Quantitative Microscopy of Angiogenesis in Zebrafish Embryos

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Abstract

Angiogenesis is the process by which new blood vessels are formed from the pre-existing vasculature. This is a complex and controlled process, and all aspects of it, including endothelial cell migration, proliferation, and activation are under tight regulation of factors that either promote or inhibit angiogenesis. Angiogenesis plays a crucial role in many conditions including embryonic development, tissue repair, and disease. While inadequate vessel growth leads to tissue ischemia, excessive vascular growth promotes cancer and inflammatory disorders. Enhanced angiogenesis as a reparative therapy is a major goal of regenerative medicine, especially as pertaining to ischemic diseases such as myocardial infarction. On the other hand, inhibition of angiogenesis is an important area of interest for the field of cancer biology. Previous research has shown that tumor angiogenesis is required for the growth and metastasis of solid tumors. As such, inhibition of vessel formation is a potential target for cancer therapy. Many of the pathways involved in embryonic development are implicated in adult human disease. Zebrafish vascular development is an excellent example of this. Zebrafish have a closed circulatory system, and the form of the developing vasculature, the processes used to assemble vessels, and the molecular mechanisms underlying vessel formation are very similar to those in humans. Furthermore, the zebrafish offers many unique advantages for studying vascular development in vivo. Zebrafish embryos develop externally, making them easily accessible to manipulation. Also, their optical transparency facilitates high-resolution imaging of blood vessels in the developing embryo.

Materials and Methods

Zebrafish maintenance
Zebrafish were maintained according to standard conditions. Adult zebrafish, both male and female, were mixed and maintained at 28 °C with 14/10 hour light/dark cycle. In order to collect embryos, male and female zebrafish were put into a breeding basket the night before and embryos were collected in the morning. Zebrafish embryos were kept in a 28 °C incubator in E3 media (SM0 NaCl, 0.33 mM MgSO4, 0.33 mM CaCl2, 0.17 mM KCl and 0.1% methylen blue).

VEGF-inhibitor treatment
Embryos were mounted in 1% low melt agarose and positioned in clear bottom black sided 96 well plates (Corning, Corning, NY) in a dorsal orientation. Zebrafish embryos were then treated with the VEGF receptor inhibitors SU5416 or sunitinib (Torcs, Minneapolis, MN). In one experiment, 28 hpf embryos were treated with 2.5 µg/mL, 1.25 µg/mL, 0.65 µg/mL and 0 µg/mL SU5416. In another experiment, 25 hpf embryos were treated with 100 µg/mL, 25 µg/mL, 6.25 µg/mL, and 0 µg/mL sunitinib. All treatments were diluted in E3 media and 100 µL was added to each well of the 96 well plate.

Imaging
Images were acquired using a 10x objective on the Lionheart FX Automated Microscope (BioTek Instruments, Winooski, VT) configured with a Texas Red light cube. In each well, a beacon was placed at the end of the yolk sac (Figure 3): Stacks of images were taken in both the brightfield and Texas Red channels around the beacon. For each embryo, 10 stacks were taken with a height of 17µm per stack.

Image Analysis
Focus stacking was used to process the 10 image stack into one focused image. Next, automatic background flattening parameters were used to remove background fluorescence from the Texas Red channel. No processing was needed in the brightfield channel. Object masking thresholds were then set to identify the vascular area. Images were analyzed by masking on the Texas Red channel (Figure 4).

Results

Zebrafish embryos at 1 day post fertilization (dpf) were mounted in low melt agarose in the dorsal orientation. Zebrafish embryos were then treated with a VEGF receptor inhibitor, either sunitinib or SU5416. A beacon was set for each embryo at the base of the yolk sac as shown in the image above. Image stacks were taken in both the brightfield and Texas Red channels with a 10x objective. For each embryo, 10 stacks were taken with a height of 17µm per stack. All embryos are positioned roughly in a lateral orientation and are in the center of the well.

Figure 4: Angiogenesis Analysis Workflow.

(A) Image stacks were taken at 10x. (B) Image after using focus stacking to create an in-focus image projection. (C) The resultant image was subjected to pre-processing to remove any background. (D) The vascular area per embryo was determined with the primary mask function of Gen5 software. Object Masks in yellow highlights red vascular area.

Conclusions

1. Angiogenesis is a crucial developmental process, as well as an important therapeutic target for conditions ranging from cancer to heart disease.
2. There is a dose dependent decrease in vascular area after treatment with SU5416 and sunitinib.
3. Changes in angiogenesis can be measured with a Gen5 primary mask of vascular area.
4. Analysis with Gen5 allows for consistent and unbiased determination of vascular area.