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VEGFR2-targeted Ultrasound Molecular Imaging of Angiogenesis to Evaluate Liver Allograft Fibrosis

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Supplemental Materials and Methods

In vitro specific attachment of MB_{VEGFR2} to HUVEC

Human Umbilical Vein Endothelial cells (HUVEC) with a relatively high expression of VEGFR2 were used to confirm specific targeting ability of MB_{VEGFR2} *in vitro*. HUVECs were routinely cultured in Endothelial Cell Medium (ECM) containing 10% fetal bovine serum (FBS) at 37 °C in a

humidified atmosphere. After the cells reached the confluence of 70%, HUVECs were seeded in 6-well plates at a density of 1×10^5 cells per well and incubated overnight in complete growth medium. Meanwhile, to locate MB_{VEGFR2}, DiI marked MB_{VEGFR2} or MB_{CON} were prepared. 0.5 ml of medium containing either MB_{VEGFR2} or MB_{CON} were incubated to the cells for 30 minutes. After washing with PBS for 3 times, all cells were stained in a solution of 2 μg/mL DAPI for 30 minutes to mark the nucleus. Afterwards, the cells were observed under a fluorescent microscope (Nikon, Japan) at magnitude of 400×. 3 samples of each group were observed in this experiment.

In vivo localization of MB_{VEGFR2} with in liver

To determine the location of MBs to the target (VEGFR2) *in vivo*, we used confocal laser scanning microscopy (CLSM) to observe tissue slides. Rat LAF models (7 d after IRI surgery) were established. A total of 2.0×10^7 bubbles/mL of DiI-labeled MB_{VEGFR2} or MB_{CON} were injected into the tail vein of each rat (n = 3 of each group). To clear the labeled bubbles from circulation, the heart was perfused with 200ml 0.9% normal saline 1 h after bubble injection. Liver tissues were immediately extracted for sectioning into 5-μm slices. Frozen sections underwent immunofluorescent staining for VEGFR2. Briefly, after incubation with 0.5% Triton for 10 minutes, the sections are treated with 10% normal goat serum for 1 h at room temperature for antigen blocking. VEGFR2 (Abcam, Cambridge, MA) antibodies were then added to the sections for 1 h at 37 °C. Finally, after being washed with PBS, all sections were incubated with with FITC-conjugated anti-rat secondary antibodies (eBioscience, San Diego, CA) at room temperature for 30 min. The frozen

slides were observed using an CLSM (Zeiss, Germany) at a 630× magnification.

The Destruction–Replenishment Approach

Targeted US imaging was obtained via a destruction–replenishment approach (Figure S1): Firstly, 60 s of continuous imaging of sufficient affiliated and freely circulating MBs was obtained. Afterwards, all MBs in the region were destroyed by high mechanical index for 1 s using a "flash" function. Subsequent post-destruction images were recorded for 10 s to capture freely circulating MBs.

Supplementary Figures

Fig S1

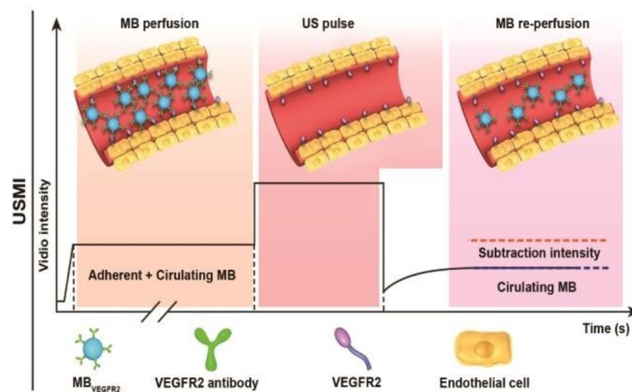

Fig S2

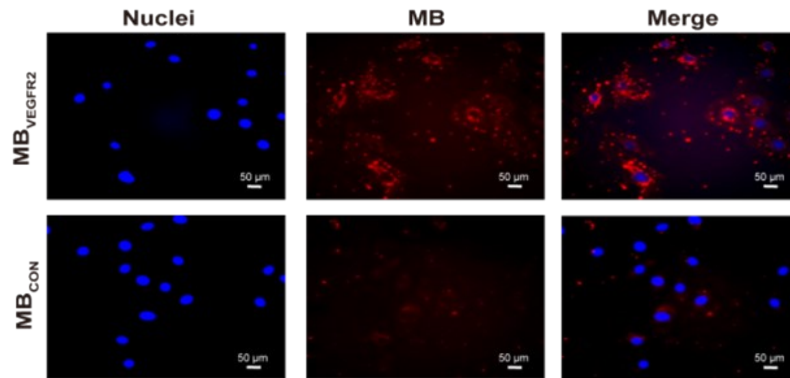

Fig S3

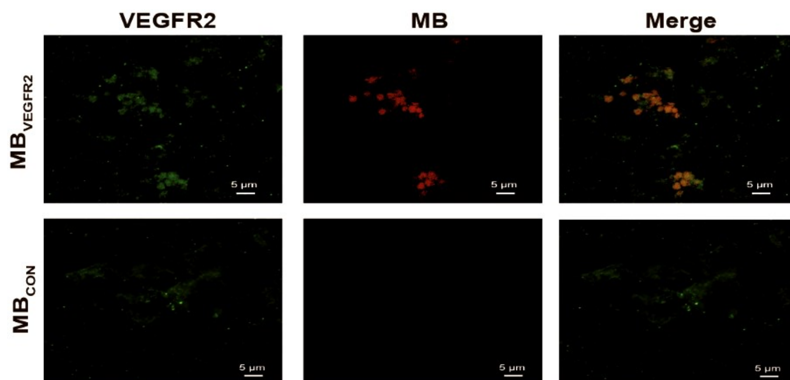

Fig S4

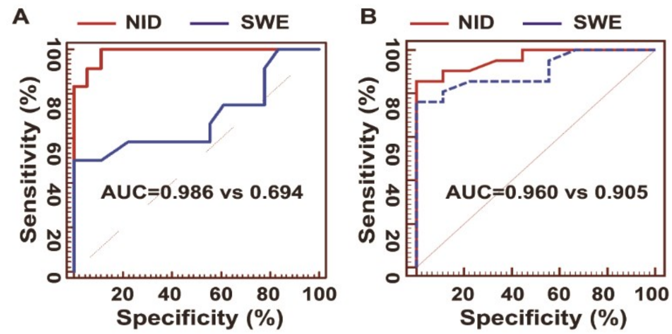

Fig S5