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Original article

SLIT3–ROBO4 activation promotes vascular network formation in human engineered tissue and angiogenesis in vivo ☆

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ABSTRACT

Successful implantation and long-term survival of engineered tissue grafts hinges on adequate vascularization of the implant. Endothelial cells are essential for patterning vascular structures, but they require supportive mural cells such as pericytes/mesenchymal stem cells (MSCs) to generate stable, functional blood vessels. While there is evidence that the angiogenic effect of MSCs is mediated via the secretion of paracrine signals, the identity of these signals is unknown. By utilizing two functionally distinct human MSC clones, we found that so-called "pericytic" MSCs secrete the pro-angiogenic vascular guidance molecule SLIT3, which guides vascular development by directing ROBO4-positive endothelial cells to form networks in engineered tissue. In contrast, "non-pericytic" MSCs exhibit reduced activation of the SLIT3/ROBO4 pathway and do not support vascular networks. Using live cell imaging of organizing 3D vascular networks, we show that siRNA knockdown of SLIT3 in MSCs leads to disorganized clustering of ECs. Knockdown of its receptor ROBO4 in ECs abolishes the generation of functional human blood vessels in an in vivo xenogenic implant. These data suggest that the SLIT3/ROBO4 pathway is required for MSC-guided vascularization in engineered tissues. Heterogeneity of SLIT3 expression may underlie the variable clinical success of MSCs for tissue repair applications.

1. Introduction

Cell-based regenerative medicine therapies such as cardiac tissue engineering for ischemic heart disease require adequate vascularization for successful implantation and long-term survival [1,2]. Pericytes are microvascular mural cells which directly interact with endothelial cells to promote endothelial growth and stabilize endothelial networks [3–5]. Importantly, pericytes and mesenchymal stem cells (MSCs) derived from bone marrow or other tissues express a similar array of cell surface markers, thus suggesting that they have significant functional and phenotypic overlap [6–8].

Like pericytes, MSCs can be combined with endothelial cells to enhance the formation of vascular networks [3,9–11]. The currently proposed mechanisms of how MSCs promote angiogenesis are thought to involve the release of angiogenic growth factors [12,13] and stabilization of vascular networks through direct contact. The pericyte function of MSCs remains poorly defined and it is not known whether MSCs release mediators of vascular endothelial cells, similar to those released by pericytes [14]. We sought to explore the contribution of MSCs to EC vascular network formation in vitro and to highly vascularized tissue constructs in vivo, as well as define the role of potential vascular mediators released by MSCs.

Abbreviations: DAPT, N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethelyl ester; EC, endothelial cell; HAEC, human aortic endothelial cell; HUVEC, human umbilical vein endothelial cell; MSC, mesenchymal stem cell or marrow stromal cell; MSC5, human MSC clonal line HS-5; MSC27a, human MSC clonal line HS-27a; VEGF, vascular endothelial growth factor.

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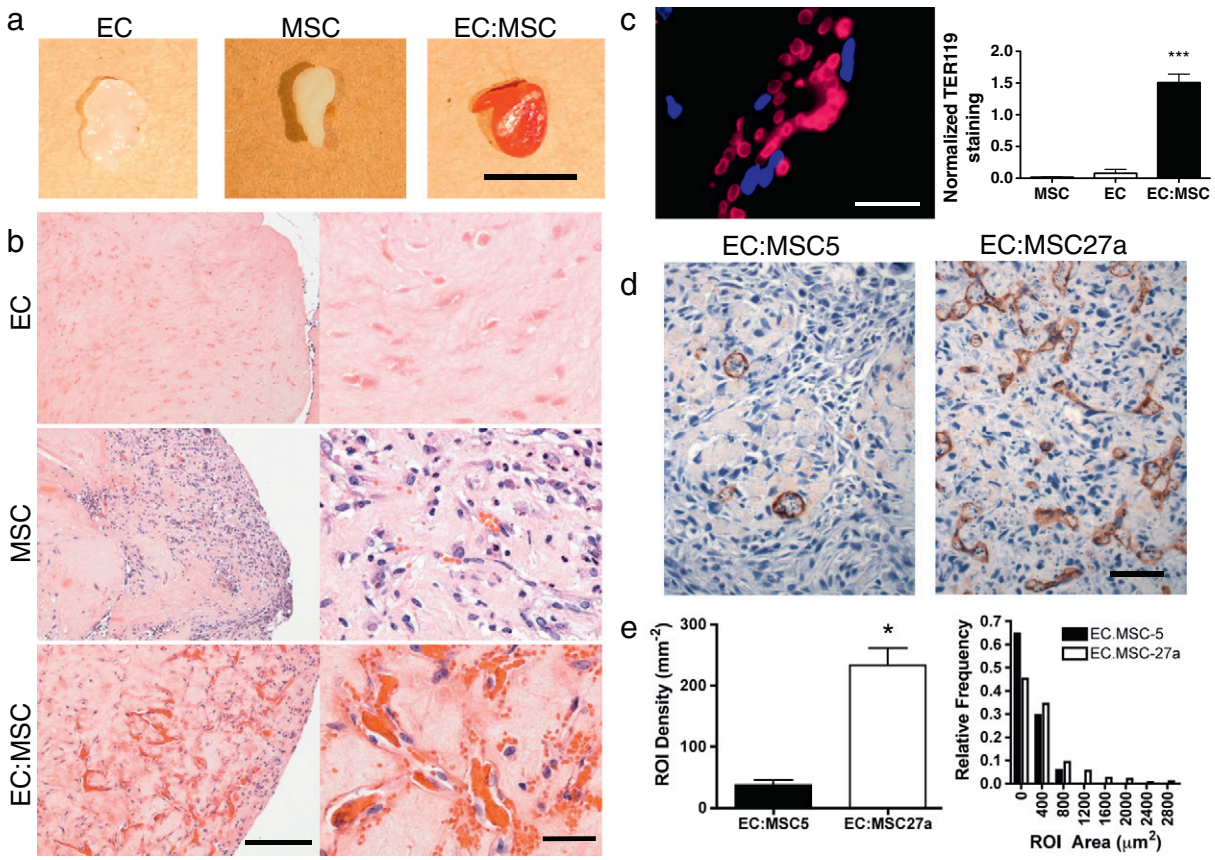

Fig. 1. Cross-talk between human endothelial cells (ECs) and human mesenchymal stem cells (MSCs) promotes the formation of vascular networks in engineered tissue and perfused blood vessels in vivo. a, Gross photographs of whole gel explants at 7 days illustrate significant vascularization of gels containing both cell types by the visible red color in EC:MSC explants (right). Scale bar: 5 mm. b, Representative hematoxylin & eosin (H&E) stainings of collagen/fibronectin gel explants after 7 days in vivo show minimal vascularization in implants containing either human ECs (top) or human MSCs (middle). Significant vascularization is observed in implants containing a mixture of the two cell types (bottom) in a 1:1 ratio, where 1×10^6 total cells were implanted per condition. Scale bar: 200 μm, left; 40 μm, right. c, Red blood cell content in vascularized EC:MSC gel plug, measured by immunostaining against TER-119 (red), is significantly increased in EC:MSC plugs as compared to either cell type alone ($p < 0.001$). Scale bar: 20 μm. Quantification of TER-119 positivity is normalized to the number of DAPI-positive nuclei within each high power field ($n = 18$ (MSC), $n = 4$ (EC), and $n = 16$ (EC:MSC)). d, Immunostaining against CD31 (brown, DAB) in representative scaffold-free engineered tissues after 8 days of in vitro culture containing ECs plus either MSC27a or MSC5 cells shows greatly increased EC networks with MSC27a co-culture. Scale bar: 100 μm. And e, Quantification of in vitro vascularization in engineered tissues by automated detection of regions of interest (ROI) from brown CD31 stain shows increased vascular density (left) and a broader spectrum of structure sizes (right, assessed by individual ROI areas and plotted as a histogram). ROI density is defined as the total number of ROI per area (mm^2). ROI area (μm^2) indicates the area of each ROI outlined (see Supplementary Fig. 1) and is binned and plotted as a relative frequency of the total number of structures (defined as 1.0). The ROI area is a surrogate measure for vessel diameter.

2. Materials and methods

2.1. Cell culture

Adult human aortic endothelial cells (HAECs, Lonza) and human umbilical vein endothelial cells (HUVECs, Lonza) were maintained in endothelial cell growth medium-2 (EGM-2, Lonza) according to the manufacturer's recommendations and no longer than passage 7. Human bone marrow-derived mesenchymal stem cells (MSCs, Lonza) were maintained in minimal essential medium (α-MEM, Invitrogen) supplemented with 16.5% FBS, 2 mM L-glutamine, and penicillin/streptomycin (final concentration 100 units/ml penicillin and 100 μg/ml streptomycin). MSCs were passaged no more than twice after being thawed before being used in experiments. Human marrow stromal cell clones HS-5 (MSC5) and HS-27a (MSC27a) [15] were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) with 5% fetal bovine serum (FBS) and 2 mM L-glutamine.

2.2. Generation of scaffold-free engineered vascular tissue

Cells were harvested and mixed in a 2:1 ratio (HUVECs:MSCs) without exogenous extracellular matrix proteins to form scaffold-free engineered vascular tissue. Tissue patches were formed in ultra-low attachment six-well plates (Corning) on a rotating orbital shaker at 40 rpm at 37 °C in 50/50 v/v EGM2 and a high-serum medium of KO-DMEM (Gibco) with 20% FBS, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, and 1% non-essential amino acids as previously described [11,16]. Disk-shaped engineered tissues (circular and about 400 μm thick) formed by hydrodynamic forces and cell adhesion within 2 days and were cultured for up to 8 days with medium changes every other day. Growth factors and small molecule inhibitors were added fresh with media changes and included: recombinant human vascular endothelial growth factor ($VEGF_{165}$, 10 or 100 ng/mL; Millipore) and/or N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethyletheyl ester (DAPT, a gamma-secretase inhibitor, 0.2, 2, or 20 μM; Tocris Bioscience). Dimethyl sulfoxide (DMSO, up to 0.1%) was used as a negative control in experiments requiring DMSO as a solvent for small molecule inhibitors.

2.3. Preparation of cellularized three-dimensional gels

For in vitro and in vivo gel experiments, 3D gels were prepared as previously described [17]. Briefly, gel components were placed on ice and combined to the following final concentrations: HEPES, 25 mM;