Revisiting the Principles of Cell Culture: A Macromolecular Crowding (MMC) Strategy to Fine-Tune Endothelial Colony-Forming Cell Phenotype for *in vitro* Tissue Engineered Heart Valve Applications

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Objectives: Endothelial colony-forming cells (ECFCs) in circulating blood represent an attractive cell source for cellularizing engineered valve scaffolds that may function as *living* valve replacements with durability and growth potential. In other cell types, media concentrated with inert macromolecules simulates *in vivo* microenvironmental conditions to enhance proliferation and collagen deposition. We previously demonstrated this in ECFCs, but it is unclear if MMC agents affect endothelial phenotype. We hypothesized that media containing these MMC agents, Dextran Sulfate 500kDa (DS) or Ficoll-400 (Fc400) would affect the endothelial characteristics of ECFCs *in vitro* during cell culture and seeding of valve scaffold materials.

Methods: N=3 ovine ECFC lines (CD31+, α-SMA+) were cultured in media containing no crowder, Fc400, or DS at four concentrations (n=576), then immunocytochemically cell scored for α-SMA, vWF, VEGFR-2, CD34, and vimentin. ECFCs were seeded onto decellularized porcine pulmonary valve scaffolds using plain, DS-, or Fc400-supplemented media and analyzed histologically.

Results: Fc400 decreased fibrotic marker α-SMA expression and increased proliferation and endothelization, representing a more valvular endothelial-like behavior. DS promoted a more valve interstitial-like behavior, with ECFC invasion into the scaffold, CD34 positivity, and enhanced collagen production. (Multiple linear regression (R^2=.38, $F(4,43)$=5.15, p=.002) revealed that Fc400 concentration (β=-.61, p=.03) and passage number (β=10.06, p=.001), but not cell density or DS concentration, predicted α-SMA positivity).

Conclusions: Results suggest that macromolecular crowding of the extracellular microenvironment fine-tunes ECFC proliferation, collagen production, and possibly endothelial-to-mesenchymal transition (EndMT). Future studies will explore translation to human ECFCs and analyze crowder effects on EndMT and scaffold pre-cellularization.