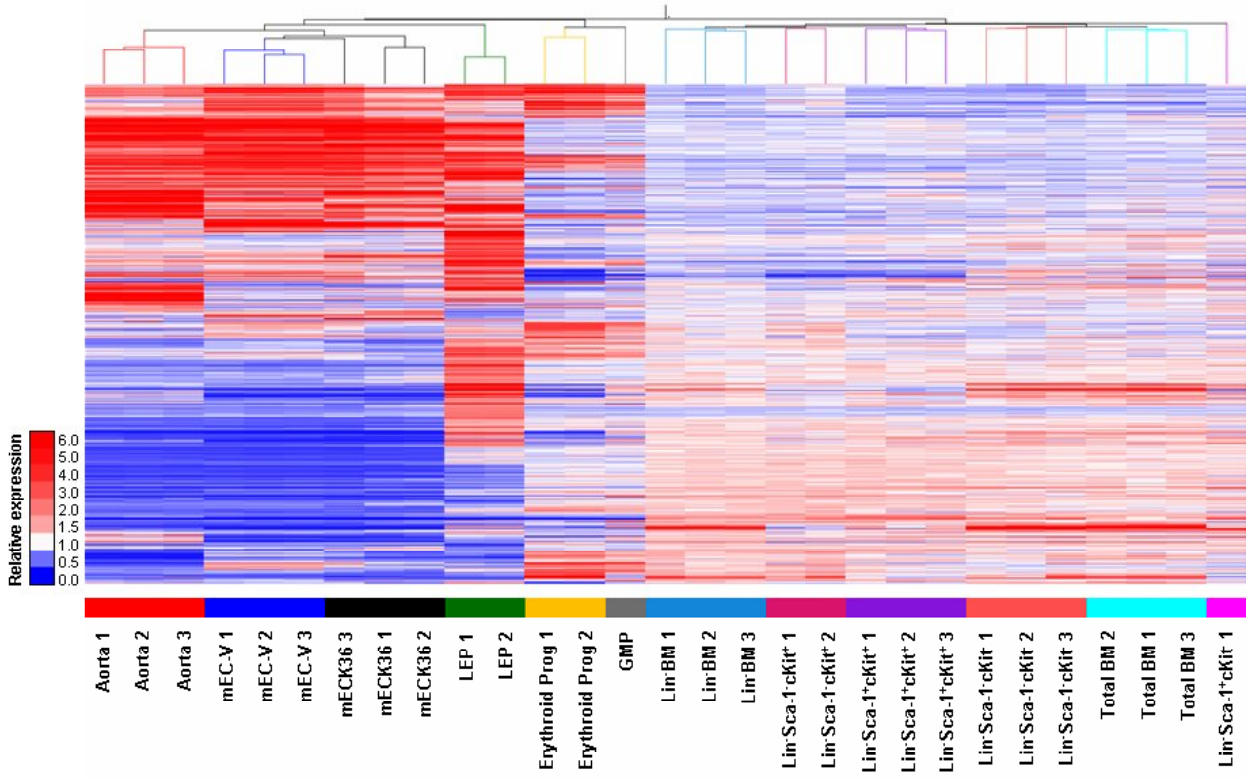

Figure S1: mECK36 transcriptome analysis points to an endothelial-lineage origin.

Hierarchical clustering using a set of 6413 genes normalized and filtered (FDR<0.01) using GeneSpring 7 package, Pearson correlation (see methods). Using the Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA), we examined gene expression

levels of triplicate samples of mECK36 and mEC-V. Raw data intensity profiles were analyzed using the GeneSpring 7 (Agilent, Palo Alto, CA) to perform microarray normalization and statistical analysis. Aorta: aorta endothelial cells. LEP: liver endothelial progenitor cells. GMP: Granulocyte-Macrophage Progenitors. Lin^-BM: Lineage negative Bone Marrow. Total BM: total Bone Marrow. Erythroid Prog: Erythroid Progenitor.