

Calif.). mRNA expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Fold increase in expression levels for each endogenic and pericyte specific gene was normalized to the expression levels of control passage 2 ASC. Fold increase in expression levels for each gene was determined by 2- $\Delta\Delta$ CT method.

[0063] Impregnation of ASC Loaded Chitosan Microspheres in PEGylated Fibrin and Collagen Gels

[0064] Loading ASC into chitosan microspheres. Chitosan microspheres (CSM) were prepared by water in oil emulsification process along with an ionic coacervation technique using our previous described protocol. Prepared CSM were sterilized using absolute alcohol and washed ($\times 3$) with sterile water to remove residual salts. ASC were loaded into CSM at 10000 cells/mg using our culture insert technique as previously described. Before culturing ASC in CSM, the cells were cytoplasmically labeled with Quantum dot (Qdot) nanocrystals 565 using Qtracker cell labeling kit (Invitrogen, Carlsbad, Calif., USA). Cells were labeled according to manufacturer's instructions, briefly; 2 μ l of labeling solution containing 10 nM of Qdots was incubated for 5 minutes at 37° C. and to this solution 200 μ l of MesenPRO media was added and vortexed. To this solution mixture 1 ml of cell suspension (1×10^6 cells/ml) was added and incubated for 45 minutes at 37° C., 5% CO₂. Following incubation labeled cell suspension was diluted with MesenPRO media to a final concentration of 5×10^4 cells/200 μ l and seeded over sterilized CSM (5 mg), spread over culture insert of 8 μ m pore size membrane (24-well format, BD Falcon, Franklin Lakes, N.J.) and incubated for 24 hours in a humidified incubator at 37° C. and 5% CO₂.

[0065] Impregnation procedure. ASC (with and without Qdot label) loaded CSM (5 mg) were collected and mixed with PEGylated fibrin gel matrix (prepared as described earlier). The PEGylated fibrin-ASC-CSM mixture was added to a 12 well plate and incubated for 10 minutes at 37° C. In another experimental setup ASC (with and without Qdot label) loaded CSM (5 mg) were collected and impregnated into collagen type I gels following our previous procedure. Briefly, type 1 collagen (5 mg/ml, Travigen, Gaithersburg, Md.) from rat tail tendon was fibrillated by adjusting the pH to 6.8-7.0 using 100 μ l of Dulbecco's phosphate buffered saline (DPBS) and 23 μ l of 1N NaOH. The fibrillated collagen-ASC-CSM mixture was added to a 12 well plate and incubated for 30 minutes at 37° C. Following complete gelation both the gels (PEGylated fibrin and collagen gels) were incubated at 37° C., 5% CO₂. Release of cells was observed for 8 days in case of PEGylated fibrin gels, while in collagen gels cells were observed for 12 days and light microscopic pictures were taken at different days using Olympus IX71 inverted microscope equipped with reflected fluorescence system. To track cells and show their release into the gels fluorescence micrographs were taken on day 6 in both the gels.

[0066] Development of Bilayered PEGylated Fibrin—(ASC-CSM)—Collagen Gel Constructs

[0067] To develop the bilayer construct, PEGylated fibrin gel was prepared as previously described and added to a 6 well culture insert. Over the surface of the PEGylated fibrin gel 5 mg of ASC-loaded chitosan microspheres (10000 cells/mg) suspended in culture media (200 μ l) was seeded onto the gel. After the microsphere have settled over the gel, fibrillated type 1 collagen, prepared as previously described was carefully applied over the PEGylated Fibrin—(ASC-CSM) platform before gelation. After which the whole construct was

placed for 30 minutes at 37° C. to achieve complete gelation of collagen matrix. The final bilayered construct consisted of PEGylated Fibrin—(ASC-CSM)—Collagen gel matrix, with collagen gel on the top surface, PEGylated fibrin gel on the bottom and the ASC loaded CSM sandwiched in the interface. The entire bilayered construct was incubated at 37° C., 5% CO₂ for 12 days, during which cells released into the gels, were observed and photomicrographs were taken at different days to assess the morphology of the released cells into the gel matrix.

[0068] Results

[0069] Undifferentiated ASC

[0070] The phenotype of undifferentiated ASC has been described with respect to cell surface marker expression measured with FACS. These cells are positive for CD49d, CD54, CD71, CD90 and STRO-1. Prior to the utilization of a particular cell population the positive expression of these five markers was confirmed using immunocytochemical staining. An example result for the cell populations used in this study is shown in FIG. 13.

[0071] ASC Growth Characteristics within PEGylated Fibrin

[0072] Much like bone marrow-derived MSCs, ASC demonstrate the ability to proliferate and express a characteristic phenotype within PEGylated fibrin gels. FIG. 14 shows the dependency of cell seeding density and culture time on the resulting cell morphology. ASC began to exhibit cellular extensions by day 3. These extensions were more pronounced in cultures with $>10,000$ cells/ml. Over time the cellular extensions progressed with the formation of dense multicellular networks. By day 7, ASC at all seeding densities demonstrated extensive network formation which was greatest at the highest cell density. Proliferation was assessed over the same timecourse using the MTT assay. (FIG. 5) Proliferative activity increased over the seven day study for all seeding densities and was dependent on the initial density.

[0073] ASC Phenotype and Genotype within PEGylated Fibrin

[0074] The endothelial cell markers, CD31 and vWF were used to establish the identity of cells expressing an endothelial cell genotype and phenotype. RT-PCR demonstrated that over the 11 day timecourse, there was a dramatic upregulation of endothelial cell markers relative to the housekeeping gene. Specifically, CD31 was upregulated 25 fold over controls while vWF was up 42 fold over controls. (FIG. 15) The immunohistochemical staining confirmed the presence of the expressed protein for both CD31 and vWF. (FIG. 7A-7D) Confocal images of stained sections confirm that both markers are expressed on multicellular networks generated from day 11 samples. What was demonstrated is that the CD31 is more closely associated with the cell nucleus than the vWF. This may be due to the fact that CD31 is expressed on endothelial cell membranes, where vWF may be secreted from the cell and maintained within the fibrin network. The pericyte markers, NG2, PDGFR β and α -smooth muscle actin were used to track the differentiation of ASC towards a pericyte, or mural cell, phenotype. RT-PCR after 11 days in culture demonstrated that the markers, NG2 and PDGFR β , were upregulated by 6 fold and 9 fold, respectively, relative to controls. It is important to note here that undifferentiated ASC also express a basal level of PDGFR β which was approximately 5 fold greater than controls. This value declined as the culture progressed reaching a minimum at day 7 prior to an increase at day 11. Immunohistochemistry revealed that multicellular