MATERIALS AND METHODS

ES Cell Culture and In Vitro Differentiation

Wild-type (WT) and *flt-1*$^{-/-}$ (gift of G.H. Fong) mouse ES cell maintenance and differentiation was as described.[1] The γ-secretase inhibitor IX, DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester, Santa Cruz Biotechnology, Inc.), was resuspended in DMSO (Sigma), and added to ES cell cultures at 8 μM on dy 5 and 7. DAPT doses above 8 μM did not increase the overall response of the ES cell cultures (Figure I and IIB in online-only Data Supplement), and in some cases, led to deleterious effects (data not shown). Recombinant human VEGF-A (Peprotech) was reconstituted in PBS/0.1% BSA and added on dy 5 and 7 at 30 ng/ml. The recombinant Dll4-Fc protein inhibitor (Adipogen, mouse extracellular Dll4:human Fc) was resuspended in PBS/0.1% BSA, and added at 1 μg/ml every other day from dy 3 onward.

ES Cell Differentiation Culture Antibody Staining and Quantitative Analysis

Fixation and staining of dy 7 and 8 ES cell cultures was as described.[2] Primary antibodies used were: rat anti-mouse PECAM-1 (BD Biosciences) at 1:1000, and rabbit polyclonal anti-phospho-Histone H3 (ser10, EMD Millipore) at 1:500. Secondary antibodies were donkey anti-rat AlexaFluor488 (IgG; H+L) at 1:1000 (Invitrogen), and donkey anti-rabbit AlexaFluor568 (IgG; H+L) at 1:500 (Invitrogen). Incubation with DAPI for 30 min followed all staining.

ES cell cultures were imaged with a confocal microscope (Leica TCS SP5) using a ×40 objective (HCX PL APO oil), and 6-10 z-axis confocal scans were acquired and flattened. Vessel images were traced using the ArtStudio v5.0 application (Lucky Clan) for an Apple iPad, and traces were processed using ImageJ plugins (BoneJ/Skeletonise 3D, Skeleton/AnalyzeSkeleton (2D/3D)) to generate branch point density values. ImageJ was used to count phospho-Histone H3+ (PH3+) mitotic endothelial cells and total endothelial cells, to yield a mitotic index (PH3+ and PECAM+ cells per total PECAM+ cells) for each image. Vascular area was also measured using ImageJ, and pixel intensity threshold values used to determine total PECAM+ area/total image area.

Zebrafish Husbandry and Experimentation

Zebrafish (*Danio rerio*) embryos were bred as previously described.[3] Transgenic adults, *Tg(hsp70l:vegfaa,myl7:EGFP,kdrl:GFP)nc2a* [ref. [4], referred to as *Tg(hsp70l:vegfaa)*], were maintained as heterozygotes, and WT and transgenic embryos were genotyped by PCR upon conclusion of each experiment. Heat-shock treatment occurred 30 hr post-fertilization (hpf) for 1 hr at 42°C. DAPT was administered at 14 and 30 hpf at a final concentration of 100 μM in 1% DMSO.

Zebrafish Imaging and Quantification

Zebrafish vessels expressing GFP were imaged at 48 hpf with a confocal microscope (LSM 5 PASCAL, Carl Zeiss, Inc.) using a 10× objective (Carl Zeiss, Inc.), acquiring 6 μm thick optical slices through the z-axis. For DAPT experiments without VEGF over-expression, the percentage of embryos with abnormal intersegmental vessels (ISVs) or an aberrant caudal vein plexus (CVP) was determined by scoring each embryo as

having perturbed vessels or not, and dividing the total number of defective embryos by the total number evaluated. In heat-shock VEGF experiments with Notch inhibition, ISV defect penetrance and severity were determined. Penetrance was calculated as the percent of embryos with irregular ISV formation among all embryos observed. Defect severity was found by determining, for each embryo with affected ISVs, the percent of somites containing malformed ISVs, and the percentages for each embryo were then averaged.

Endothelial Cell Enrichment

Endothelial cells were enriched from ES cell cultures using magnet-assisted cell sorting (MACS) (Miltenyi Biotec). Briefly, cultures were digested in 2 mg/ml collagenase in HBSS for 45 min at 37°C with repeated, vigorous pipetting. Dissociated cells were passed through a 70 μm filter, centrifuged 5 min at 2000 rpm, and re-suspended in autoMACS® Running buffer with a mouse Fc receptor-blocking reagent (Miltenyi Biotec) on ice for 10 min. Rat anti-mouse PECAM-1 linked to phycoerythrin (PE, BD Bioscience) was added for 15 min. Cells were centrifuged 5 min at 2000 rpm and re-suspended in buffer with magnetic beads coated with anti-PE antibodies for 15 min on ice. After another 5 min spin at 2000 rpm, cells were re-suspended in buffer, passed through a 30 μm filter, and put over a magnetized column. Columns were washed 3X, removed from the magnetic field and placed into new tubes; new buffer was added, and the isolated cells collected.

Real-Time Quantitative PCR Analysis

Endothelial cell-enriched preps were digested in TRIzol (Invitrogen), and RNA was extracted using 1-Bromo-3-chloropropane (Sigma) and ethanol. Following purification using an RNeasy Plus Kit (Qiagen), 0.3 μg of RNA was retro-transcribed using a SuperScript II RT kit (Invitrogen). Real-time quantitative PCR was performed in triplicate using TaqMan Universal Master Mix II (Applied Biosystems) and the Applied Biosystems 7900HT Fast Real-Time PCR System. PCR primers for TATA binding protein (tbp), Flt-1, Hey1, Dll4, and Nrarp were obtained from Applied Biosystems. The tbp values were used for normalization, and the comparative CT method was used to determine expression changes.

Protein Analysis

Western blot analysis was performed as previously described.[2] Enriched endothelial cell preps were incubated in ProteoJet mammalian cell lysis buffer (Fermentas). Following protein separation on a 10% SDS-polyacrylamide gel, samples were transferred to a polyvinylidene fluoride membrane (GE Healthcare). Primary antibodies used were: rabbit anti-Hey1 (5 μg/ml, Millipore), goat anti-actin (1:5000, Santa Cruz Biotechnology), goat anti-Dll4 (1:500, Abcam), and goat anti-GAPDH (1:5000, Abcam). The horseradish peroxidase (HRP)-tagged secondary antibodies included anti-rabbit (GE Healthcare) and anti-goat (Santa Cruz Biotechnology), and were visualized with enhanced chemiluminescence (GE Healthcare).

Statistics

Statistical comparison of ES cell-derived vessel branch points, vessel area, and endothelial RNA levels was done using the Student's two-tailed t-test, as was zebrafish

ISV penetrance and severity. Mitotic index values and DAPT effects on zebrafish ISVs and CVPs were compared using χ^2 analysis.

REFERENCES

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