SUPPLEMENTAL INFORMATION FOR TWARDOWSKI ET AL.:

METHODS:

Two Dimensional Fluorescent Image Acquisition and Analysis

The fibrin hydrogel constructs were cultured for 7-10 days for media studies or 10 days for varied co-culture studies, after which high-resolution images of the hemispheres from each condition were captured on an Olympus IX81 microscope at 10x and 20x magnifications. The length of each sprout was measured in the XY projections from segments of each EC sprout (defined as branch point to branch point) using ImageJ (NIH, Bethesda, MD). The vast majority of the sprouts lacked a significant curvature, thus a straight line was drawn from one branch point to another branch point. The average sprout length for each construct condition was determined and plotted against the ratio of support cells to ECs for each support cell condition. In order to determine whether the ECs sprouts were multicellular, the number of EC nuclei in each sprout was also counted. Individuals blinded to the conditions of the experiment carried out the measurements using ImageJ.

Conditioned Media Study:

To assess growth factor release and paracrine signaling from the support cells, a conditioned media study was carried out. Rat cardiac fibroblasts were cultured in fibroblast media (DMEM, 15% FBS and 1% penicillin-streptomyocin) and MSCs were cultured in MSC media (α-MEM, 20%FBS, 1% penicillin-streptomyocin) for 24-48 hours. Five milliliters of conditioned media was collected from a T75 flask, into a 15mL conical and allowed to settle for 5 minutes at room temperature. The purpose of allowing the media to settle is that any dead cells mixed with the media could potentially be removed. The conditioned media contained 30% Support Cell conditioned media, 20% fresh support cell medium and 50% Endothelial Cell media (EBM-2 + BulletPack), as illustrated in Table 1 below. The experiment was conducted in

a 24 well plate with 2.5 x 10^3 ECs per well. EC media was used as a control for n=6 wells. As a control for each support cell media type, a 1:1 ratio of Support Cell media: EC media was placed in wells for each support cell type (n=3) while conditioned media for each support cell type was added to 3 wells, each. Each well of the 24 well plate received 1mL of media. Cells were cultured for 4 days.

Media Type	EC : Support Cell Media	EC:CF Conditioned Media	EC:CF Conditioned Media
EC Media	2.5 ml EC media	2.5 ml EC media	2.5 ml EC media
Support Cell Media	2.5 ml either CF or MSC media	1 ml CF media	1 ml MSC media
Conditioned Media		1.5 ml CF conditioned media	1.5 ml MSC conditioned media

Table S1: Conditioned media experimental set-up

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Western Blotting for VEGF Expression:

RESULTS:

Flow Cytometry of Constructs in Varied Co-Culture Ratios:

Prior to encapsulating the ECs and support cells in constructs, flow cytometry was conducted in order to validate that our chosen markers were cell type specific. Figure 6 demonstrates the percent of ECs in 2D culture stained with CD31 (Fig. S1A) and CD90 (Fig. S1B). The ECs demonstrated a strong staining for CD31 with 99.4% of the events marking for CD31 in 2D culture, while only 1.4% of the ECs demonstrated a CD90 binding. Moreover, the MSCs did not stain for CD31 (Fig. S1C) and had a strong stain for CD90 (~91%, Fig. S1D). Since there is no true marker for CFs and the CFs did not stain for CD31 (<1%, Fig. S1E), all cells that were $CD31^+$ in the CF-EC co-culture were assumed to be ECs.

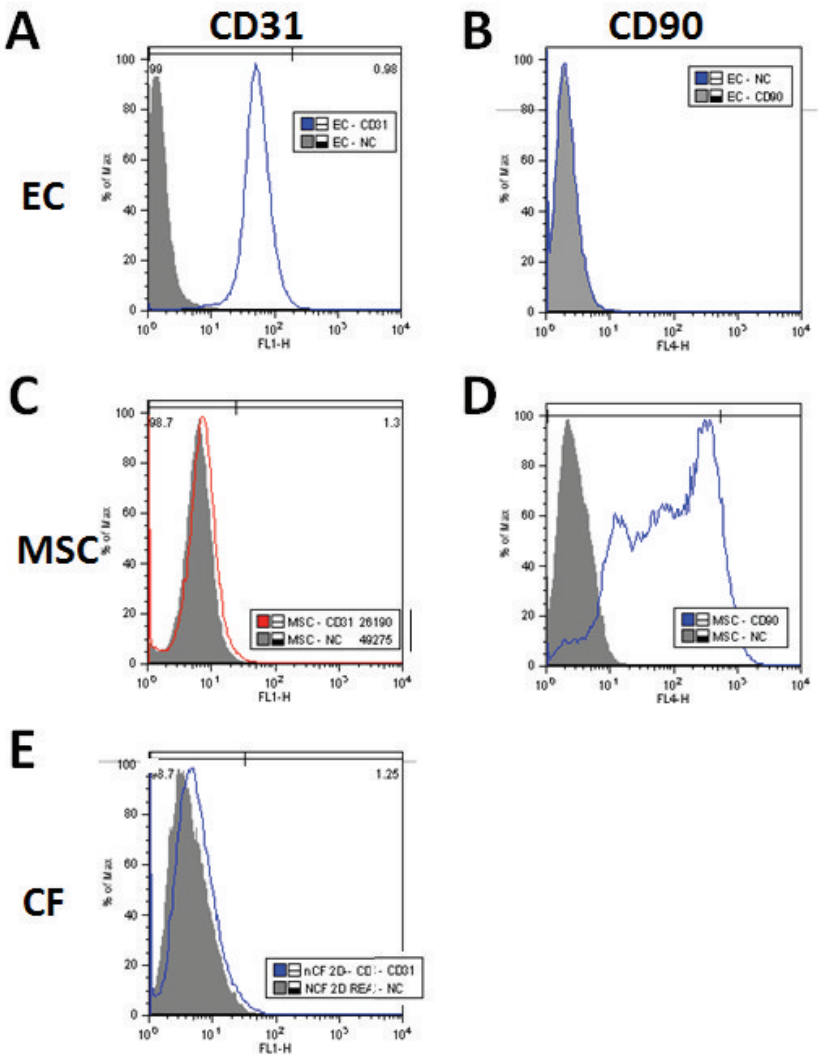

Figure S1: Flow data and support cell varied co-culture study. A-B) ECs stained with CD31 (A) and CD90 (B). C-D) MSCs stained with CD31 (C) and CD90 (D). E) CFs stained with CD31.

Endothelial Cell Conditioned Media Study:

To ascertain whether CFs and MSCs secrete growth factors that induce or inhibit endothelial cell proliferation, ECs were cultured in 2D culture with fresh EC: Support Cell media and the conditioned media of neonatal, fetal and adult CFs and MSCs. Initial results of the EC conditioned media study indicated that EC proliferation is greatest in the unconditioned EC:MSC media, and is also slightly greater in the MSC and CF condition media. The increase in proliferation in the conditioned media groups could be a result of paracrine signaling between growth factors in the conditioned media and the endothelial cells. Growth factors that can influence EC proliferation include VEGF and an upregulation of the Notch signaling pathway.

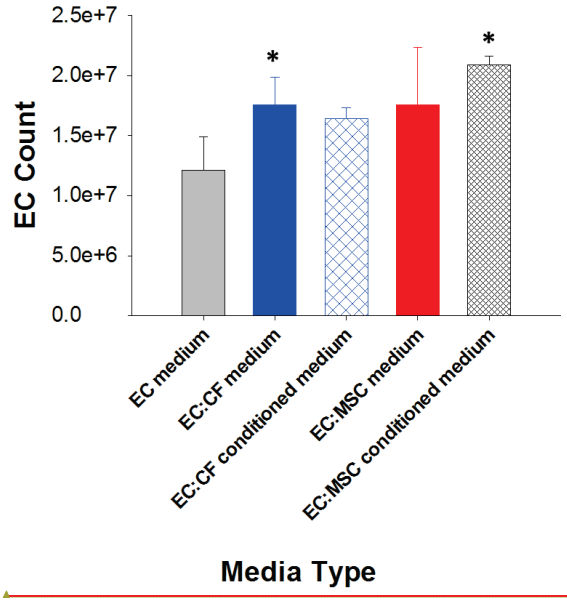

Figure S2: EC count after 4 days in various culture medium. Note that * represents $p<0.05$ compared to EC medium condition.

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Co-localization Images:

Samples of the 1:1 ratio of ECs to CFs and MSCs were created with support cells that were pre-labeled with the membrane stain PKH26. Samples were cultured in the respective 50-50 medium for 7 days and then fixed and histologically stained as described in the paper for vWF. Representative images are shown in Figure S3 below. Note that in the case of the CFs we see some co-localization (yellow arrow Figure S3A) while with MSCs we see multinucleated sprouts without the appearance of any MSCs in contact (yellow arrow Figure S3B)

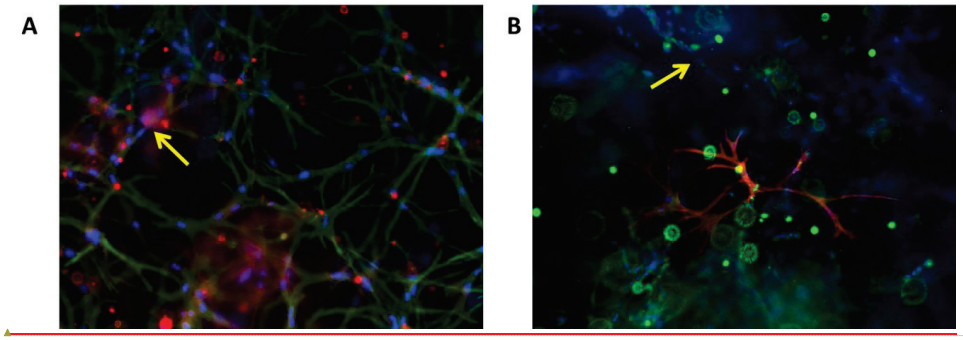

Figure S3: Co-localization of PKH26 (red) labeled CFs (A) and MSCs (B) with ECs stained in green for vWF.

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