# Optimization of endothelial progenitor cells for pulmonary regeneration: Agarose cocooning for enhanced cell survival and targeted engraftment

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**Statement of Purpose:** Recent studies have highlighted the potential of autologous cell-based therapies to regenerate the damaged pulmonary vasculature[1]. Despite the success of these studies, current cell therapy strategies can be limited by poor retention of transplanted cells within the target tissue, likely linked to both the failure of the cells to engraft and anoikis (cellular apoptotis resulting from a lack of extracellular matrix contacts). To overcome these limitations we have developed a cocooning strategy to enclose single cells in an agarose gel enriched with provisional matrix proteins. We hypothesized that the presence of integrin binding partners, such as fibrinogen and fibronectin, in the agarose matrix will maintain cell survival and stimulate targeted engraftment upon cell migration from their cocoons.

Initial studies found that the presence of integrin-matrix contacts increases the survival of isolated cells in suspension. In vivo, agarose entrapment enhances the retention of therapeutic bone-marrow stromal cells (BMSCs) in the rat lung[2]. Subsequent mechanistic investigations have suggested that increased cell survival results from enhanced signaling through β1 and β3 integrins.

With these successes, our focus has shifted towards optimization of the isolation, expansion, and cocooning of human endothelial progenitor cells (EPCs), a potentially regenerative cell in models of pulmonary hypertension[3]. EPCs derived from blood or bone marrow are believed to promote neovascularization either by differentiating into new vessel endothelium or by exerting a paracrine effect to promote angiogenesis. In this study we describe methods to culture human EPCs from peripheral blood (leukapheresis samples), develop early and late outgrowth cultures, and cocoon these cells to enhance survival upon transplantation (potentially mediated by increased Integrin-linked kinase (ILK) activity).

**Methods:** All human research was performed under Research Ethics Board approval. Human peripheral blood-derived EPCs were obtained from leukapheresis samples. In brief, healthy volunteers underwent an MNC collection procedure on a Cobe Spectra (Gambro BCT, Denver, CO) to collect 100 ml of enriched white cells with a hematocrit of 3-8%. The samples, containing approximately 50 billion leukocytes were enriched in blood mononuclear cells (10-20% of total). Washed samples were cultured on fibronectin-coated plates in EGM-2MV medium containing 20% human serum (both Cambrex, Walkersville, MD). Cells were characterized by flow cytometry based on the presence or absence of particular cell-surface markers.

For cocooning, cells were entrapped in 2.5% ultra-low gelling point agarose by vortexing an aqueous cell-agarose suspension in an organic phase of dimethylpolysiloxane oil. The agarose droplets formed by vortexing were subsequently gelled at 0°C and filtered to remove droplets larger than 70 μm. In accordance with a Poisson statistical distribution, this entrapment method produces a non-uniform population of single, multiple cellular and empty capsules. A method of Ficoll (Amersham Biosciences, Piscataway, NJ) density gradient centrifugation was developed to isolate a single-cell encapsulated population.

Integrin-linked kinase (ILK) expression and activity was measured from EPC lysates using Western blotting and immunoprecipitation followed by a radiolabeled kinase activity assay.

**Results and Discussion:** Two distinct populations of peripheral blood-derived human EPCs were obtained from the cultured leukapheresis samples. The first population, termed early EPCs, appeared within a few days of culture and disappeared by 3 weeks. These cells characterized as positive for CD14 and KDR and negative for CD34. The second population, termed late-outgrowth EPCs (L-EPCs), appeared after 2 weeks in culture. Like endothelial cells, L-EPCs grow in a cobblestone pattern, express CD34 and KDR and are subject to contact inhibition. These cells are easily expanded in culture and display a more adherent phenotype, making them ideal for cocooning. However, their role as a therapeutic cell is yet to be determined.

Previous in vivo experiments required the sequential isolation of cocooned cells from empty agarose droplets using flow-activated cell sorting. Although very precise, producing a nearly homogeneous single cell cocooned population, this process was time consuming (>8 hrs per $1.5x10^6$ cells) and total cell recovery was low (25%). Ficoll centrifugation allowed for the recovery of 42%+/-5.1 of the entrapped cells, at a purity of 80% +/-3.6. This batch method allows for the efficient separation of several million cocooned cells in roughly 30 minutes. Such yields will be essential for the scale-up of this technique for use in either large animal models or clinical trials.

Analysis of ILK activity revealed that entrapment of L-EPCs in agarose supplemented with fibrinogen and fibronectin increased ILK activity relative to basal activity levels. This result suggests that ILK is a mediator of cocooned cell survival.

**Conclusions:** Single-cell agarose cocooning is a promising and novel approach to enhance the retention and survival of therapeutic cells in the pulmonary vasculature.

**References:**

1. Campbell *et al*. *Circulation.* (2001) **104(18):**2242-8
2. Karoubi *et al*. *Circulation.* (2004) Suppl., AHA Scientific Sessions
3. Zhao *et al*. *Circ Res.* (2005) **96(4):**442-50