Supplementary Information

Conversion of vascular endothelial cells into multipotent stem-like cells

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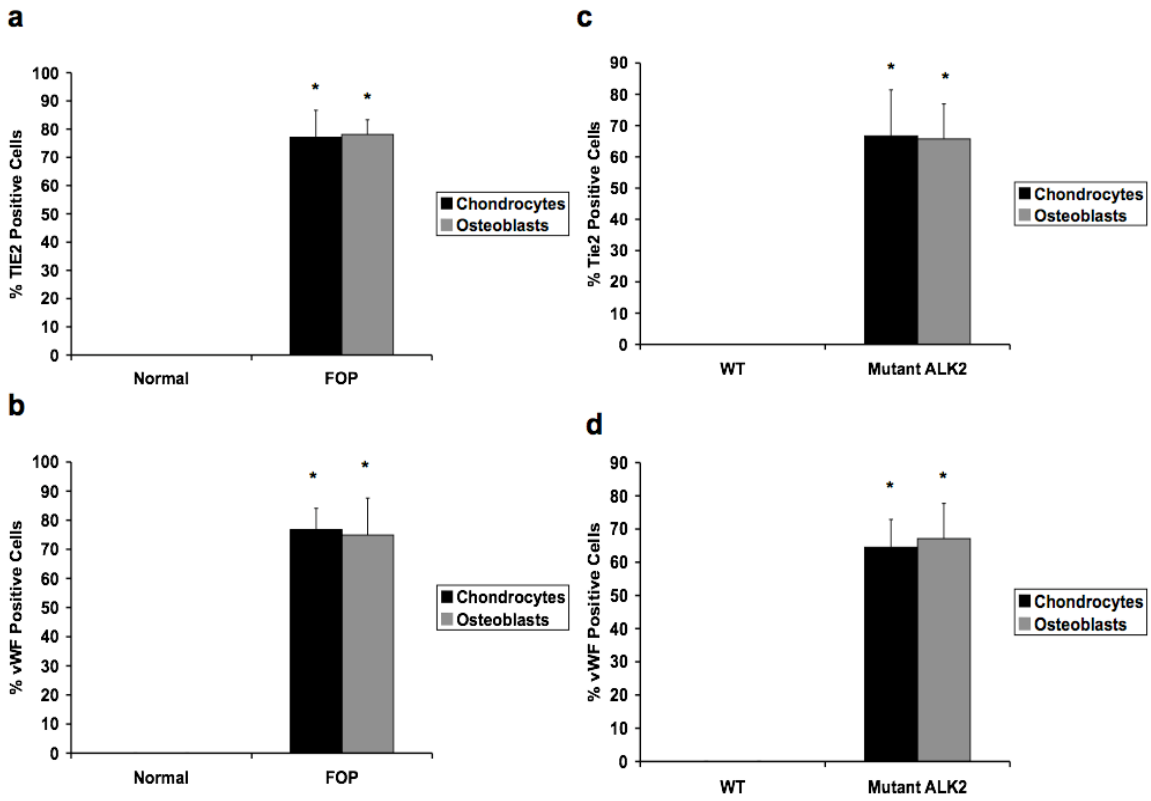
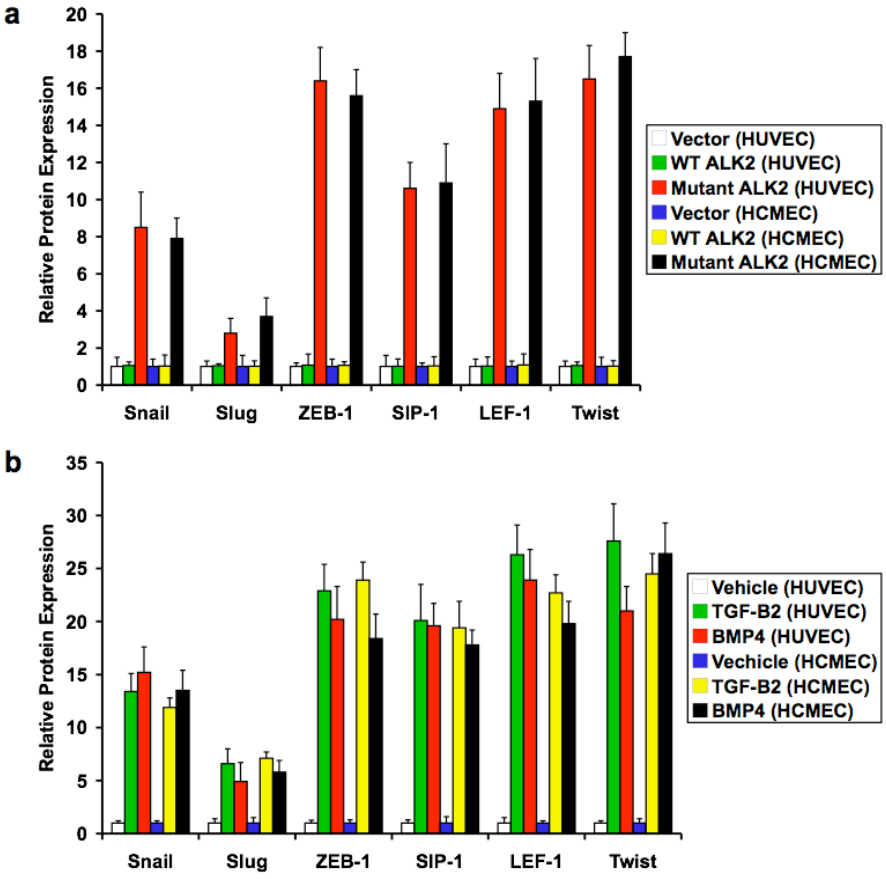
Supplementary Figure 1



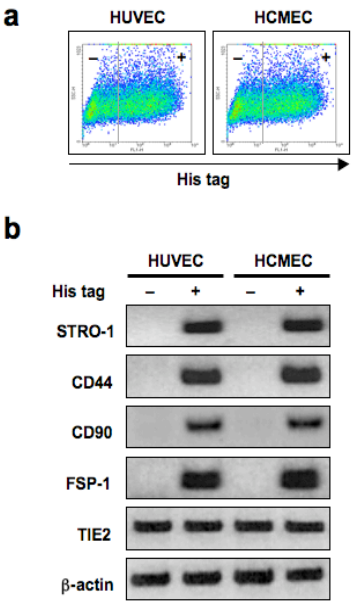
Supplementary Figure 1 Quantification of TIE2 and vWF positive osteoblasts and chondrocytes in heterotopic bone and cartilage. (**a–d**) Graphic representation of the percentage TIE2 (a,c) and vWF (b,d) positive cells in normal human bone and cartilage from the hip joint (n=3) vs. heterotopic bone and cartilage from FOP patients (n=3) and wild-type (WT) bone and cartilage from the mouse knee joint (n=3) vs. heterotopic bone and cartilage from Cre-dependent mutant ALK2 transgenic mice (n=7). Data represent mean ± s.d.; *P<0.01 (student's t test) compared to normal or wild-type tissue.

Supplementary Figure 2



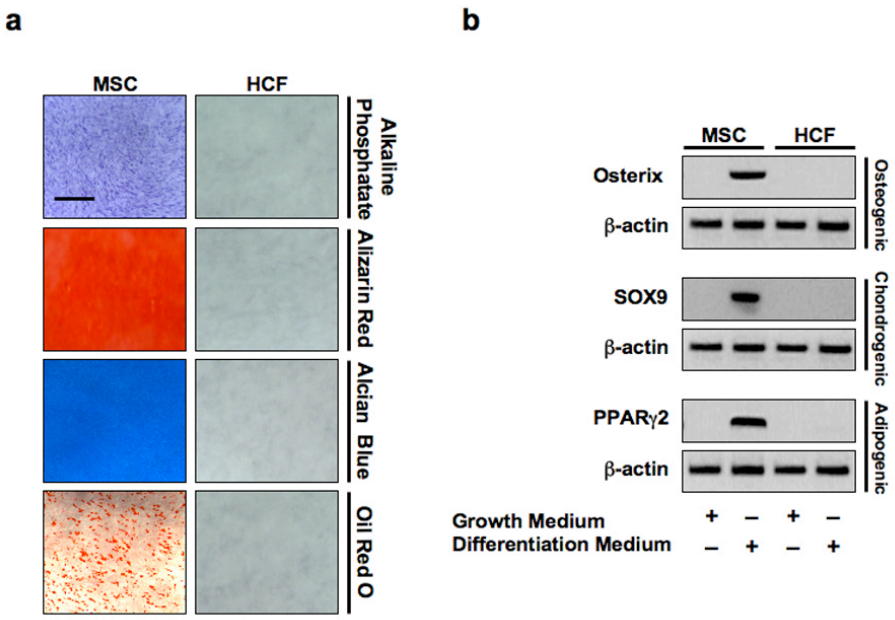
Supplementary Figure 2 Expression analysis of EndMT-inducing transcription factors. (**a,b**) Multiplex ELISA analysis showing increased expression of Snail, Slug, ZEB-1, SIP-1, LEF-1, and Twist in cells expressing mutant ALK2 compared to wild-type (WT) ALK2 or vector (a), and in cells stimulated with TGF-β2 or BMP4 compared to vehicle (b). Data represent mean (n=3) ± s.d.; P<0.05 (ANOVA) for all mutant ALK2 expressing cells compared to WT ALK2 expressing cells and all TGF-β2 or BMP4 treated cells compared to vehicle treated cells.

Supplementary Figure 3



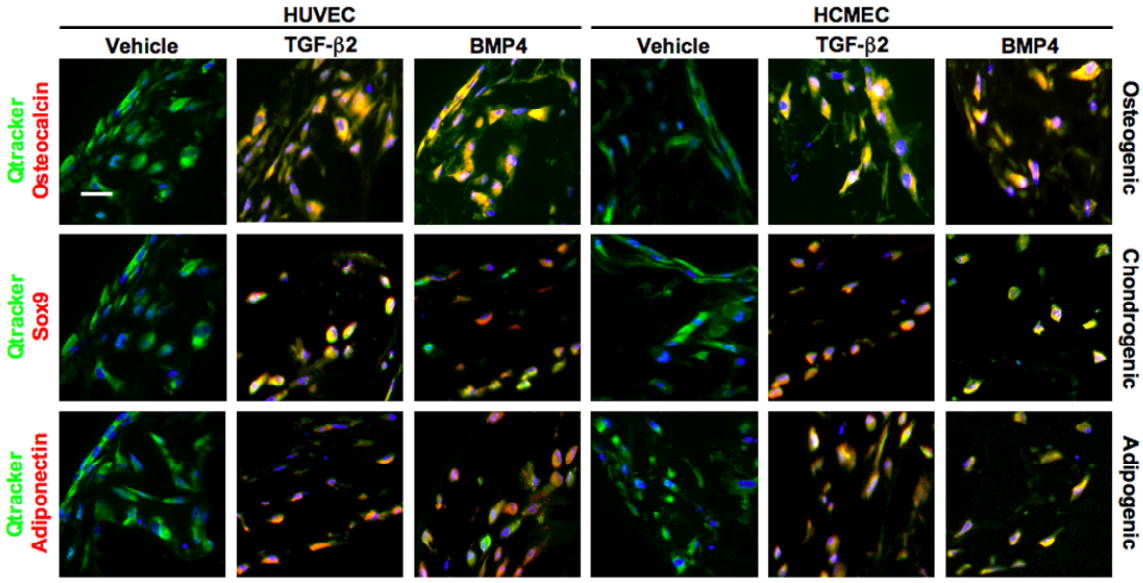
Supplementary Figure 3 Mutant ALK2 induces expression of mesenchymal stem cell markers in endothelial cells. (**a**) Fluorescence activated cell sorting (FACS) separating mutant ALK2 treated endothelial cells that express the His tag (+) from those that do not (–). (**b**) Immunoblotting of endothelial cell fractions isolated by FACS showing that only cells containing the mutant ALK2 His tag express the mesenchymal marker FSP-1 and the stem cell markers STRO-1, CD44, and CD90.

Supplementary Figure 4



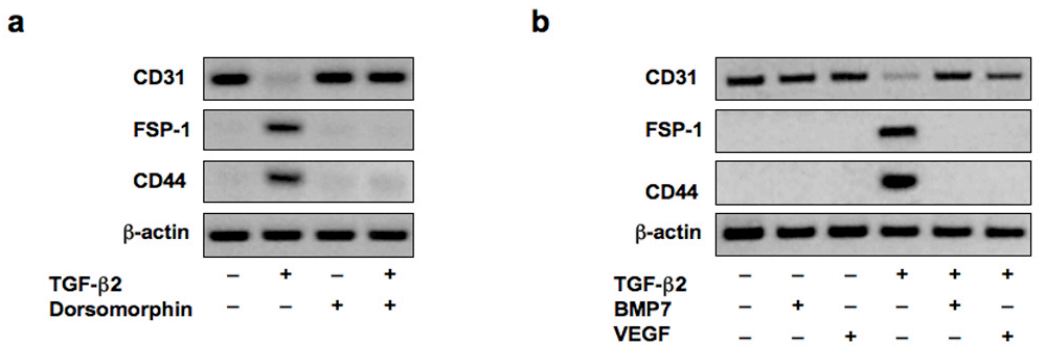
Supplementary Figure 4 Differentiation potential of human bone marrow derived mesenchymal stem cells (MSC) and human corneal fibroblasts (HCF). (**a**) Positive staining for osteoblast (alkaline phosphatase and alizarin red), chondrocyte (alcian blue), or adipocyte (oil red O) products in MSC cultures grown in osteogenic, chondrogenic, or adipogenic culture medium. No positive staining was found for HCFs. Scale bar, 100 μm. (**b**) Immunoblotting showing expression of osteoblast (osterix), chondrocyte (SOX9), or adipocyte (PPARγ2) markers in MSCs, but not in HCFs, exposed to osteogenic, chondrogenic, or adipogenic culture medium. When cultured in growth medium, MSCs did not differentiate.

Supplementary Figure 5



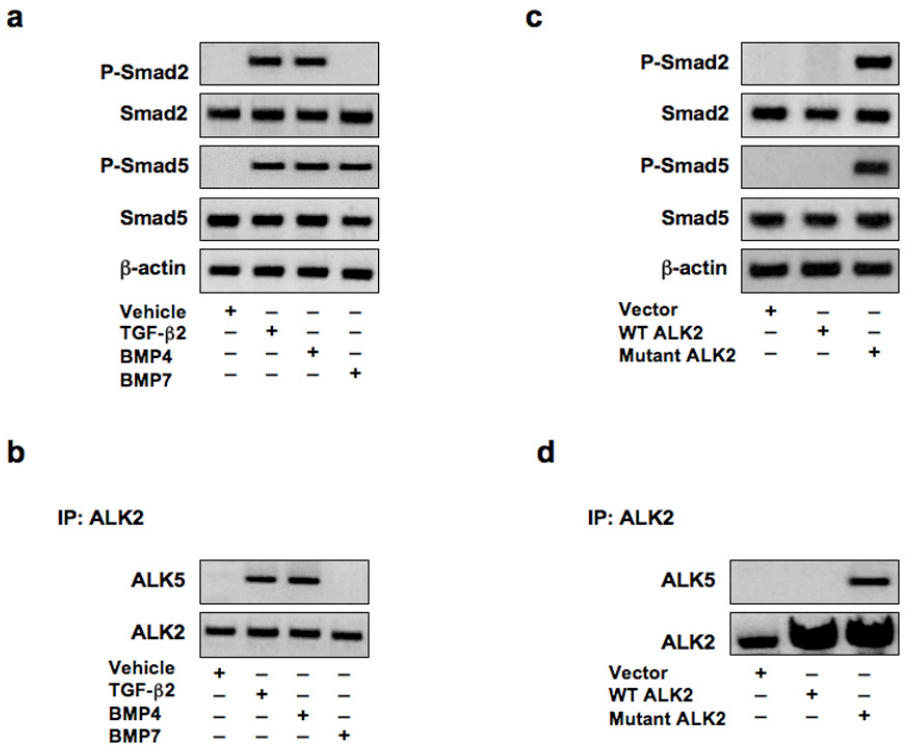
Supplementary Figure 5 Tracking endothelial derived stem-like cell differentiation in vivo. Immunohistochemistry of sections of polylactic acid scaffolds containing endothelial cells pre-labeled with fluorescent quantum dots (Qtracker) and treated with vehicle, TGF-β2, or BMP4 and subcutaneously implanted into nude mice followed by local injection of differentiation medium every 72 h for 6 weeks. The data demonstrate co-expression of the Qtracker with osteocalcin, SOX9, or adiponectin in implants of cells treated with TGF-β2 or BMP4, but not with vehicle, and injected with osteogenic, chondrogenic, or adipogenic medium, respectively. Scale bar, 10 μm.

Supplementary Figure 6



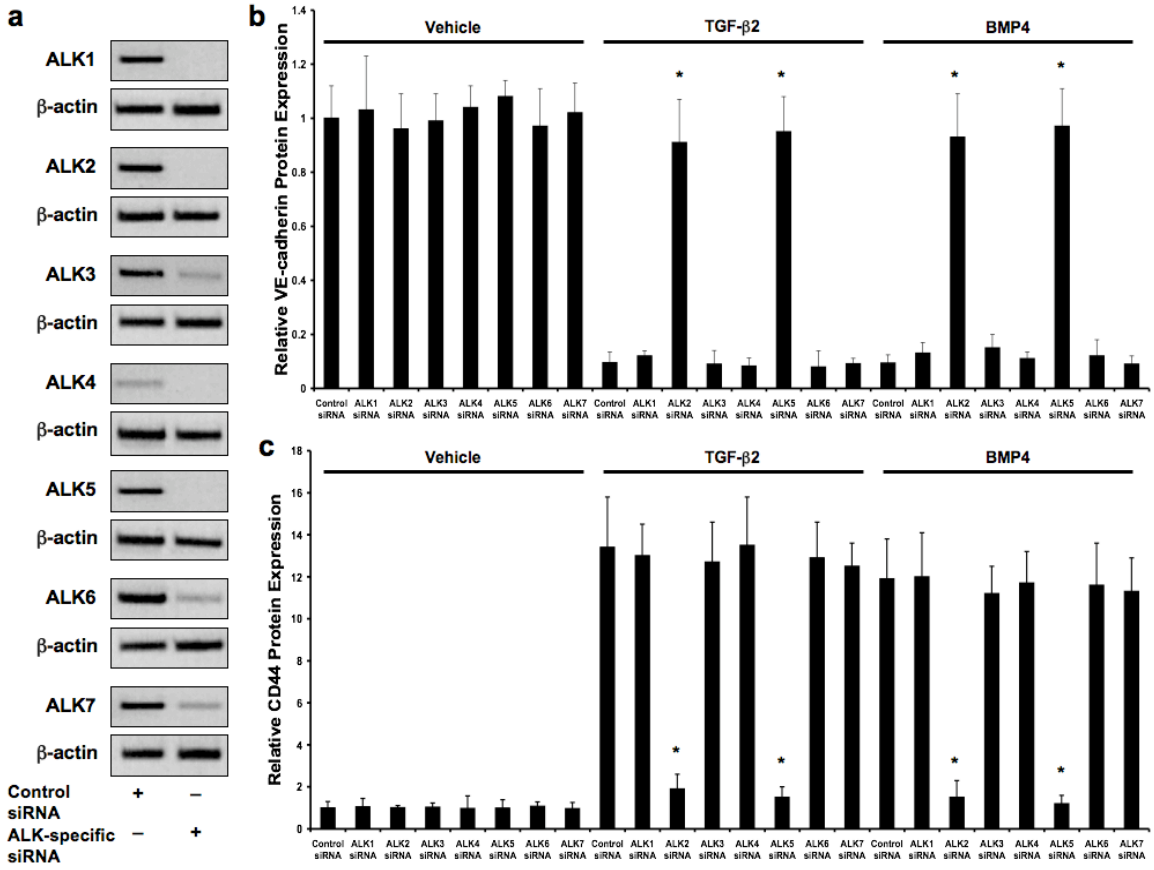
Supplementary Figure 6 Assessment of EndMT inhibitors. (**a,b**) Immunoblotting showing inhibition of TGF-β2-induced decreases in CD31 and increases in FSP-1 and CD44 expression by dorsomorphin (a), BMP7 or VEGF (b) in HUVECs.

Supplementary Figure 7



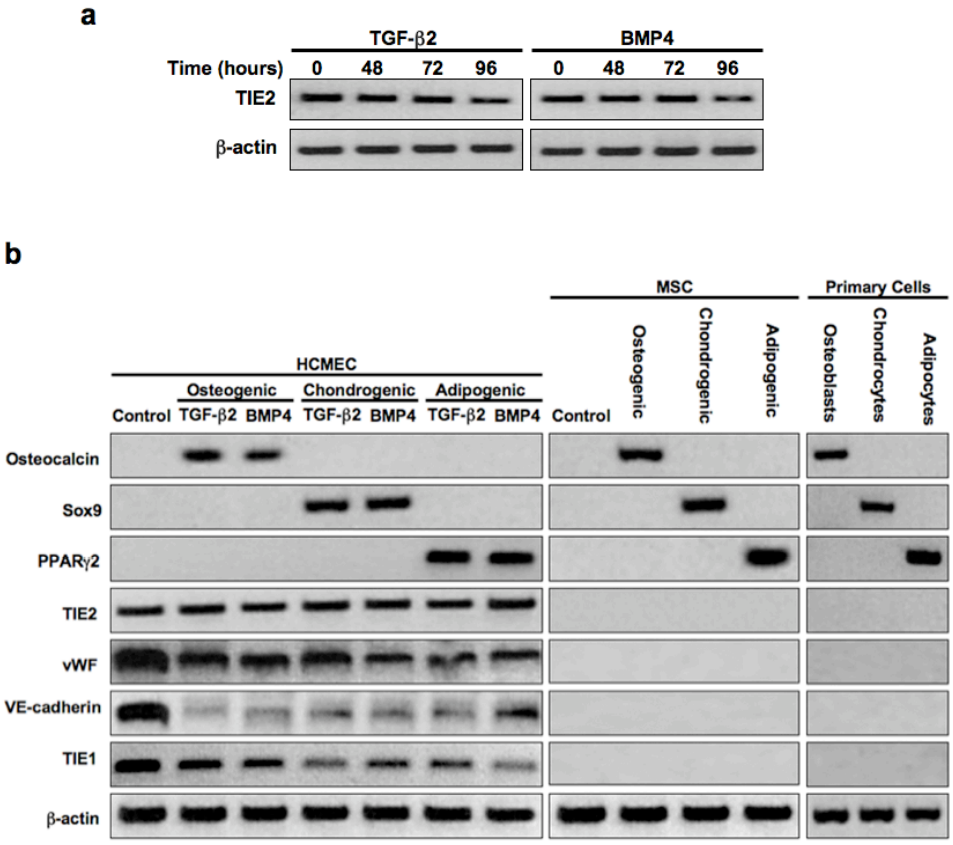
Supplementary Figure 7 Ligand specificity in ALK2 signaling. (**a**) Immunoblotting demonstrating phosphorylation of Smad2 (P-Smad2) and Smad5 (P-Smad5) in HUVECs treated with TGF-β2 or BMP4 for 1 h, but phosphorylation of only Smad5 in those treated with BMP7. (**b**) Immunoprecipitation of ALK2 showing the presence of ALK5 in precipitates of lysates from cells treated with TGF-β2 or BMP4 for 15 min. No ALK5 was observed in precipitates of lysates from cells treated with vehicle or BMP7. (**c**) Immunoblotting showing that HUVECs expressing the mutant ALK2 protein found in FOP have phosphorylation levels of both Smad2 and Smad5. (**d**) Immunoprecipitation demonstrating the presence of both ALK2 and ALK5 in lysates from cells expressing mutant ALK2, but not vector or wild-type ALK2.

Supplementary Figure 8



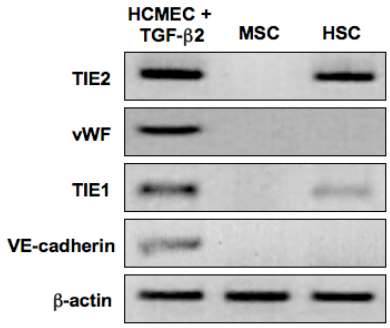
Supplementary Figure 8 ALK receptor specificity in mediating EndMT. (**a**) Immunoblotting showing expression knockdown of all ALK receptors (ALK1-ALK7) in HUVECs using siRNA duplexes specific for each receptor. (**b,c**) ELISA analysis showing that TGF-β2- or BMP4-dependent decreases in VE-cadherin (b) and increases in CD44 (c) are inhibited by ALK2 siRNA or ALK5 siRNA, but not siRNA specific for ALK1, ALK3, ALK4, ALK6, or ALK7. Data represent mean (n=3) ± s.d.; *P<0.01 (student's t test) compared to control siRNA.

Supplementary Figure 9



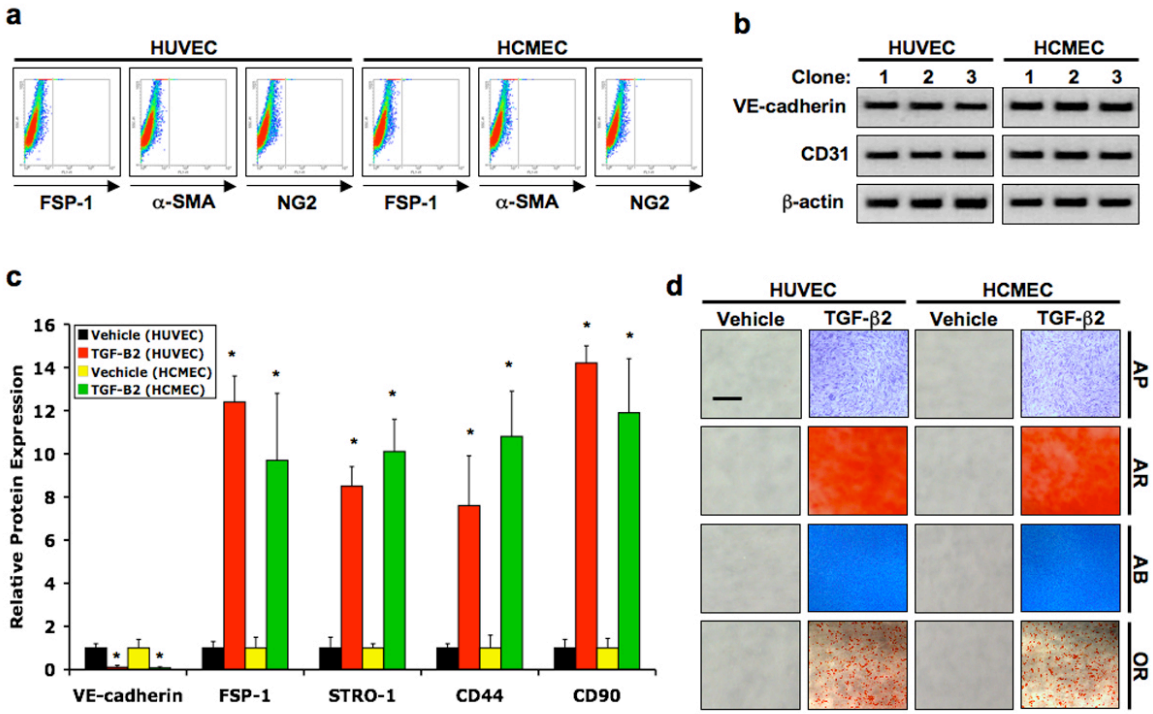
Supplementary Figure 9 Retention of endothelial markers after EndMT. (**a**) Immunoblotting showing a slight decrease in expression of TIE2 in HCMECs after 96 h of exposure to TGF-β2 or BMP4. (**b**) Immunoblotting showing that bone marrow-derived mesenchymal stem cells (MSC) do not express the endothelial markers TIE2, vWF, VE-cadherin, and TIE1, nor do osteoblasts, chondrocytes, or adipocytes (as identified by expression of their characteristic molecular markers osteocalcin, SOX9, and PPARγ2) derived from MSCs grown in appropriate differentiation medium, or primary human osteoblasts, chondrocytes, or adipocytes. Osteoblasts, chondrocytes, and adipocytes derived from endothelial cells stimulated by TGF-β2 or BMP4, followed by exposure to appropriate differentiation medium, do express these markers.

Supplementary Figure 10



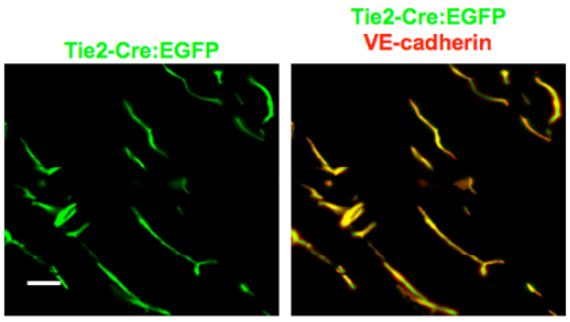
Supplementary Figure 10 Expression analysis of endothelial markers in stem cells. Immunoblotting showing that bone marrow derived hematopoietic stem cells (HSC) express TIE2 and trace amounts of TIE1, but not other endothelial markers (vWF and VE-cadherin) expressed in endothelial derived stem-like cells (HCMEC) transformed by TGF-β2. Bone marrow derived mesenchymal stem cells (MSC) do not express any of these markers.

Supplementary Figure 11



Supplementary Figure 11 Homogeneity and clonality of HUVECs and HCMECs. (**a**) Flow cytometry analysis of HUVEC and HCMEC populations demonstrating no positive staining for fibroblast (FSP-1), smooth muscle cell (α-SMA), or pericyte (NG2) markers. (**b**) Immunoblotting for VE-cadherin and CD31 using lysates from three clonal populations of HUVECs and HCMECs expanded from single endothelial cells. (**c**) Multiplex ELISA analysis demonstrating decreased expression of VE-cadherin and increased expression of FSP-1, CD44, and CD90 in the three clonal populations of HUVEC and HCMEC treated with TGF-β2 for 48 h. Data represent mean (n=3) ± s.d.; *P<0.05 (student's t test) compared to vehicle treated cells. (**d**) Representative images of one of three clonal populations of HUVEC and HCMEC transformed by TGF-β2 staining positive for osteoblasts (alkaline phosphatase [AP] and alizarin red [AR]), chondrocytes (alcian blue [AB]), or adipocytes (oil red O [OR]), after exposure to osteogenic, chondrogenic, or adipogenic culture medium, respectively. Scale bar, 100 μm.

Supplementary Figure 12



Supplementary Figure 12 Positive control images of Tie2-Cre:EGFP localization. Immunohistochemistry of sections of muscle tissue from Tie2-Cre;IRG mice showing EGFP expression localized in blood vessels and confirmed by staining with antibodies specific for the endothelial marker VE-cadherin. No leakage or aberrant expression of the Tie2-Cre reporter was observed. Scale bar, 20 μm.