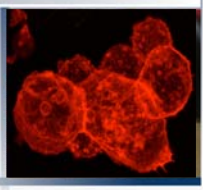
ILLINOIS
UNIVERSITY OF ILLINOIS AT URBANA-CHAMPAIGN
1867

Laboratory of Comparative Genomics

A Three Dimensional Culture System for Directed Differentiation of Porcine Mesenchymal Stem Cells



L.R. Trump[1], U. Mirsaidov[2], G. Timp[2], and L.B. Schook[1]

[1]Institute of Genomic Biology, University of Illinois

[2]Beckman Institute, University of Illinois, Urbana, IL.

http://www.swinegenomics.com

Abstract

Mesenchymal stem cells (MSCs) have been targeted for use in cell-based therapies, regenerative medicine, and tissue engineering. For MSCs to be utilized in these applications, further elucidation of environmental and biochemical stimuli directing differentiation is needed. A limitation to studying directed MSC differentiation is that traditional culture systems represent a heterogeneous population. The objective of this study is to understand specific environmental cues and signals directing differentiation. To achieve this goal, we created a three-dimensional poly (ethylene glycol) diacrylate (PEGDA) based microenvironment that supports cell and signal placement controlled by laser tweezers. PEGDA environments allow transport of small molecules, demonstrate biocompatibility, and are easily modified. Laser tweezers manipulate cells and place them in any desired location. Coupled with photopolymerizable hydrogels, they provide control of the cellular environment by specific placement of cells and signals. U937 cells were used to develop hydrogel microenvironments since they are easily differentiated into macrophages by small molecules (phorbol 12-myrisate 13-acetate; PMA). Visual monitoring systems utilizing fluorescent reporter vectors were created to analyze cellular activity by transfecting a GFP reporter gene controlled by a CMV promoter. Differentiation was monitored by transfecting a DsRed gene driven by PMA induced TNFα and osteopontin promoters. RT-PCR analysis shows that 50 nM PMA stimulation results in upregulation of TNFα and osteopontin expression within 2 and 4 hours, rapidly detecting differentiation. Optimal microenvironments have adequate PEGDA pore size, molecule diffusion, and minimal cell death from UV exposure, photoinitiator, or laser manipulation. Microenvironments were optimized by placing U937 cells in 5%-10% of 400 mW or 3.4 kDa hydrogel with 0.1%-0.2% photoinitiator and polymerized with UV light for 10-15 seconds. Fluorescence microscopy analysis of GFP expression and Molecular Probes Live/Dead kit indicate that cellular metabolic activity was optimized with 5% 3.4 kDa PEGDA, 0.2% photoinitiator and 15 seconds UV exposure. Lower PEGDA concentrations (5% 3.4 kDa) displayed higher metabolic activity (45%) compared to 8-10% PEGDA concentrations (30%) 48 hours after trapping in hydrogel. These data demonstrate that U937 cells remain metabolically active in hydrogel for sufficient time to monitor differentiation. After development of optimal microenvironments, cells were manipulated with laser tweezers into 3x3 arrays and monitored for cellular activity. One hour after trapping, all cells remained metabolically active. After trapping, cells remained 55% metabolically active after 48 hours, reflecting similar metabolic activity to non-arrayed cells in hydrogel (45%) and reflecting that laser manipulation does not affect cellular activity. Lastly, differentiation in hydrogel was demonstrated by adding 50 nM PMA to the culture medium and monitored by visualization of DsRed fluorescence. After complete optimization of microenvironments using U937 cells, MSCs will be encapsulated in hydrogel and cultured with lineage-specific differentiation signals. In conclusion, we have created a cellular microenvironment system where the use of laser trapping in conjunction with a PEGDA microenvironment provides a platform for studying directed differentiation of MSCs.

Introduction

Multipotent adult mesenchymal stem cells (MSCs) have a potential of treating several diseases via cell based therapies. A rate limiting step to obtaining full clinical potential of MSCs is obtaining a homogeneous population of differentiated stem cells to inject in the patient. Stem cell differentiation has been well established in traditional culture, however cell signals in this environment are heterogeneous. Signals from surrounding cells, other cell types, and other unknown factors released into the culture medium can affect differentiation. To study cellular differentiation on a single-cell basis, we have created a three dimensional cell culture system where cells and signals can be placed in desired locations with sub micron precision via laser manipulation[1]. Laser trapping and hydrogel environments for cell culture and differentiation are being optimized with the human lymphomic cell line U937. We chose U937 cells because they can endure severe culture conditions, and are easily and visibly differentiated into the macrophage lineage[2]. Hydrogel microenvironments are composed of poly (ethylene glycol) diacrylate (PEDGDA). Signals to induce differentiation can be presented to the cells in several manners: bathing the hydrogel in media containing differentiation signals, placing signals in microcapsules that can be manipulated to release signals at desired time points, and placing cells secreting signals into the environment. Laser and culture systems have been optimized using U937 cells, and we are now further investigating U937 cell signaling and modifying parameters for porcine MSCs. At the conclusion of this study we will establish methodologies for trapping and differentiating cells in a 3D scaffold for investigating cellular differentiation.

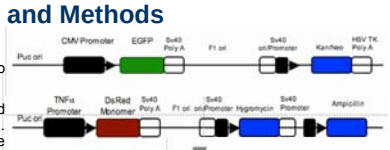
Objectives

- To establish a three dimensional hydrogel culture system capable of supporting metabolic activity and differentiation of U937 and MSC cells.
- To laser assemble U937 and MSCs into desired position in hydrogel without loss of viability or differentiation capabilities.
- To effectively differentiate laser captured cells in hydrogel microenvironments.

Materials and Methods

Vector Construction and Cell Transfection

- The pEGFP-N1 plasmid was transfected into cells as received
- TNFα and OPN-DsRed constructs were created by excising the CMV promoter from the plasmid. TNFα and OPN promoter sequences were amplified by PCR with primers containing restriction sites for proper ligation into the plasmid. Plasmids were transfected into U937 cells with the Amaxa nucleofector kit V.



Laser Trapping

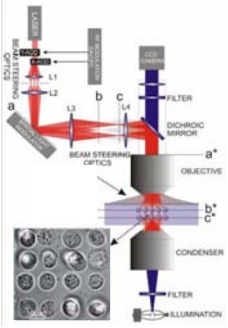
- U937 and MSCs were trapped in a 3x3 array using a Zeiss Neo-Fluor 100X, 1.25NA objective at λ=900nm with a time-averaged power per trap at the sample of 8mW/trap.

Hydrogel Formation

Cells were trapped in 5-10% 400mW, 3.4kDa or 20 kDa poly (ethylene glycol) diacrylate solution with 0.1-0.4% 2-hydroxy 2-methylpropiophenone as a photoinitiator. Hydrogels were crosslinked by exposing solution to a focused UV beam at 6mw/cm2 for 5-20 seconds.

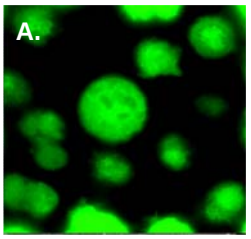
U937 Differentiation

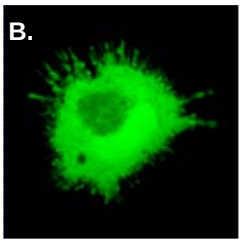
Cells were washed in PBS and resuspended in RPMI 1640 supplemented with 100 nM PMA at a concentration of 1x10^6 cells/ml.

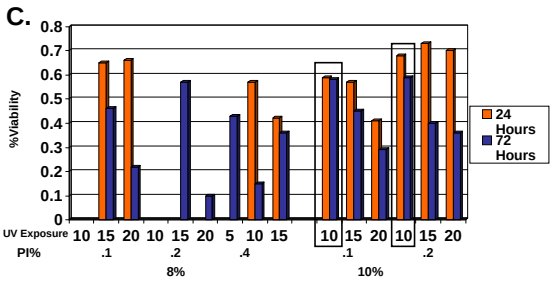


Results

U937 cells readily undergo differentiation into macrophages. U937 cells were induced to differentiate with the addition of 100 nM PMA in the culture medium for 48 hours. Prior to differentiation, U937 cells have rounded morphology and are non-adherent **(Panel A).** Fully differentiated cells are larger in size and adherent to the culture dish **(Panel B).**



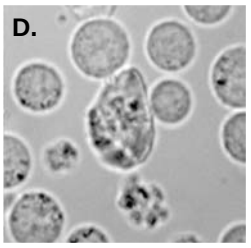


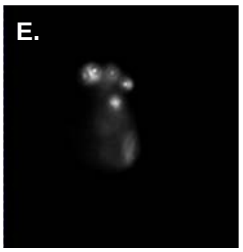


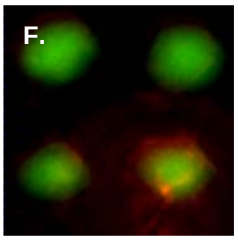
Viability Assays U937 Cellular viability in hydrogel was measured using the Molecular Probes live/dead kit. Cells were placed in 3.4 or 20 kDa hydrogel. Results displayed are from 20 kDa experiments. Optimal viability was found in 10% hydrogel with 0.1-0.2% PI and 10 seconds UV exposure. Viability assays in 3.4 kDa displayed similar results, whereas cells in 400 mW hydrogels displayed poor viability (~10%).

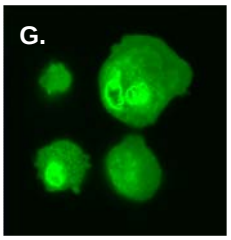
Fluorescent Reporter Activity

TNFα-DsRed Transfected U937 Cells were induced to differentiate with 100 nM PMA. Fluorescent microscopy was performed 4 hours after the addition of PMA. **Panel D:** Transmitted Image of a Differentiating U937 Cell **Panel E:** Fluorescent image of the cell in **D.** Red fluorescence indicates that TNFα is being produced in response to differentiation stimuli.









U937 and porcine MSCS can be successfully manipulated by laser tweezers into a hydrogel array. Panel F: GFP-U937 cells were trapped with laser tweezers into a 2x2 array in 5% 3.4 kDa hydrogel and imaged 24 hours after trapping. Cells are stained with the molecular probes live/dead kit. Cell metabolic activity is similar to non-arrayed cells, indicating the laser has little effect on viability. **Panel G:** Porcine MSCs were trapped in a 2x2 array in 5% 3.4 kDa hydrogel. Cells were stained with Syto-9 and imaged 2 hours after trapping.

Ongoing Research

Hydrogel Modifications Poly (ethylene glycol) hydrogels presents a 'blank' cellular environment. Nuttleman et al. Has shown that human MSCs in PEDGA environments have very low cellular viability, most likely due to the lack of adhesion molecules in hydrogel[3]. We plan to modify hydrogels to provide cells with signals such as RGD peptides to increase MSC viability.

U937 and MSC Differentiation in Hydrogel We plan to study cell differentiation by administering differentiation signals via microspheres or nearby cells releasing signals in hydrogel. Differentiation reagents in microspheres are released by heating the microspheres allowing for specific control over differentiation signals. Cell signaling and quorum sensing in hydrogel have been demonstrated with *E. coli* bacterium, and we are beginning signaling experiments with U937 cells.

Conclusions

- U937 cells can successfully differentiate into the macrophage lineage upon the addition of PMA.
- U937 cells can be laser manipulated into desired positions in hydrogel.
- Reporter systems easily monitor differentiation in real time in hydrogel.
- Cells in 3.4 kDa and 20 kDa hydrogels show similar viability.
- Methodologies for placement of MSCs into a 3D scaffold are being established.

References

[1]Akselrod, G., W. Timp, U. Mirsaidov, Q. Zhao, C. Li, R. Timp, K. Timp, P. Matsudaira, and G. Timp, "Laser-Guided Assembly of 3D Heterotypic Living Cell Microarrays," *Biophys. J.* **9**: 3456-73.;

[2] Larrick, J.W., D.G. Fischer, S.J. Anderson, and H.S. Koren, "Characterization of a Human Macrophage Like Cell Line Stimulatied In Vitro: A Model of Macrophage Functions" *J Immunol.*, 125: 6-12 (1990).

[3] Nuttleman, C.R.,Tripodi, M.C., Anseth, K.S. "Synthetic Hydrogel Niches that promote hMSC viability" *Matrix Biology*. **24**: 208-18 (2005).

Acknowledgements

LT was supported by Initiative for Future Agriculture and Food Systems Grant no. 2001-52100-11527 from the USDA Cooperative State Research, Education, and Extension Service.