Title
Gene Expression Profiling of Human Mesenchymal Stem Cells for Tissue Engineering

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Publication Date
2014

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Gene Expression Profiling of

Human Mesenchymal Stem Cells for Tissue Engineering

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Biomedical Engineering

by

Thanmayi Ranganath

2014

ABSTRACT OF THE THESIS

Gene Expression Profiling of

Human Mesenchymal Stem Cells for Tissue Engineering

by

Thanmayi Ranganath

Master of Science in Biomedical Engineering

University of California, Los Angeles, 2014

Professor Andrea M. Kasko, Chair

Tissue engineering is a combination of advanced technologies with a goal to fabricate better materials for the repair or replacement of damaged tissue. This is accomplished with the help of cells, biomaterials, and other physiological and chemical cues. In tissue engineering, human mesenchymal stem cells (hMSCs) have huge potential as they can differentiate into many cell types, reduce immune response, be isolated from adult bone marrow, and are easily available. The present study analyzes the gene expression profile of hMSCs when

cultured under different conditions: two-dimensional (2D) versus three-dimensional (3D) culture systems; presence or absence of radicals, and exposure to ultra violet (UV) radiation. In our experiments, cells were cultured on 2D substrates and in 3D hydrogel systems fabricated with and without free-radicals. Total RNA was extracted from cells cultured under each condition and the differential expression of genes was analyzed using the Human U133 plus 2.0 Affymetrix gene chip. We observed that the largest difference in gene expression occurs in cells cultured in 3D as compared to 2D systems. UV radiation did not have a significant effect on gene expression but, when combined with the free-radicals generated during fabrication, significant variations in the hMSC gene expression profile were observed. Therefore, can conclude that, while all three factors (2D vs. 3D, free-radicals, and UV light) can influence the gene expression profile of hMSCs, switching from 2D to 3D cell culture system results in the largest change in gene expression.

The thesis of Thanmayi Ranganath is approved.

Min Lee

Tatiana Segura

Andrea M. Kasko, Committee Chair

University of California, Los Angeles

2014

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ACKNOWLEDGEMENTS:

I am deeply thankful to Dr. Andrea M. Kasko, not only for her guidance, but also for the inspiration and encouragement during my thesis work. Dr. Kasko mentored me and taught me how to plan my research step-by-step. She was my constant source of encouragement and she has definitely helped me become a better researcher. My special thanks to Dr. Darice Wong, who was a constant source of support and advice to me throughout my two years in the lab. Her optimism kept me working hard on my experiments. I am also grateful for my committee members, Dr. Min Lee and Dr. Tatiana Segura for their time and guidance with my thesis.

I would like to thank all my lab mates, especially Dr. Ken Lin and Jason Kerr, for their unwavering support and understanding during my failures, and their enthusiastic celebrations for my successful experiments.

Lastly I am indebted to my family, especially my parents Dr. Ranganath and Pramila, for their unwavering support and patience. They have instilled in me a passion to learn and have always stood by me through the highs and lows.

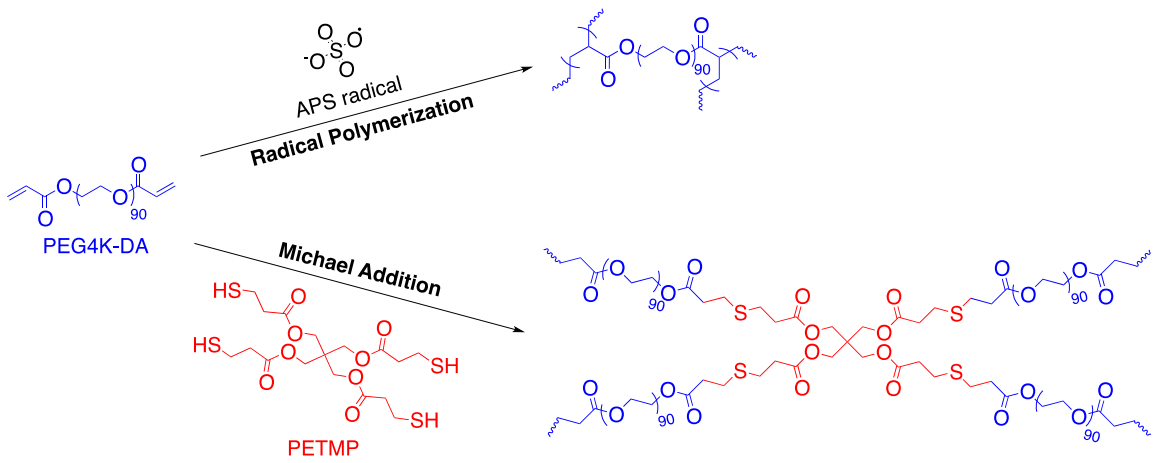
It has been a great pleasure and honor working in Dr. Kasko's lab in UCLA. I am glad I had this opportunity and I thank everybody for believing in me.

1 INTRODUCTION

Tissue engineering is an emerging field that aims to engineer complex tissues or organs *in vitro*.[1] A key milestone required for success is engineering the stem cell niche which is required for the differentiation of stem cells into a desired lineage. Optimization parameters include biomaterials, cell source, and chemical cues.[1] Cells are commonly cultured *in vitro* on 2D substrates such as tissue culture polystyrene (TCPS) that gives a very basic interpretation about growth conditions and differentiation potential.[2] However, it has been shown that cells behave differently when they are removed from their 3D tissue and cultured in a monolayer. For example, Bissel et al., demonstrated that human breast epithelial cells develop tumor like characteristics when excised from their native 3D tissue.[3] Additionally 2D substrates are inherently unnatural for most cell types, as the cells adhere abnormally and grow in a monolayer. This monolayer culture assures that cells are uniformly exposed to the culture medium and to any other external factors, but this is not representative of *in vivo* conditions. Furthermore, it is extremely difficult to recreate higher-order cellular processes like morphogenesis, tissue remodeling, and cancer cell migration in a 2D system, as these processes occur when cells alter their microenvironment, and cells are unable to alter the TCPS 2D culture system.[4] Therefore, researchers designed various 3D culture systems to better recreate native tissue conditions in order to bridge the gap between native tissue structure and 2D *in vitro* culture conditions. Our interest lies in understanding how 2D versus 3D culture systems affects gene expression in hMSCs, and how fabrication of 3D culture systems impacts hMSCs.

In tissue engineering, a common 3D cell culture system is a hydrogel, which is a water swollen cross-linked polymer network.[5] Hydrogels mimic the body's physiological

conditions since they have similar water content, tunable elasticity, and are biocompatible.[6] Fabrication of hydrogels for tissue engineering applications can be accomplished via physical or chemical cross-linking. Chemical cross-linking is commonly accomplished by free-radical polymerizations or by conjugate addition of macromers. Radical polymerization utilizes radical initiators [e.g. ammonium persulfate/N,N,N',N'-tetramethylethylenediamine (APS/TEMED), Irgacure 2959][7,8] that attack the monomer, resulting in the chain growth of a polymer. However, free-radicals generated can place oxidative stress on the cell systems. On the other hand, there is no radical generation during fabrication of hydrogels using conjugate addition for cross-linking.[7] Conjugate addition allows reaction with a large number of functional groups (such as acrylate or maleimide)[9], however, the reaction rate could be slow compared to radical polymerization. Additionally, many cross-linkers are hydrophobic and require non-biocompatible organic solvents. The mechanisms for cross-linking by both free-radical polymerization and conjugate addition are depicted in Scheme 1. We aimed to investigate two different 3D systems, formed with and without radicals, in order to determine the extent of damage that free-radical encapsulations may cause.



Scheme 1: Radical and Non-Radical Conjugation: Hydrogels can be fabricated using PEG4K-DA as the monomer unit. Cross-linking of PEG4K-DA can occur by free-radical initiator or by cross-linkers such as pentaerythritol tetrakis(3-mercaptopropionate) (PETMP) (non-radical fabrication)

Photoinitiation is an attractive tool for hydrogel fabric ation since light allows both spatial and temporal control. Although many wavelengths have been reported, the most common conditions utilized are low intensity (5-20 mW/cm^2), long wavelength UV light (365 nm).[10–12] Photoencapsulation typically utilizes a photoinitiator that cleaves to produce free-radicals which react with unsaturated bonds in the macromers. In addition to cell encapsulation, UV radiation is also utilized in various other tissue engineering techniques like photouncaging[13], photopatterning[11], and photodegradation[10,12]. Drugs and other small molecules can be entrapped or covalently attached in the hydrogel and can be subsequently released around cells and tissues.[10,14,15] In these cases UV light may or may not generate free radicals. Therefore, it is imperative to determine the effect of UV light alone on cells.

Previous experiments conducted have reported the cytocompatibility of long wavelength UV in the presence of photoinitiators[11,16] and after photodegradation[10]. This was characterized by viability assays like MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium

Bromide] and Live/Dead.[10,11,16] Although viability is one measure of compatibility, undesired changes can occur in cells that are viable. Viability studies do not provide sufficient information on the possible changes occurring in addition to cell death. Specifically, no study exists that systematically explores the effect of commonly used long wavelength UV radiation on cells.

hMSCs are of tremendous research interest due to their extensive capacity for self renewal and a broad potential for differentiating into diverse cell/ tissue types.[15,17] In our experiments, we investigate the effects of long wavelength UV on the gene expression of cells. It is necessary to know if there are any gene expression changes after exposure to UV light and, other than cytotoxicity, what other functional pathways change as a result of changes in gene expression. We cultured cells in both 2D and 3D environments, including two variations in the 3D culture systems fabricated with and without radicals, to isolate gene expression changes due to exposure to free radicals. This will help us understand the effect of radicals alone and in combination with UV exposure on the stem cell gene expression, as well as the effect of UV light alone. We used the human U133 plus 2.0 Affymetrix gene chip for gene expression profiling of hMSCs cultured and exposed to different experimental conditions.

2 BACKGROUND

2.1 Cell Culture Conditions

Cells can be cultured in either 2D or 3D systems; until recently, most experiments were conducted with 2D cell culture systems, with cells being cultured on glass/plastic surfaces.[4] Therefore, most of the field's understanding of various cellular activities (differentiation, migration, and adhesion) is based on experiments conducted in 2D cell culture system.[4] 2D cell culture systems are popular because they are simple, accessible, easy to design, set up, and maintain; however, *in vivo* cells are surrounded by a complex environment where there are dynamic interactions between various cell types and extracellular matrix components.[4] Such complex interactions cannot be recapitulated in 2D cell culture systems. 2D experiments might not be successful in capturing the physiological behavior of cells *in vivo*.[4] Figure 1 illustrates some of the cues such mechanical, soluble, and topographical cues which cells are exposed to *in vivo* but cannot be recapitulated *in vitro* in the 2D systems.[18] Therefore, despite 2D system's convenience to observe morphology and cell characteristics under a microscope, it is no longer considered an accurate representation of the cell in *in vivo* environment and cannot be used to predict the complex behavior of biological systems.

Previous research has shown that the same cell types behave differently when cultured in 2D versus in 3D systems. For example, consecutive 2D culturing of rabbit chondrocytes leads to a loss in phenotype and changes in the collagen and proteoglycan production.[19] In contrast, when the chondrocytes were encapsulated in 3D agarose gels, they re-expressed the differentiated phenotype. In addition to differences in protein production, cells typically adopt different morphologies in 2D systems as compared to 3D systems.[20] For example,

Figure 3 depicts the remarkable differences in cell morphology, fibronectin matrix organization (blue), and architecture of α_5 integrin-positive adhesion structures (white) of human fibroblasts cultured in 2D and 3D systems.[21]

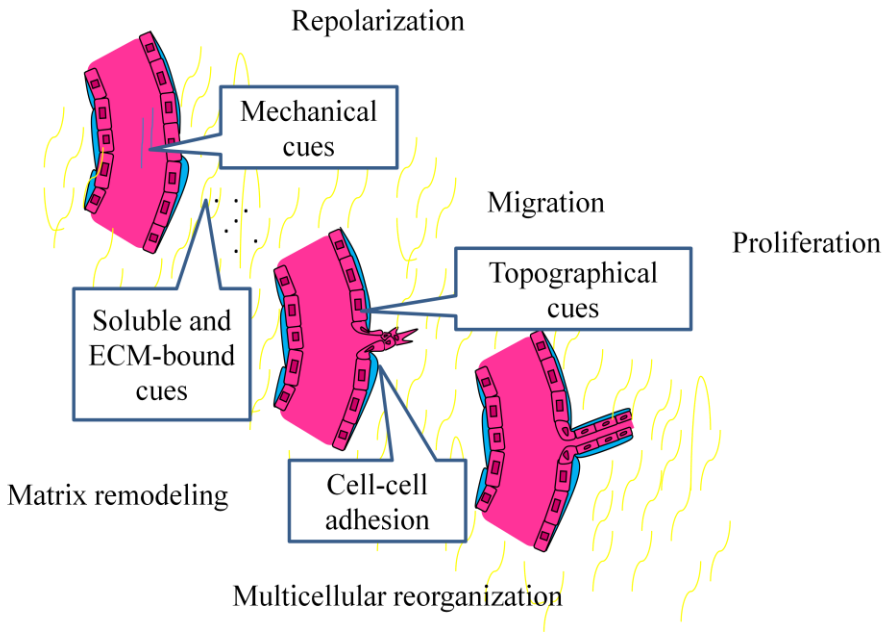


Figure 1: Cellular Phenomena Controlled by Different Cues (adapted from4) : Epithelial cells in vivo experience mechanical cues from the flow of blood through vessels, as well as soluble and ECM-bound cues from the surrounding tissues. These cells have specific polarization and topographical cues from naturally existing in monolayer lining the lumen of a vessel, but other cell types have different cues from their natural state.

Researchers are relying more extensively on 3D culture systems to validate the previous results from 2D systems. However, 3D systems also vary in their ability to mimic the native tissue. Cells encapsulated in 3D systems are not exposed to uniform conditions since they experience different stress levels, growth factor, and medium concentrations, due to diffusion-mediated gradients inherent in 3D systems. These might be difficult to quantify. Figure 2 gives a pictorial representation of the striking differences between a collagen coated 2D substrate and a collagen hydrogel which forms the 3D culture system.[4]

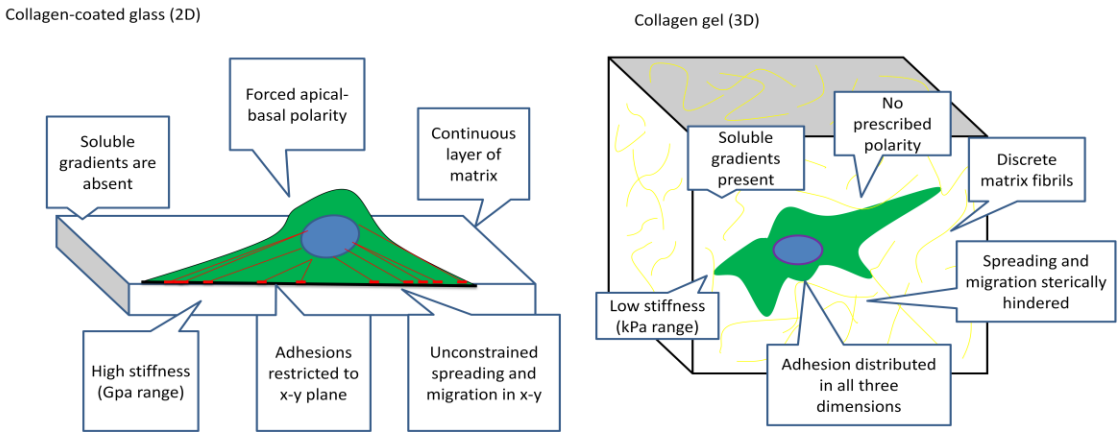


Figure 2: Adhesive, Topographical, Mechanical, and Soluble Cues in 2D and 3D are very Different (adapted from4)

Since both 2D and 3D systems are still widely used in research literature, it is necessary to know how each culture condition affects cells at the genetic level. This will be accomplished by quantifying the differences in gene expression levels based on the cell culture system.

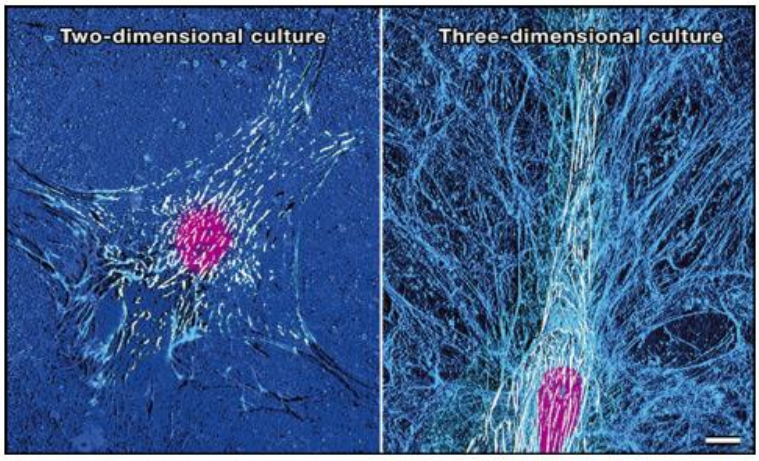


Figure 3: Human Fibroblasts In 2D versus 3D Cell Culture (Reproduced from reference[21] with permission from Elsevier): Fibroblasts cultured in the agarose gels produce more adhesion structures than when they are cultured in 2D systems.

2.2 Culture System Description

Researchers over the past few years have developed various substrates for 2D and 3D cell culture systems. 2D cell culture systems use glass, plastic, or patterned hydrogel surfaces as

common substrates for cell growth.[22] For 3D systems, polymeric scaffolds such as hydrogels and other porous scaffolds are utilized due to their ability to mimic physiological conditions.[6]

Hydrogels are an important class of biomaterials in tissue engineering due their easily tunable properties (modulus, mesh size, and volumetric swelling ratio) all of which are heavily influenced by the cross-link density.[8,23] A common method to fabricate hydrogels is to covalently bond monomers to form a cross-linked network.[5] Covalent cross-links can be generated by free-radicals or by utilizing multifunctional cross-linkers. For radical polymerization, possible ways to generate radicals are via redox systems (e.g. APS/TEMED)[10] and photoinitiators (Irgacure 2959, using UV radiation at 365 nm, 10 mW/cm^2 for 10-20 minutes)[11]. However, free radicals in solution have been known to cause oxidative stress and mediate cellular damage.[24] Therefore, we are interested in determining the changes in gene expression in cells exposed to these common encapsulation conditions (UV light and radicals).

UV radiation is also used for various other tissue engineering techniques since light gives us both spatial and temporal control in both 2D and 3D systems.[25] It has been shown that hMSCs can be encapsulated in hydrogels polymerized with the use of photoinitiator Irgacure 2959 without loss in cell viability (I_o=4 mW/cm^2, t=10 min, λ=365 nm).[26] Bryant et al., photoencapsulated chondrocytes using Irgacure 2959 as the photoinitiator (I_o=8 mW/ cm^2, t=10 min, λ=365 nm).[16] Almost all the chondrocytes survived the encapsulation process.[16] Photodegradation of hydrogels by exposure to 365 nm UV at 10 mW/ cm^2 for 8 minutes to release encapsulated hMSCs has been reported by Kloxin et al. and Griffin et al., while maintaining good cell viability of released cells.[10,12] In these previous studies, the most

commonly considered cellular response to UV radiation is cytotoxicity, usually assayed Live/Dead or MTT for mammalian cells.[10,12,16,27] However, across these studies, there is no information on the effects of UV radiation on the gene expression of cells.

Previous studies have demonstrated that the entire spectrum of UV radiation damages the biological system by heating it, altering chemical bonds in molecules, and forming reactive oxygen species.[28] Stress responses activated due to DNA damage by UV radiation could lead to growth rate changes, signal transduction changes, inflammation, gene repair, mutagenesis, recombination, and induction of variety of genes associated with growth control.[29] Ultimately, effects on gene expression may change with the intensity and the duration of UV exposure on hMSCs. In our experiments, we chose to expose hMSCs at 365 nm, 4 mW/cm^2 for 25 minutes. This intensity is comparable to the total flux commonly used for cytocompatible exposure in literature for various experiments like cell encapsulation, photodegradation, and photouncaging.[10,12,16,27]

Monomers and cross-linking molecules for hydrogel fabrication can be chosen from a wide range of biocompatible materials. One of the attractive precursors for polymerization is poly(ethylene glycol) (PEG). It is a hydrophilic molecule which forms hydrogels with easily tunable properties having soft tissue-like characteristics.[30] PEG hydrogels are non-toxic, non-immunogenic, and are resistant to protein adsorption and cell adhesion.[31,32] They can be used to culture cells such as chondrocytes which do not need any extra signals for growth.[31,32] If needed, PEG macromers can be functionalized with various cell adhesion peptides like RGD which help promote cell adhesion.[33,34] Drugs such as dexamethasone can be covalently tethered to a functional group on PEG and, can be polymerized in the presence of a photoinitiator resulting in a controlled drug release system.[35] Due to these various advantages

PEG hydrogels are commonly reported in tissue engineering applications. We use acrylated PEG as the monomer for our hydrogel systems and form cross-linked networks via radical initiation as well as non-radical Michael addition. We did not investigate a degradable 3D system since we were only interested in the effect of light and 2D versus 3D, not the additional effect of a dynamically changing system.

To achieve an understanding of the effects of UV light on gene expression of hMSCs, we expose cells cultured in 2D and 3D culture systems (fabricated with and without radicals). This will facilitate our understanding of the effect of radicals, effect of UV radiation, and the combined effect of radicals and UV radiation on the gene expression of hMSCs.

2.3 Human Mesenchymal Stem Cells (hMSCs)

2.3.1 Background Information

MSCs are non-hematopoietic stromal cells which contribute to the regeneration of variou[36–38] s mesenchymal tissues such as ligament, bone, tendon, cartilage, muscle, and adipose. hMSCs are not immortal but are capable of expanding up to many passages in culture while retaining their multipotency.[36,37] hMSCs are primarily isolated from the adult bone marrow and are easily available.[39,40] hMSCs have a varied morphology when cultured in 2D and 3D systems. For example, hMSCs are long and thin when cultured on a 2D surface and have a rounded morphology when encapsulated in PEG hydrogels with a tight mesh [Figure 4].[41] hMSCs can be stimulated to differentiate *in vitro* into various cell types and are relatively easy to expand in culture; making this cell line an ideal use in tissue engineering.[42]

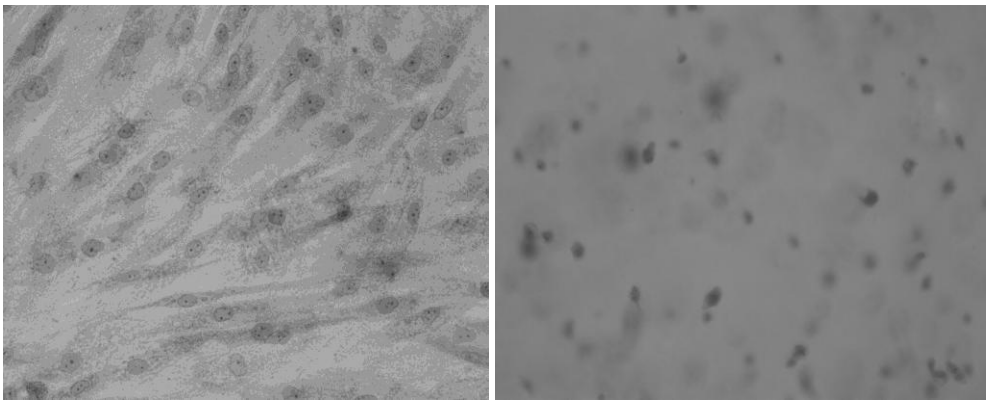


Figure 4: Morphology of hMSCs Cultured in 2D and 3D Culture Systems: hMSCs cultured on tissue culture polystyrene (TCPS) have a long and thin morphology. However, when encapsulated in PEG hydrogels, they show a rounded morphology due to the tight mesh size.

Another significant property of hMSCs is that, when transplanted *in vivo*, they are capable of migrating toward the site of injury.[42] hMSCs are also reservoirs for the production of

cytokines, chemokines, and the extracellular matrix that all support cell survival and proliferation.[43]

2.3.2 Reasons For Use

The ability to control differentiation is a reason for the tremendous research on hMSCs for tissue engineering. hMSCs differentiate into specific cell types on exposure to different growth factors, chemicals, and changes in the culture microenvironment [Figure 5].[44] For example, hMSCs can be stimulated to undergo chondrogenesis with the use of transforming growth factor-β3 (TGF-β3) and by varying the hydrostatic pressure.[45] Growth/differentiation factors 5, 6, and 7 from the TGF-β family have been implicated in tendon formation from MSCs.[46] hMSCs can be induced to enter the osteogenic lineage *in vitro* with the use of synthetic glucocorticoid dexamethasone.[47,48] Various biomaterials have been designed over the past few years for the encapsulation and delivery of hMSCs for orthopedic therapeutic applications and cartilage regeneration.[49,50] Therefore, an understanding of the variations in the gene expression of hMSCs due to culturing conditions becomes imperative. We use hMSCs as the model system to investigate the influence of 2D versus 3D cell culture, radicals in the cell culture system, and exposure to long wavelength UV radiation.

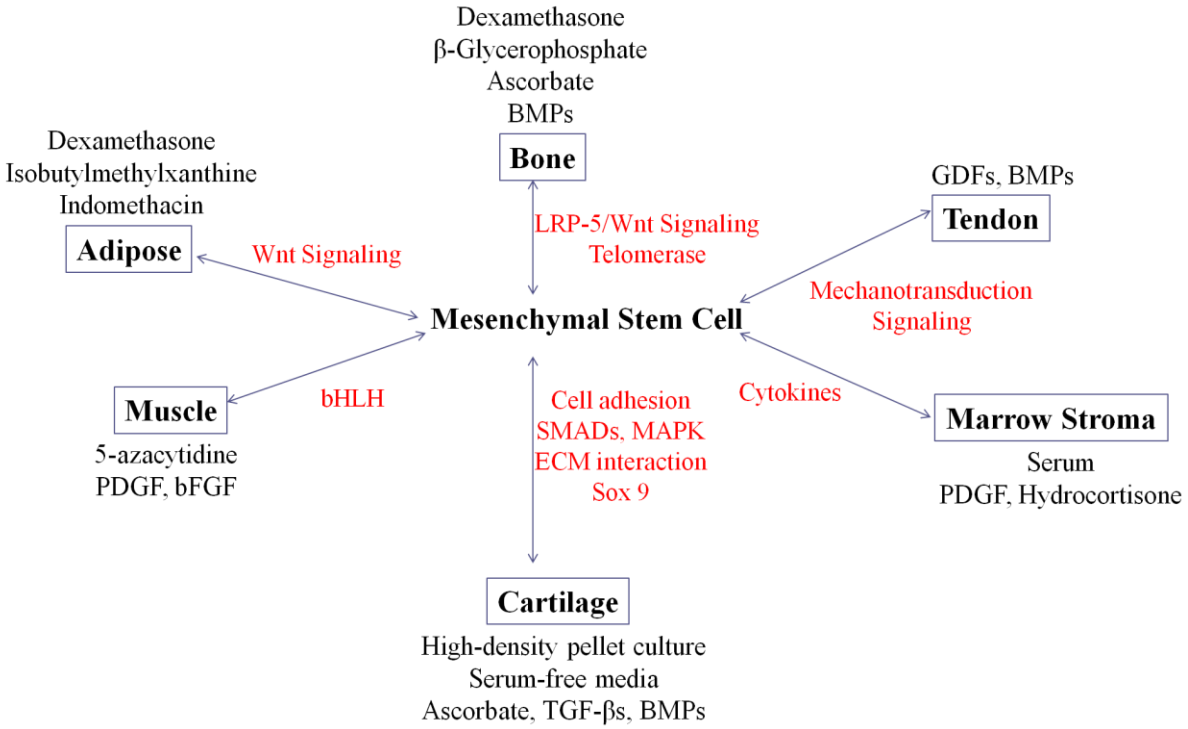


Figure 5: Lineage Potential of Adult hMSCs (adapted from[44]): In vitro hMSCs can be differentiated using various growth factors and signaling conditions into multiple lineages.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemical Materials

Acryloyl chloride (Alfa Aesar, 96%), ammonium persulfate (APS) (Amresco), chloroform (Alfa Aesar, 99%), dichloromethane (DCM) (Acros Organics, 99%), ethanol (Decon Laboratories), ethyl ether anhydrous (Fisher Scientific), poly(ethylene glycol) (PEG4K; M_n = 4000) (Mallinckrodt), triethylamine (TEA) (Alfa Aesar, 99+%), tetrahydrofuran (THF) (DriSolv), N,N,N',N'-tetramethylethylenediamine (TEMED) (Calbiochem), dimethyl sulfoxide (DMSO) (Fisher Scientific).

DCM and TEA were distilled from CaH_2 under N_2 and stored under N_2 in a dry, air-free flask. All other chemicals were used as received.

3.1.2 Biological Materials

Fetal bovine serum (Atlanta Biologicals), L-glutamine (Hyclone), MEM Richter's Modification Medium (Hyclone), penicillin streptomycin (MP Biologicals), phosphate buffered saline (PBS) (Corning Cellgro), pure link™ DNase (Invitrogen), pure link™ RNA mini kit (Life Technologies) trypsin (Hyclone, 0.05%), TRIzol® (Life Technologies).

3.2 Methods

3.2.1 Acrylation of PEG4K

Polyethylene glycol (10 g, 2.5 mmol) (M_n 4000) was dissolved in 250 mL THF and triethylamine (1 mL, 7.5 mmol) in a round bottom flask and cooled in an ice bath prior to adding acryloyl chloride (470 µL, 5.83 mmol) in 10 mL THF drop-wise to the round bottom

flask. The solution was stirred under nitrogen and allowed to warm at room temperature overnight. Reaction was monitored by ^{1}H NMR. Triethylamine salts were removed by filtration followed by removal of THF by rotary evaporation. The product poly(ethylene glycol)-4000 diacrylate (PEG4K-DA) was collected by filtration and dried under vacuum overnight. Characterized of the product by ^{1}H NMR which showed complete acrylation of PEG and no TEA salts. ^{1}H NMR spectra (δ ppm) were recorded on a Bruker Biospin Ultrashield 300 MHz NMR Spectrophotometer.

Yield: 58.6%

^{1}H NMR ($CDCl_3$): δ=6.43 (d, C**H$_2$**CH$_1$C(O)), δ=5.71 (d, C**H$_2$**CH$_1$C(O)), δ6.18 (d, CH$_2$C**H$_1$**C(O)), δ=3.63 (H-4), δ=4.39 (d, CH$_2$C**H$_1$**C(O)), δ=3.63 (m, PEG's -C**H$_2$**-C**H$_2$-),** δ=3.42 (t, PEG-CH$_2$C**H$_2$**OC(O)CH$_2$CH$_2$).

3.2.2 Human Mesenchymal Stem Cell Culture

Human mesenchymal stem cells (hMSCs) were obtained from Texas A&M Health Science Centre College of Medicine through a grant obtained from NCRR of the NIH (P40RR017447). The frozen cells were recovered overnight and passaged (seeding density: 60 cells/cm^2) the next day. The cell culture medium used was MEM Richter's Modification medium with 20% Fetal Bovine Serum, 1% L-Glutamine and 1% Penicillin-Streptomycin. Media was changed once in 3 days. Once the cells reached ~80% confluency, they were trypsinized, counted and used for sample preparation (passage 2 hMSCs). For the scope of this project, we were interested in only analyzing the initial effects of free-radicals, UV exposure, and culture systems. Therefore, we did not test the effect of cell age (by using cells

in different passage numbers) on free-radical sensitivity, UV exposure , and culture systems. All our sample sets had hMSCs in passage 2.

3.2.3 2D cell culture (Day 1)

Freshly trypsinized hMSCs (500,000 cells) were plated in 75 cm^2 Corning tissue culture flasks with 20 mL fresh media and placed in the incubator.

3.2.4 Encapsulation of hMSCs in PEG Hydrogels (Day 1)

Optimal cell density in the hydrogels was determined to be 3,000,000 cells/ 100 µL. Freshly trypsinized cells were centrifuged down to a cell pellet, which was resuspended in fresh medium. Cells were counted using a hemocytometer. Aliquots having 3,000,000 cells were centrifuged in separate tubes at 0.5 rcf for 10 minutes. The resulting cell pellets were encapsulated in hydrogels which were fabricated as described below.

3.2.4.1 Radical Encapsulation

The hMSC cell pellet (3,000,000 cells) was resuspended in PEG4K-DA solution (0.01 g PEG4K-DA in 80 µL PBS) in an eppendorf tube. 20 µL of 2.5 M APS and 20 µL of 1.18 M TEMED was added to the cell+PEG4K-DA solution. This resulted in a 10 wt% PEG4K-DA solution with a final concentration of 5 mM APS and 2.5 mM TEMED in PBS. The solution was swiftly mixed and the gels were cast in constructs that resulted in cylindrical hydrogels. After 12 minutes the gels were transferred to fresh medium and the medium was changed after 24 hours. Cell viability was quantified using LIVE/DEAD assay. Fluorescence was detected using a Zeiss AxioObserver Inverted Fluorescent microscope equipped with AxioVision software and cells were counted manually.

3.2.4.2 Michael Addition (Non-Radical) Conjugate Addition

Pre-reaction: PETMP was pre-reacted with PEG4K-DA in a 2:1 PETMP to PEG4K-DA molar ratio (0.03 g of PETMP with 0.125 g PEG4K-DA). The pre-reaction was carried out for 2 hours in 1000 µL deuterated dimethyl sulfoxide. Completion of the reaction was monitored via ^{1}H NMR. The product was then precipitated into 200 mL cold diethyl ether, filtered, and dried under vacuum. The pre-reaction product (PEG4K-DA-PETMP) was dissolved in 1000 µL PBS. 25 hydrogels were made from this sample, each with a 100 µL volume.

To achieve an equivalent 2:1 PEG4K-DA to PETMP in the final product, 0.375 g of PEG4K-DA was dissolved in 875 µL PBS. To fabricate one hydrogel, 45 µL of the 0.092 M pre-reaction product and 35 µL of 0.10 M PEG4K-DA solution were mixed. 3,000,000 hMSCs pellet was resuspensed in this solution to fabricate the one hydrogel. 25 µL of 0.8 M TEMED solution was added to obtain a final concentration of 0.2 M TEMED in the polymer solution to catalyze conjugate addition of the samples. All samples were cured in 24 well plates. After 7 minutes the gels were transferred to fresh medium and the medium was changed after 24 hours.

3.2.5 UV Exposure Conditions (Day 2)

UV lamp for radiation source was a Black Ray UV Bench Lamp 365 nm, 115V 60 Hz, 0.68 Amps from UVP, LLC. Samples were exposed to UV radiation at 365 nm 4 mW/cm^2 intensity for 25 minutes. The intensity was measured and recorded using a ILT950 spectroradiometer.

3.2.6 RNA Extraction by TRIzol® Method and Spin Column Purification (Day 3)

Total RNA was extracted using the commercially available TRIzol® guanidinium-phenol based reagent followed by spin column purification using Ambion PureLink kit.

3.2.6.1 Homogenization

2D Samples: Plated cells were trypsinized and centrifuged at 0.5 rcf for 10 minutes. 1000 µL TRIzol® was added to the pellet and was pipette homogenized.

3D Samples: Gels were removed from the medium and washed with PBS. They were coarsely homogenized using a plastic tissue homogenizer and then flash frozen in liquid nitrogen. Each sample was thawed in 1000 µL TRIzol®. Fine homogenization was performed on ice using an electric tissue homogenizer (Tissue Tearor from Biospec Products, Inc) for 1 minute at speed 35.

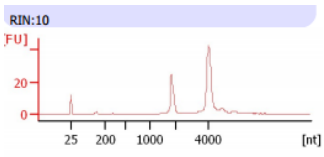
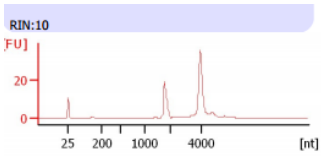
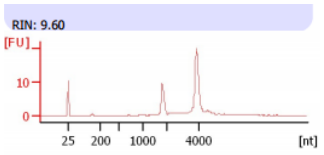
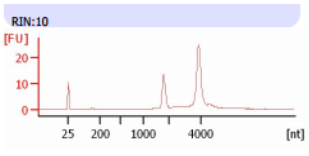
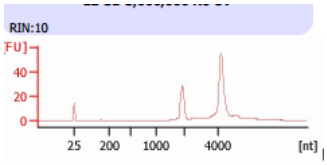
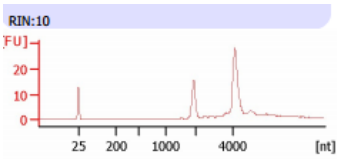
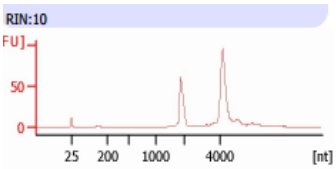
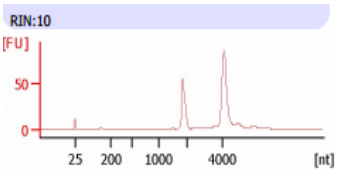
3.2.6.2 Phase Separation, Precipitation and Purification:

Samples in TRIzol® were incubated at room temperature for 5 minutes before further processing. 200 µL chloroform was added to each sample and incubated at room temperature for 3 minutes before centrifuging at 4˚C for 15 minutes 12,000xg for phase separation. The aqueous layer was collected and an equal volume of 70% ethanol was added. This solution was centrifuged multiple times through the spin column with wash buffer I and II (protocol by Life Technologies). DNA impurities were removed with DNase bought commercially. In the final step, RNA was eluted in RNase free ultra pure water and stored at -80˚C for further analysis.

3.2.7 RNA Integrity Analysis by Agilent 2100 Bioanalyzer

RNA integrity analysis was performed by the Clinical Microarray Core (CMC) facility located in UCLA. Analysis was performed using the Agilent 2100 bioanalyzer. The bioanlayzer generated electropherograms and RNA integrity number (RIN). Table 1 shows representative electropherograms for each sample set. The samples processed for microarray experiments need to have RIN > 7. All our samples had distinct 18s and 28s RNA subunit peaks and RIN > 9.40.

Table 1: RNA Integrity Analysis: Representative electropherograms for each sample set with average RIN.

Sample Set	Electropherogram	RIN
A [2D UV- R-]		10.0 ± 0.0
A1 [2D UV+ R-]		10.0 ± 0.0
B [3D UV+ R-]		9.6 ± 0.2
B1 [3D UV+ R-]		10.0 ± 0.0
C [3D UV- R+]		10.0 ± 0.0
C1 [3D UV+ R+]		10.0 ± 0.0
D [2D UV- R-]		10.0 ± 0.0
D1 [2D UV+ R-]		10.0 ± 0.0

3.2.8 Gene Chip Microarray

As demonstrated by the RNA integrity analysis, all samples were suitable for microarray analysis. Total RNA samples were sent to the CMC for microarray profiling. Human U133 plus 2.0 Affymetrix gene chip was used for profiling each sample. Figure 6 shows a general workflow overview of the experiments conducted during gene profiling.

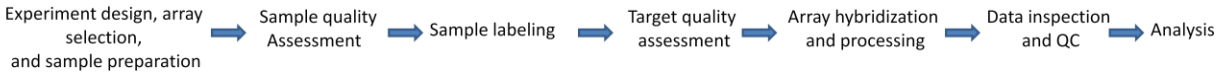


Figure 6: Workflow in a Microarray Core Lab (adapted from[51])

3.3 Experimental Flow

Figure 7 shows a pictorial representation of the experimental set up with a description of sample sets and time points.

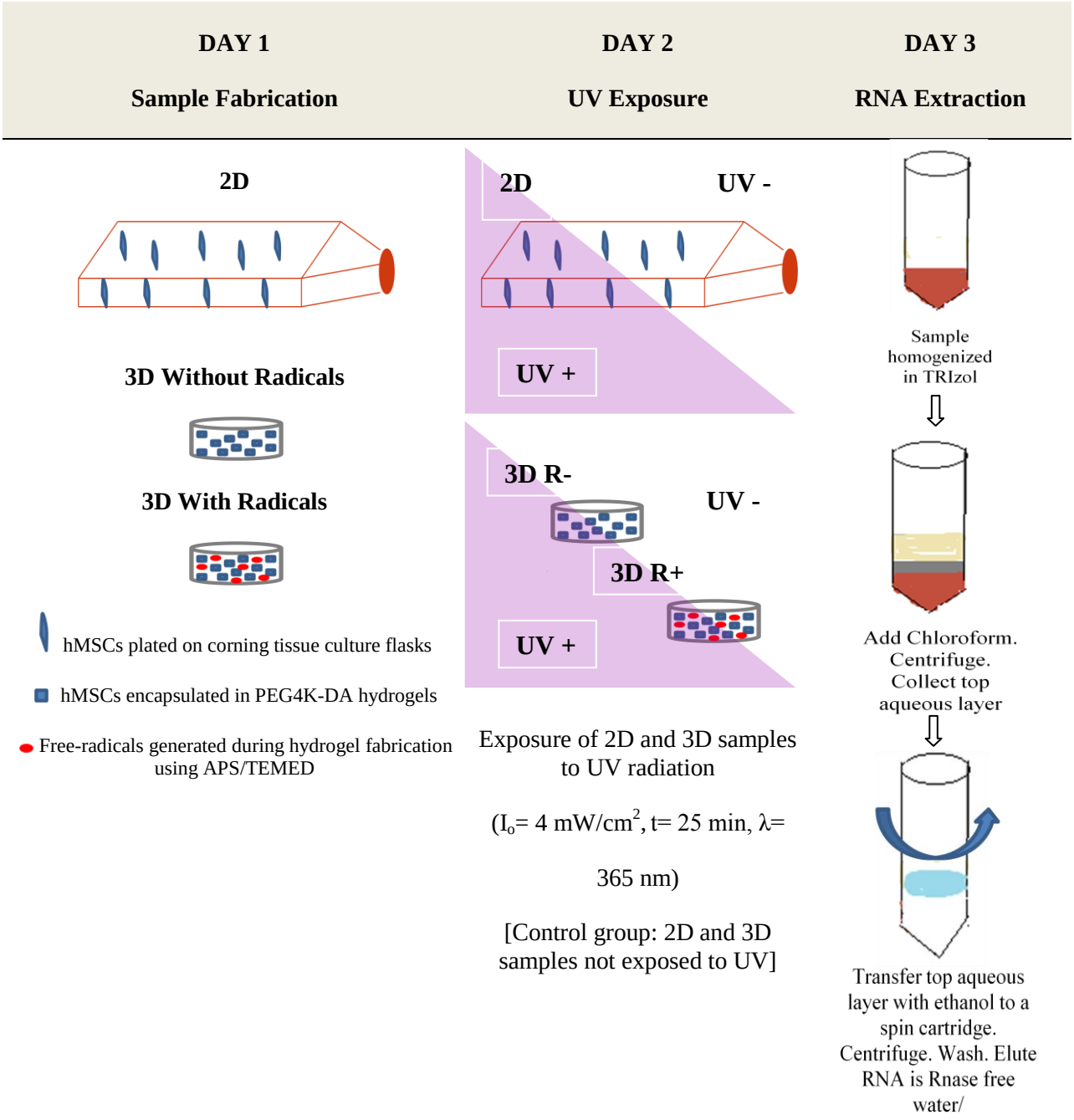


Figure 7: Sample Set Fabrication and Experimental Flow: After sample fabrication (Day 1) we placed 2D and 3D samples overnight in the incubator to give them enough time to recover from trypsinization, plating and encapsulation. On day 2, we exposed half the sample sets to UV radiation after the 24 hour recovery period. After UV exposure, we gave the cells 24 hours to divide and show probable changes in gene expression after UV exposure. Therefore, we extracted total RNA on Day 3 after UV exposure.

4 OPTIMIZATION OF SAMPLE SETS

4.1 2D Sample Preparation

2D sample set (without radicals) was achieved by plating 500,000 hMSCs in a Corning tissue culture flask. 2D samples were not exposed to radicals as it was not representative of experimental conditions they experience (i.e., cells are exposed to radicals when they are encapsulated but not in 2D)[14,16,52,53]. Betz et al., showed that hMSC metabolic activity was significantly reduced after 3 hours of exposure to 20 mM APS/TEMED.[53] Higher concentrations applied to hMSCs result in a loss of viability, as described below. Therefore, we analyzed the effect of radicals in 3D systems only.

4.2 3D Radical Polymerization

Free-radicals are known to cause cell damage, so we first needed to optimize the free-radical mediated cell encapsulation in order to maintain $\geq$ 90% viability of hMSCs. We systematically varied the concentration of the initiating system (APS/TEMED) to determine the minimum concentration needed to form viable 3D constructs in 15 minutes or less. A 2:1 mol: mol ratio of APS/TEMED was used based on the mechanism of radical generation.[54] The concentration of PEG4K-DA was held constant at 10 wt%. Table 2 summarizes the results.

Table 2: Conditions for Radical Polymerization Optimization

APS concentration (mM)	TEMED concentration (mM)	Polymerization time (minutes)	LIVE/ DEAD Assay- 48 hour time point
100	50	2-3	
50	25	3-4	70%-90% cells dead (likely due to high concentration of radicals)
10	5	11	
1	0.5	Gelation did not occur 30 minutes	----
5	2.5	12	~90% cells alive.

We observed that high concentrations of APS/TEMED resulted in rapid polymerization, but cells did not remain viable. We did not measure the actual number of radicals generated but correlated our decrease in cell viability to the relative concentration of APS/TEMED. The lowest concentration tested, 1 mM APS and 0.5 mM TEMED, was too low to achieve sufficient gelation. However, the next lowest concentration, 5 mM APS and 2.5 mM TEMED, resulted in polymerization within 12 minutes and ~90% hMSCs remained viable. hMSC viability was quantified using LIVE/DEAD assay. The optimal encapsulation conditions using radical initiation were determined to be: 10 wt% PEG4K-DA gels polymerized with 5 mM APS and 2.5 mM TEMED and 12 minutes polymerization time.

4.3 Cell Density for Encapsulation

Next, we systematically varied the cell density to have > 50 ng concentration (minimum concentration needed for the bioanalyzer and gene array experiments), of the extracted total

RNA. We systematically varied the cell density from 100,000 hMSCs to 5,000,000 hMSCs per 100 µL gel. We observed that the hydrogels with ≤ 2,500,000 cells did provide the required amount of total RNA (extracted total RNA concentration ~20 ng/µL). The optimal cell density for 3D samples, 3,000,000 hMSCs in a 100 µL gel resulted in > 50 ng/µL concentration of the extracted total RNA.

4.4 3D Non-Radical Conjugate Addition

Just as we optimized the free-radical polymerization, we also needed to optimize the non-radical hydrogel fabrication via Michael addition. In this case, we held the cell density constant based on our previous results (3,000,000 cells per 100 µL gel). Initial attempts to cross-link by varying the PEG4K-DA concentration was unsuccessful since we discovered the cross-linker PETMP was too hydrophobic to allow encapsulation in aqueous medium. Therefore, we pre-reacted PETMP with PEG4K-DA to increase its solubility in aqueous solutions. The presence of excess acrylates during the pre-reaction causes gelation. To avoid this issue, a 2:1 mole ratio of PETMP:PEG4K-DA (4:1 mole ratio of thiol:acrylate) was reacted in deuterated dimethyl sulfoxide and followed by ^{1}HNMR.

Table 3: Conditions for Non-Radical Polymerization Optimization

Condition	Values	Observations
Macromer (PEG4K-DA) weight percentage with PETMP (2:1 mole ratio)	10 to 50 wt% PEG4K-DA	Gelation did not occur since PETMP was too hydrophopic.
Pre-reaction time	1 to 12 hours	Pre-reaction > 4 hours resulted in the formation of a viscous product which could not be successfully precipitated. With pre-reaction < 2 hours, reaction did not go to completion and product could not be isolated.
TEMED concentration	0.2 to 0.4 M	0.2 M TEMED resulted in ~90% cell viability and a gelation time of ~7 minutes

After pre-reaction of PETMP with PEG4K-DA, all encapsulation components became water-soluble and we could proceed with hMSC encapsulation. We used a molar ratio of 2:1 PEG4K-DA:PETMP (1:1 mole ratio of thiol:acrylate) in PBS. The rate of the Michael addition reaction is strongly influenced by pH, with faster rates observed at higher pH.[9] We therefore added TEMED as a base catalyst to deprotonate the thiol and accelerate the encapsulation. We tested hMSC viability using LIVE/DEAD assay (as explained in 4.2) with a range of TEMED concentrations (0.2-0.4 M). Table 3 summarizes the conditions varied and the observations recorded during the optimization of non-radical cross-linking. The optimal encapsulation conditions using non-radical cross-linking were determined to be an initial pre-reaction of PETMP with PEG4K-DA (2:1 mole ratio) in deuterated DMSO for two

hours followed by precipitation, filtration, and drying of the product. In the second step, hMSCs were encapsulated in a solution of pre-reaction product and PEG4K-DA (dissolved in PBS). 0.2 M TEMED was used as a base catalyst resulting in a gelation time of ~7 minutes and ~90% hMSC viability.

4.5 RNA Extraction and Homogenization

Monolayer (2D sample) RNA extraction is well documented in the literature.[55–57] However homogenization of cells encapsulated in hydrogels for optimal RNA extraction is less common. We determined the optimal RNA extraction parameters for our systems by exploring different homogenization conditions. We experimented with four different homogenizers: Bullet blender (bead mill homogenizer), ceramic and plastic mortar and pestle for manual homogenization, and electric tissue homogenizer for automated homogenization. Our goal was to extract total RNA with integrity greater than 7 on a scale of 10 since gene array experiments require high quality RNA.[51] Table 4 lists the various combinations of homogenizers used.

Table 4: Conditions for 3D Sample Homogenization for RNA Extraction Optimization

Trial	Homogenization Condition	~ RNA Concentration (ng/ µL)	RIN
1	Bullet blender, 2.0 mm zirconium oxide beads, 20 minutes, speed 8	2.0	1.1-1.7
2	Bullet blender, 2.0 mm zirconium oxide beads in combination with 0.4 mm zirconium oxide beads for 20 minutes at speed 8	8.0	2.2-2.8
3	Electric tissue homogenizer in various combinations with 4 mm and 0.4 mm zirconium oxide beads	15.0	1.0-2.6
4	Ceramic mortar pestle in combination with electric tissue homogenizer	18.5	1.0-2.4
5	Electric tissue homogenizer for different time lengths (10 seconds- 5minutes) and different speeds (8- 35)	35.0	2.2-5.2
6	Plastic tissue homogenizer, then flash freeze samples in liquid nitrogen. Thaw in TRIzol and use electric tissue homogenizer for 1 minute speed 35 on ice.	60.0	7.4-10.0

We observed that with the use of just the bullet blender with different combinations of bead size or the plastic tissue homogenizer by itself, the hydrogel was not completely homogenized even after 8 minutes. This resulted in a low concentration of RNA extracted from encapsulated cells. With the use of ceramic mortar and pestle, we saw degradation in the extracted RNA sample, possibly due to the presence of RNases in the porous ceramic

material. The electric tissue homogenizer successfully homogenized the hydrogels but generated excess heat during the process, resulting in degradation of extracted RNA. Therefore, in order to minimize the effect of heat generated during the homogenization process, we performed the homogenization on ice. Additionally, we used the plastic mortar and pestle first to homogenize the hydrogel coarsely. This helped in reducing the homogenization time by the electric tissue homogenizer, thus minimizing the effect of heat generated during the process. The optimal RNA extraction conditions were determined to be: homogenize initially with a plastic tissue homogenizer, followed by flash freezing the sample in liquid nitrogen. Later, thaw samples in TRIzol and homogenize using the electric tissue homogenizer for one minute at speed 35 on ice. After optimizing the cell encapsulation and RNA extraction procedures, we proceeded to fabricate our sample sets as explained in chapter 3.

5 GENE EXPRESSION UNDER DIFFERENT CULTURE CONDITIONS

5.1 Summary of Sample Sets

Sample sets used in our experiments are tabulated in Table 5. UV+ and UV- indicate whether the samples were exposed to UV radiation or not respectively. R+ and R- indicated the presence or absence of radicals in the culture system respectively.

Table 5: Sample Set Description

Experimental Condition	2D R(-)	3D R (-)	3D R(+)
UV (-)	A, D	B	C
UV (+)	A1, D1	B1	C1

Samples for sets A and B were created in the first batch, and C and D in the second batch (experiments for the two batches were conducted on separate days). Sample sets A and D were used as controls between the two batches. That is, if our experiments are reproducible, we should observe no significant differences between A and D.

5.2 Principal Component Analysis, Heat Maps, and Ingenuity Pathway Analysis

5.2.1 Principal Component Analysis

Principal Component Analysis (PCA) is a statistical analysis tool which works by reducing the dimensionality of data by determining the key variables resulting in differences seen between samples.[58] The PCA map generated from this data is a visual multidimensional

representation that summarizes the changes in gene expression due to our various experimental conditions. Figure 8 is the PCA map generated for the gene expression profiles of our sample sets.

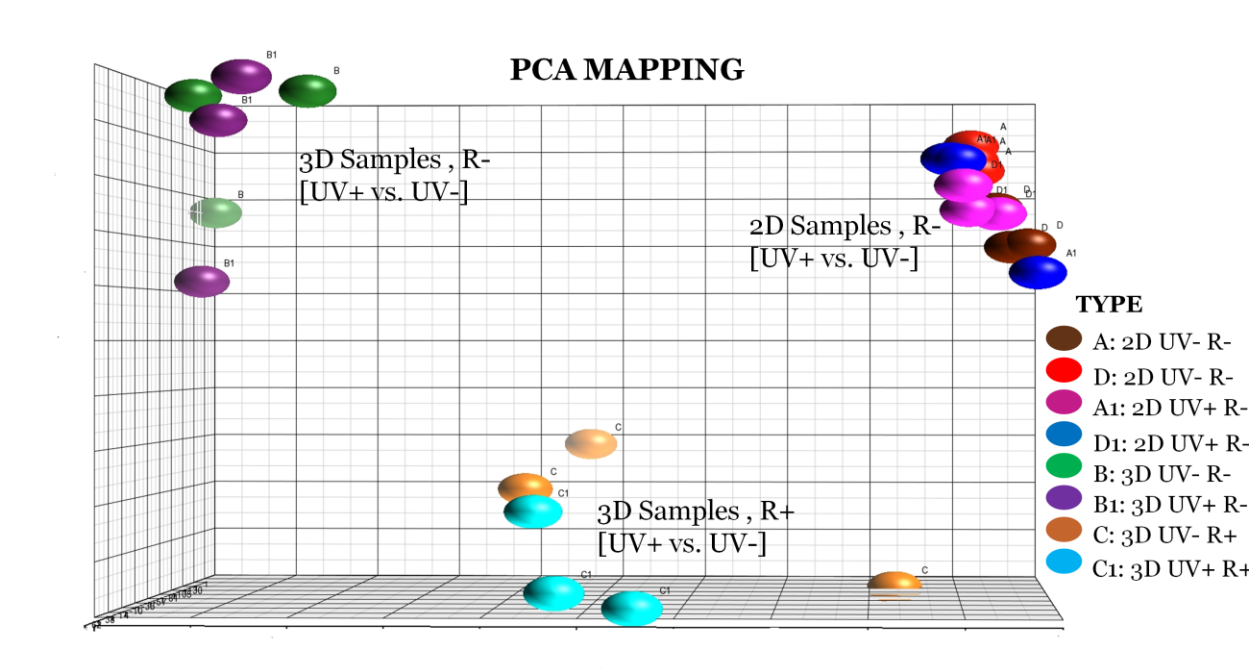


Figure 8: Principle Component Analysis Map: PCA map shows distinct clustering of all sample sets indicating the significant variation in gene expression between each sample set. We can also see that samples exposed to UV radiation have similar clustering patterns as their counterparts with no UV exposure indicating that UV exposure did not play a significant role in changing the gene expression. We can also observe that 2D and 3D sample sets cluster in different zones of the PCA indicating that culture systems play a major role in influencing the gene expression.

5.2.2 Venn Diagram of UV Comparisons Across all Sample Sets

The Venn diagram [Figure 9] provides a pictorial representation of the approximate number of genes with differential expression with each experimental condition. It also visualizes the approximate number of common genes that changes when two comparisons are combined. This will help us understand the specific effects of external factors like UV exposure, and the presence/absence of radicals in the cell culture systems.

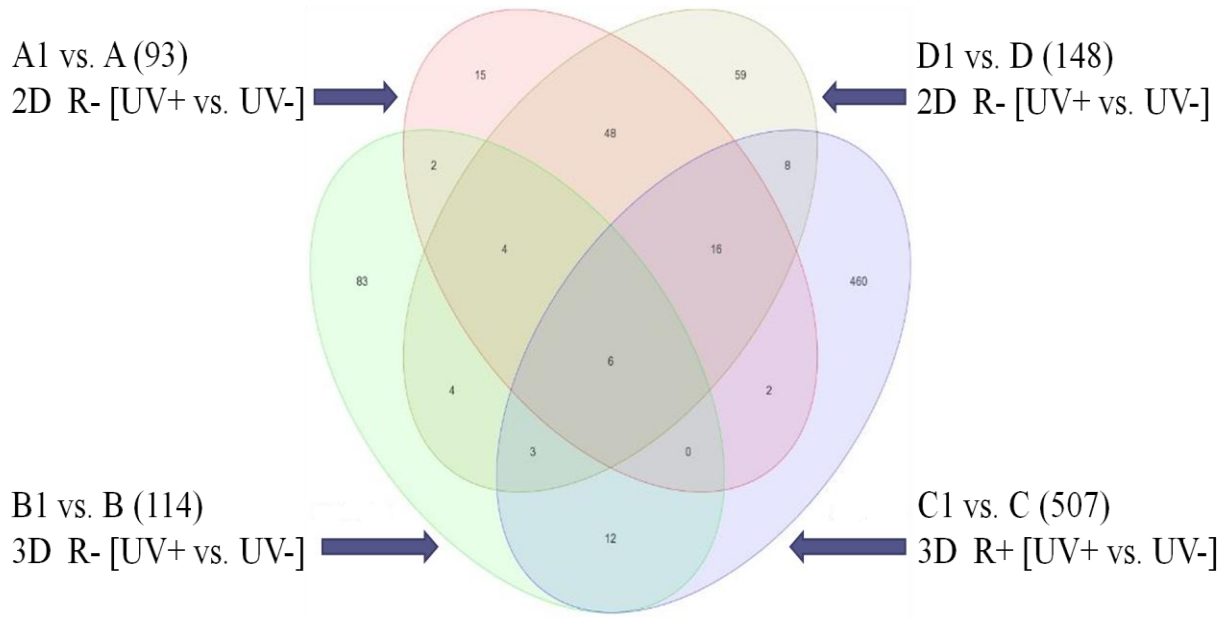


Figure 9: Venn Diagram for UV Comparisons Across all Samples: The Venn diagram depicts the number of differentially expressed genes under UV exposure across all culture conditions (2D and 3D)

5.2.3 Heat Maps

Heats maps display 2D mosaic models generated by the microarray data where the levels of gene expression are compared. Gene expression levels are correlated to a transition from red to blue where, red represents relative over-expression and blue represents under-expression. Heat maps visualize changes in the gene expression under different conditions. Data from the heat maps provide quantifiable validation for the observations made from the PCA map. Each heat map has a customized gene expression fold-change range. A minimum of two-fold differential expression of genes with a *p-value* < 0.05 is the cut-off for inclusion in the heat map.

5.2.4 Ingenuity Pathway Analysis

Ingenuity pathway analysis (IPA) helps us interpret the microarray data in the context of biological processes, pathways and networks. This analysis is very useful since we know that

genes do not work alone but have networks of interactions and their combined effects. This is also a way of potentially excluding false positive results since random gene expression would not necessarily result in specific pathway enrichment.

5.3 Reproducibility of the Experiments

The samples sets A,A1 and D,D1 [2D UV- R-, 2D UV+ R-] went through the same experimental conditions but were processed in different batches on different days. The gene expression data of these samples can be compared to demonstrate the reproducibility of our experiments. In the PCA map [Figure 9], all the four samples are clustered in the top right zone demonstrating that there is very little statistical variation of gene expression between these samples. This is further confirmed by similar expression patterns depicted in the heat map data in the following sections.

5.4 Effect of UV Radiation

The PCA map shows that the gene expression profiles do not exhibit significant variation with respect to UV exposure across all culture systems. This is seen by the clustering of the samples in three distinct zones. We can see that AD-A1D1 [2D R- (UV- vs. UV-)], B-B1 [3D R- (UV- vs. UV+)], and C-C1 [3D R+ (UV- vs. UV+)], have distinct clustering on the map indicating that the exposure to UV radiation does not play a significant role in altering the gene expression of hMSCs. However, qualitatively we can see that there is more variation in between sample replicates which were encapsulated via radical reaction. We hypothesize that this is due to the compounded effect of UV radiation in the presence of radicals generated during radical polymerization. This might be a result of UV radiation accelerating the generation of radicals present in the system. These observations show that the exposure of

hMSCs to UV radiation does not make a significant impact on the gene expression, while indicating an increased effect on the gene expression profile in the presence of radicals in the culture system.

Although no significant variation was observed between those samples exposed to UV versus those unexposed, there were 16 common genes showing variations in the gene expression profile across all sample sets. The change in expression in these 16 genes may or may not be meaningful as they represent a very small number of differentially expressed genes (< 0.1%). The change might be attributable to non-viable cells or attributable to UV exposure. The Affymetrix gene chip analyzes more than 44,000 genes. With a *p-value* < 0.05 one could potentially see as many as 2200 false positive results in differential gene expression. While this might make 16 genes look insignificant and potentially random, we cannot rule out the opposite statistical scenario that few changes occur but those that do are actually real. For validation of results for any particular differentially expressed gene, further experiments such as RT-PCR and western blot are needed. Listed below are a few of the commonly differentially expressed genes that may be related to cell health and viability. Figure 10 shows the heat map for those 16 genes across all sample sets which showed differential gene expression under UV exposure. It is seen that these sixteen genes are mostly involved in regulation of basic cellular functions like cell growth, cell division, and tissue development or in inflammatory responses.

RARRES1 and RARB are genes belonging to the retinoic acid receptors (RAR) family where these are involved in the regulation of organogenesis during tissue development.[59] Across all conditions, we observed a two-fold downregulation of the RAR family genes. FOS gene has

been shown to have an important role in cell proliferation and differentiation[60] and showed a two-fold downregulation across all sample sets after UV exposure. Most importantly we see the FOXQ1 gene with two to four-fold upregulation in expression. The FOX family of genes have been known to play a significant role in embryonic development, cell cycle regulation, tissue-specific gene expression, and cell signaling.[61] Under exposure to UV, we can see a two-fold upregulation of CCL2 and LXN. CCL2 displays chemotactic activity for monocytes and basophils. In some cases, fluctuations in CCL2 concentration depicts disease activity and/or inflammatory activity in response to cell distress.[62] LXN, which is a paralog of RARRES1 is involved in metastatic suppression and is also known to be involved in inflammatory responses.[63] Taken together, these changes point to the possibility of DNA damage in hMSCs after UV exposure followed by changes in cell proliferation and initiation of immune responses.

These changes in gene expression might indicate that some cells experience damage from the UV exposure, or might be attributed to other damage that occurs during sample fabrication. The subset of genes changing their expression is very small compared to the thousands of genes being analyzed, and this change may not be significant at all. The only condition in which > 200 genes show differential expression under UV exposure was in the presence of radicals formed during the encapsulation procedure. When > 200 genes show differential expression between two samples, this allows us to do pathway analysis to determine specific signaling pathways that might change as a result of the experimental conditions.[64–66] Although changes in fewer than 200 genes between samples may still be significant such a change is relatively small compared to the large number of genes interrogated. Figure 10

gives the summary of the number of genes differentially expressed due to UV exposure under each cell culture condition.

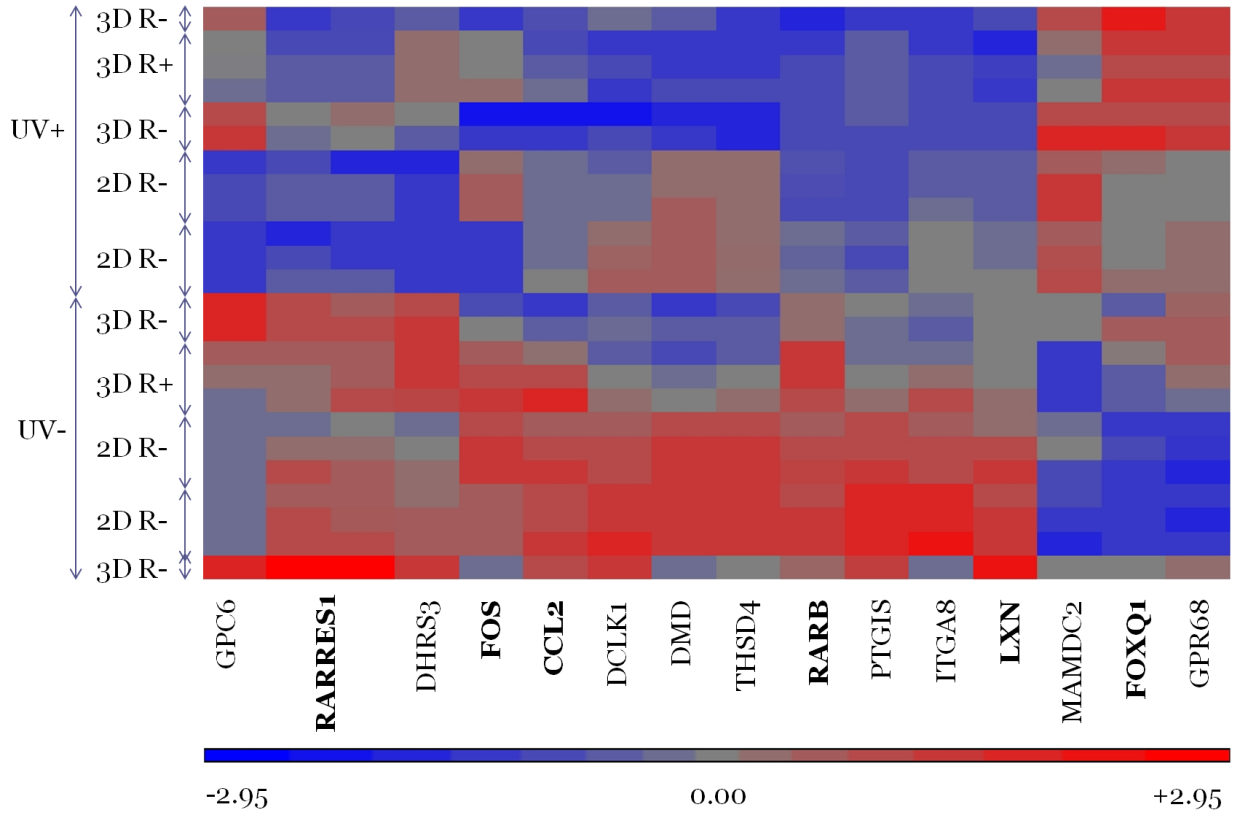


Figure 10: Heat Map of Genes Involved in UV Exposure Across All Comparisons: The heat maps depicts 16 common genes which showed differential expression across all UV exposure conditions. The genes discussed are highlighted in the heat map.

5.5 Effect of Cell Culture System

The second experimental condition for comparison was the culture system 2D versus 3D. From the PCA maps, we can observe that the gene expression profiles of hMSCs cultured in different systems change significantly since they are segregated in different zones of the map. This demonstrates that the culture conditions significantly affect the gene expression, which is no surprise given the ample evidence in literature.[67,68]

The PCA map shows a large difference in the gene expression of hMSCs cultured in different systems. Quantitative analysis shows a two-fold change in gene expression for a total of 5164 at a *p-value* < 0.05. Out of the 5164 genes in the list, we investigated a few differentially expressed genes. RASD1 plays a role in the alterations in cell morphology, growth and cell-extracellular matrix.[69] This gene showed a 103-fold change in 3D systems as compared with the 2D counterpart. ADAMTS1 has been referenced as being necessary for normal growth, fertility, and organ morphology and function.[70] Differential expression of this gene had been implicated in the migration and invasion of human breast tumor cells.[71] In our experiments, we saw a 25-fold upregulation from 2D to 3D system in the expression of ADAMTS1 gene. GDF15 is involved in the regulation of tissue differentiation and maintenance.[72] When cultured in 3D systems, we saw a four-fold upregulation of GDF15 as compared to the 2D system. We also saw a 12-fold downregulation of GAS1 gene. GAS1 gene is a growth arrest specific gene which is involved in growth suppression.[73] Table 6 has a list of differentially expressed genes with maximum fold-change occurring due to change in the cell culture system. Taken together, changes in this collection of genes implies that hMSCs cultured in a tissue-like 3D system have significant differential expression of genes involved in cell growth and morphology.

The heat map generated to depict the gene expression change for this comparison is shown in Figure 11. By comparison of the number of genes being differentially expressed, we can observe that the culture system more significantly affects gene expression as compared to UV exposure.

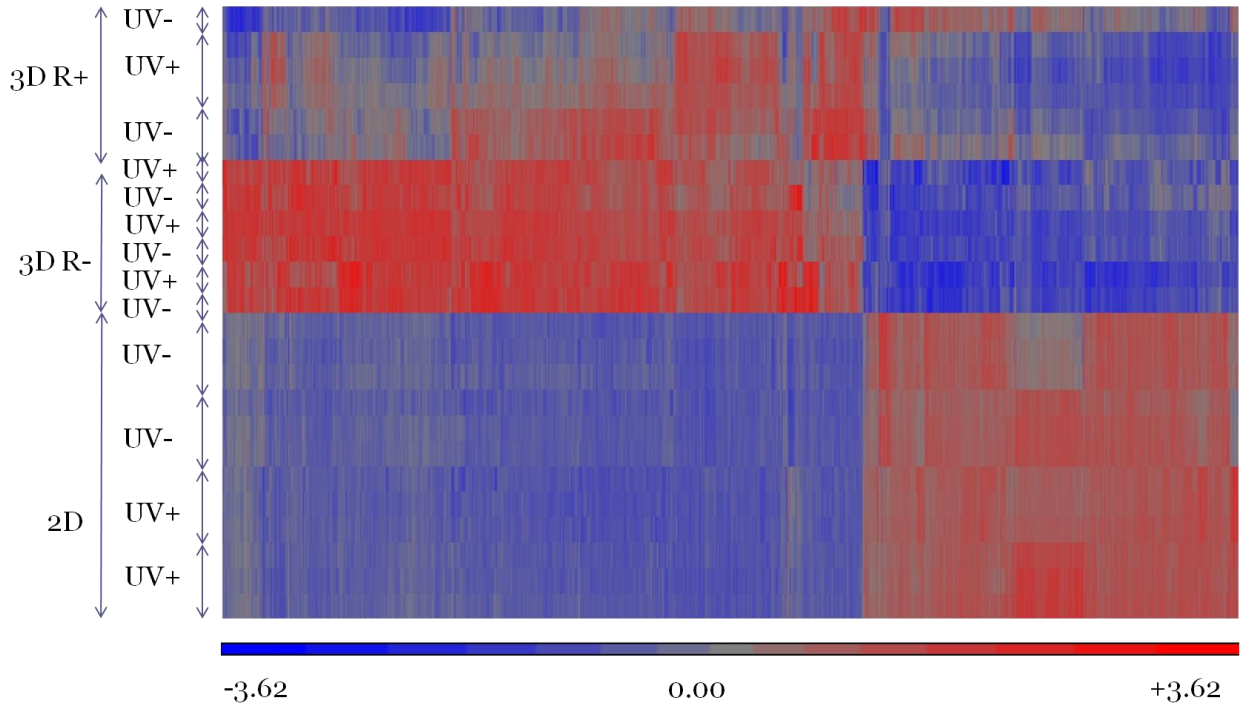


Figure 11: Heat Map for Effect of Cell Culture Systems of Gene Expression: The heat map depicts the differential expression of 5164 genes in cells cultured under 3D conditions as compared to cells cultured in 2D systems.

Table 6: Differentially Expressed Genes with Maximum Fold-Change Due to Change in Culture System

Gene Name	Fold Change	Gene Function
CEBPB	+112	Important transcriptional activator in the regulation of genes involved in immune and inflammatory responses.[74,75]
RASD1	+102	This gene may play a role in dexamethasone-induced alterations in cell morphology, growth and cell-extracellular matrix interactions.[69,76]
SAT1	+97.2	Involved in the regulation of the intracellular concentration of polyamines and their transport out of cells. Defects in this gene are associated with keratosis follicularis spinulosa decalvans.[77]
FBXO32	+44.5	Probably recognizes and binds to phosphorylated target proteins during skeletal muscle atrophy.[78,79]
SLC8A1	+43.9	Rapidly transports Ca^{2+} during excitation-contraction coupling.[80]
C11orf96	+40.4	Uncharacterized
GABARAPL1	+35.7	Facilitating anterograde intracellular trafficking of the receptor. Involved in formation of autophagosomal vacuoles.[81]
GNRH1	-12.9	Stimulates the secretion of gonadotropins; it stimulates the secretion of both luteinizing and follicle-stimulating hormones.[82]
TRIM59	-13.4	Diseases associated is asphyxiating thoracic dystrophy. GO annotations related to this gene include ubiquitin protein ligase activity and zinc ion binding.[83]
PIWIL2	-13.4	Plays an essential role in meiotic differentiation of spermatocytes, germ cell differentiation and in self-renewal of spermatogonial stem cells. When overexpressed, acts as an oncogene by inhibition of apoptosis and promotion of proliferation in tumors.[84]
NPR3	-13.7	May regulate diuresis, blood pressure and skeletal development.[85,86]
SNORD114-3	-14.1	RNA gene, and is affiliated with the snoRNA class and is involved in acute promyelocytic leukemia.[87]
GPRC5B	-16.9	This protein may mediate the cellular effects of retinoic acid on the G protein signal transduction cascade.[88]
CDCA2	-17.8	Regulator of chromosome structure during mitosis required for condensin-depleted chromosomes to retain their compact architecture through anaphase.[89,90]

In the literature, we have seen that 3D systems are used to better mimic body-like conditions and can be tailored to differentiate stem cells to desired lineages. This indicates that the culture conditions change the expression levels of genes involved in particular pathways which lead to the desired phenotypic expression. We processed the differentially expressed genes using IPA to understand the functional pathways that were affected by culturing the hMSCs in 2D vs. 3D systems. Figure 12 shows selected pathways affected to a large extent due to changes in the culture system. The majority of genes that changed were involved in the cell cycle pathway followed by cellular growth and proliferation, cell development, and cell cycle. The results help us visualize the important pathways affected when cells are cultured in 2D versus 3D systems. We can see that the culture systems plays an integral role in the genetic expression of hMSCs. Therefore, it becomes imperative to understand the consequences of culture conditions on the cells since the process may itself alter gene expression.

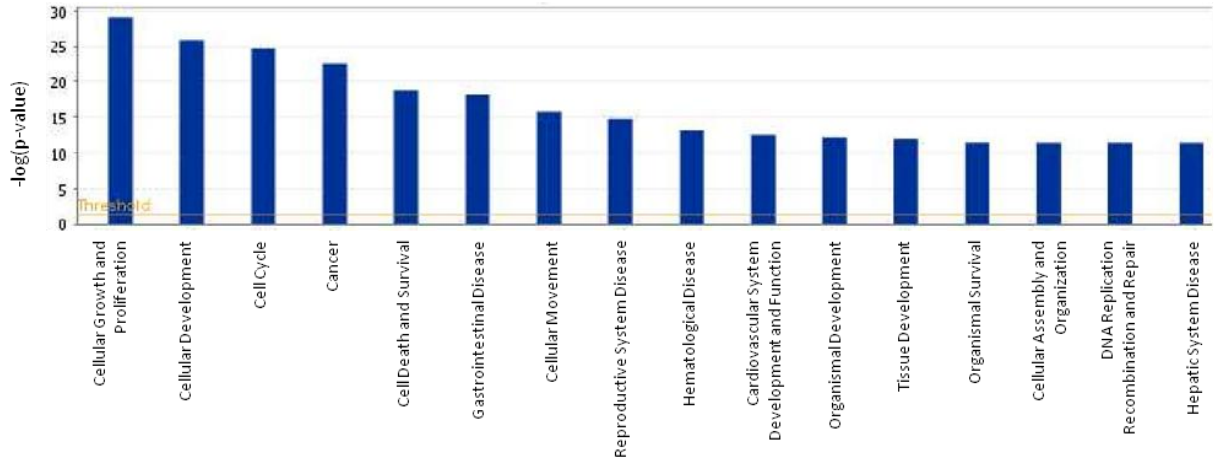


Figure 12: IPA Analysis of the effect of cell culture system 2D vs. 3D: Differentially expressed genes under 2D vs. 3D condition were analyzed using IPA to understand the cellular pathways affected.

5.6 Effect of Radicals

There is significant variation between the gene expression of both the 3D cell culture systems- B,B1 [3D R- (UV-, UV+)] versus C,C1 [3D R+ (UV-, UV+)] due to the mode of fabrication. Quantitative analysis shows that there was a change in gene expression for a total of 3030 genes with two-fold expression difference at a *p-value* < 0.05. It has been demonstrated previously that radicals damage the cell membrane, form DNA dimers and in general activate stress response in cells.[29,91–93] Our experiments show the differential expression of genes involved in these pathways. GADD45b and GADD45g are kinase inhibitors which are involved in cell cycle checkpoints induced by genotoxic stress.[94] CDC20 is a cell division cycle 20 gene acting as a regulatory checkpoint in the cell cycle.[95,96] In the presence of radicals, we saw a six-fold upregulation in the expression of these genes indicating that the cells were under genotoxic stress. This implies damage to cellular DNA. CXCL3 is a cytokine that controls immune response by mediating the migration of monocytes and interacting with various cell surface chemokine receptors.[97,98] A six-fold upregulation of this gene under the influence of radicals indicates repair mechanisms being activated inside the cell. TK1 is the thymidine kinase gene which is upregulated in rapidly proliferating cells and downregulated in resting cells.[99,100] Usually used as a marker for detecting cancer cells, it was shown to have a four-fold upregulation in cells exposed to radicals. This indicated the damaging potential of radicals to the cells since upregulation is known to cause unnatural rapid cell proliferation.[99,100]

Exposure to radicals in the cell culture system also caused differential expression in genes involved in the immune regulatory pathway, DNA repair and cell apoptosis pathways. The CCL26 gene regulates the production of proteins involved in immunoregulatory and

inflammatory processes.[101,102] A four-fold upregulation of gene expression in cells exposed to radicals implies damage to cells eliciting the activation of immune response pathways. The THBS1 gene codes for an adhesive glycoprotein which mediates cell-to-cell and cell-to-matrix interactions.[103] In cells exposed to radicals we saw a three-fold increase in the THBS1 gene expression. This could be due to the damage caused by the radicals to the cell membrane leading to changes in the cell-cell and cell-matrix interactions. CASP4 and SIVA1 play central roles in the execution-phase of apoptosis.[104–107] Exposure to radicals causes a three-fold downregulation of these genes. Downregulation of genes involved in the apoptotic pathway has been shown to cause cancer cell survival, resistance of cells to drugs, and other unnatural behavior of cells.[108,109]

The heat map generated to depict the gene expression change for this comparison is shown in Figure 13. Table 7 has a list of differentially expressed genes with maximum fold-change occurring due to the presence of free-radicals in the 3D cell culture system.

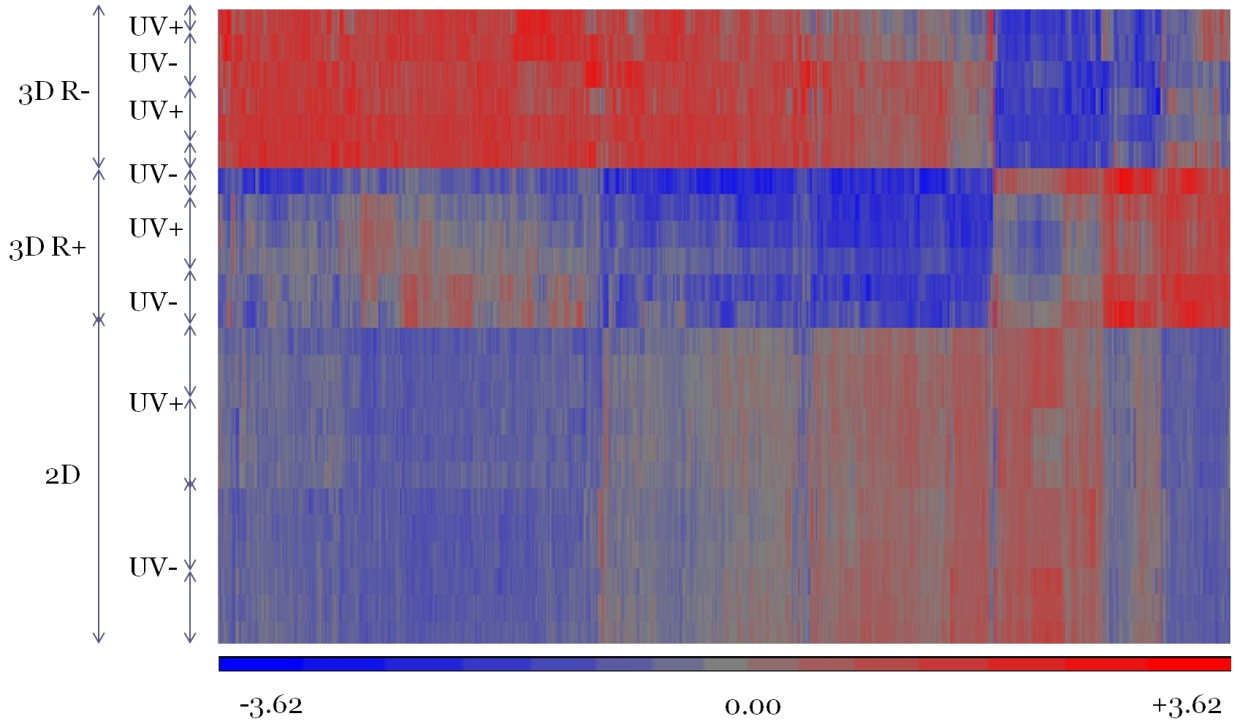


Figure 13: Heat Map for the Effect of Radicals: The heat map depicts the differential expression of 3030 genes in cells cultured in the presence of radicals.

Table 7: Differentially Expressed Genes with Maximum Fold-Change Due to Presence of Free-Radicals in the 3D Culture System

Gene Name	Fold Change	Gene Function
HSPA6	+112	Codes for proteins that bind to peptide segments exposed during translation and membrane translocation, or following stress-induced damage.[110]
ANGPTL4	+97.2	Codes for a protein which acts as an apoptosis survival factor for vascular endothelial cells and can prevent metastasis by inhibiting vascular growth and tumor cell invasion.[111]
CXCL2	+35.6	Involved in proteins produced by activated monocytes and neutrophils and expressed at sites of inflammation.[112]
RRAD	+31.5	May play an important role in cardiac anti-arrhythmia.[113]
GADD45b	+25.9	Involved in the regulation of growth and apoptosis.[114,115]
PDK4	+20.5	Plays a role in the generation of reactive oxygen species and has a role in cell proliferation.[116]
BHLHE40	+19.9	The encoded protein is believed to be involved in the control of cell differentiation.[117]
PLA2R1	-13.0	Induces cell death of cancer cells by miochondrial stress.[118]
COL3A1	-13.7	Involved in regulation of cortical development.[119]
ZNF117	-14.1	May be involved in transcriptional regulation of inflammation and apoptosis.[120]
PLXDC1	-15.5	Plays a critical role in endothelial cell capillary morphogenesis.[121]
IBSP	-17.8	Probably important to cell-matrix interaction. Promotes Arg-Gly-Asp dependent cell attachment.[122]
CHI3L1	-20.6	Involved in defense against pathogens and/or tissue remodeling. Plays an important role in cellular response helping cells cope with changes in their environment.[123]

To have a better context for understanding the data, IPA analysis was performed on these differentially expressed genes. Figure 14 shows selected pathways affected to a large extent from exposure to radicals. The majority of genes that changed were involved in the cancer

pathway followed by cellular assembly, cell cycle, and cell development. It is very interesting to observe that the majority of the differentially expressed genes are involved in cancer pathways. This can be correlated back to various studies conducted which demonstrate that radicals cause oxidative stress on cells, initiating DNA repair pathways, and if the damage cannot be repaired, initiating pathways leading to cell apoptosis.[124,125]

The IPA analysis for analyzing the effect of radicals generated during the polymerization process shows the importance of understanding the effects of fabrication methods on the gene expression of cells being encapsulated. We can see that culturing cells in the presence of radicals changes the expression of genes involved in cancer pathways, cell development, cell proliferation, and survival. Therefore, it becomes imperative to understand the consequences of our encapsulation methods on the cells.

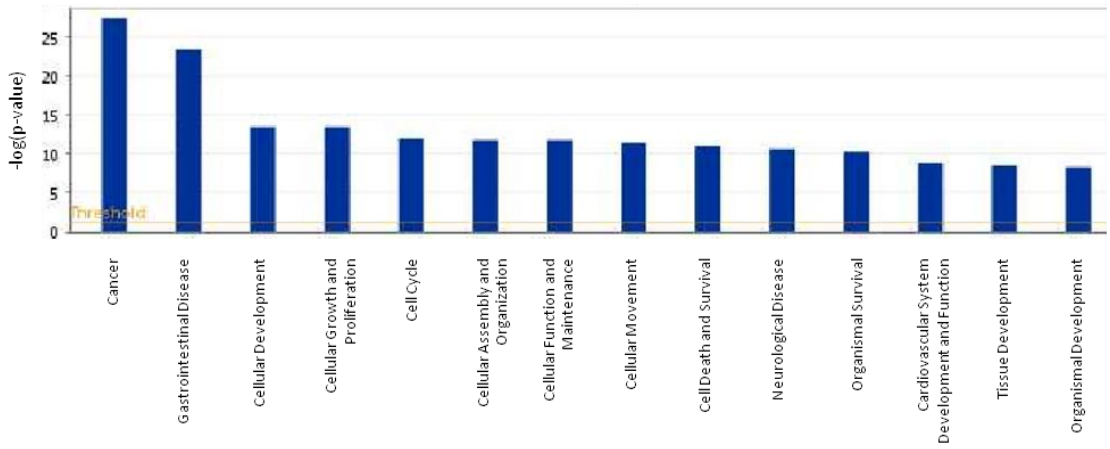


Figure 14: IPA Analysis of Effect of Radicals in the 3D Systems: Differentially expressed genes under radical vs. non-radical condition were analyzed using IPA to understand the cellular pathways affected.

5.7 Effect of UV Radiation in Conjunction with Radicals

The PCA map shows a relatively greater effect of UV radiation in the system that was fabricated by free-radical polymerization [C1 vs. C: 3D R+ (UV- vs. UV+)] as compared to samples fabricated by non-radical polymerization [B1 vs. B: 3D R- (UV- vs. UV+)] This is probably due to the acceleration in the generation of radicals thereby increasing the concentration of radicals in the system. Quantitative analysis shows a change in gene expression for a total of 507 genes with greater than two-fold differential expression at a *p-value* < 0.05 in the 3D radical system as compared to a differential expression of 117 genes in the 3D non-radical system.

CYGB gene encodes a globin protein which is produced in response to oxidative stress.[126] We see a three-fold upregulation of this gene which is indicative of the cells response to oxidative stress due to the exposure to free-radicals. FOXQ1 (related to embryonic development, cell cycle regulation, tissue-specific gene expression, and cell signaling) was upregulated two-fold indicating a possible consequences of UV exposure on tissue development.

GADD45b (inhibitor involved in cell cycle checkpoints induced by genotoxic stress)[94] is dowregulated two-fold in hMSCs exposed to UV radiation in conjunction with radicals. A downregulation for this gene might imply that the cell divison continued after DNA damage resulting in the formation of mutated cells.[108,127] GAS1 gene is a growth arrest specific gene which is involved in growth suppression.[73] We saw a two-fold downregulation of this gene which implies variations in cell growth mechanisms.

The final response against cellular damage is initiation of apoptotic factors. ALPK2 is involved in DNA-repair mechanisms and apoptosis.[128] BCL2-like11 is an apoptosis facilitator.[127,129] A four-fold upregulation in the expression of these genes is indicative of increased chances of apoptosis due to cell damage. Table 8 has a list of differentially expressed genes with maximum fold-change occurring due to the combined effect of free-radicals and UV radiation exposure in a 3D culture system. The heat map generated to depict the gene expression change for this comparison is shown in Figure 15.

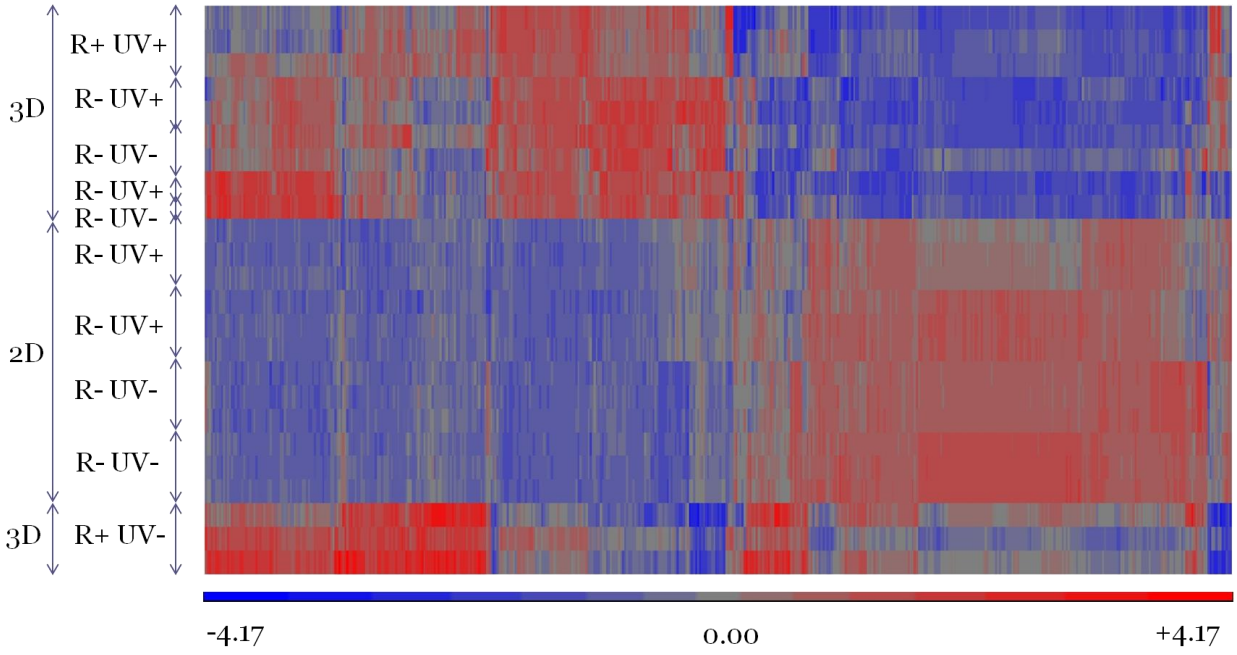


Figure 15: Heat Map for Effect of UV Radiation in Combination with Radicals: The heat map depicts the differential expression of 507 genes in cell cultured in the presence of radicals in combination with UV exposure.

Table 8: Differentially Expressed Genes with Maximum Fold-Change Due to the Combined Effect of Free-Radicals and UV Exposure

Gene Name	Fold Change	Gene Function
CHI3L1	+4.98	Involved in defense against pathogens and/or tissue remodeling. Plays an important role in cellular response helping cells cope with changes in their environment.[123]
ARHGAP29	+4.59	Plays an essential role in blood vessel tubulogenesis.[130]
BCL2L11	+4.26	Induces apoptosis and anoikis.[131]
RGS2	+4.06	May play a role in leukemogenesis and is involved in inhibition of the translation of mRNA into protein.[132]
SNED1	+3.76	Associated with islet cell tumor.[133]
QK1	+3.57	Codes for RNA-binding protein that plays a central role in myelinization. Regulates oligodendrocyte differentiation and maturation in the brain. Can act as a translational repressor.[134]
SLC16A6	+3.51	Responsible for catalyzing the rapid transport across the plasma membrane of many monocarboxylates.[135]
NUPL1	-4.22	Codes for a protein which is a component of the nuclear pore complex, a complex required for the trafficking across the nuclear membrane.[136]
UFM1	-4.32	Ubiquitin-like modifier protein which binds to a number of target proteins, such as DDRGK1.[137]
GNRH1	-4.34	It is associated with ovarian disease and myoma.[138]
ZNF786	-4.44	May be involved in transcriptional regulation.[139]
TAF9b	-4.48	Essential for cell viability and involved in gene regulation with apoptosis.[140]
CENP BD1	-5.14	Involved in DNA binding.[141]
CCNL1	-7.37	Transcriptional regulator which participates in regulating the pre-mRNA splicing process during cell cycle.[142,143]

We processed the differentially expressed genes using IPA to understand the functional pathways they affected. Figure 16 shows selected pathways affected to a large extent from the UV exposure and the radicals. The majority of genes that changed were involved in the

cell cycle pathway followed by cellular assembly, DNA replication, DNA repair, and cancer. This can be correlated back to various studies conducted in the past which show that oxidative stress on cells induces DNA damage repair pathways, and if the damage cannot be repaired, pathways leading to cell apoptosis are initiated.[124,125]

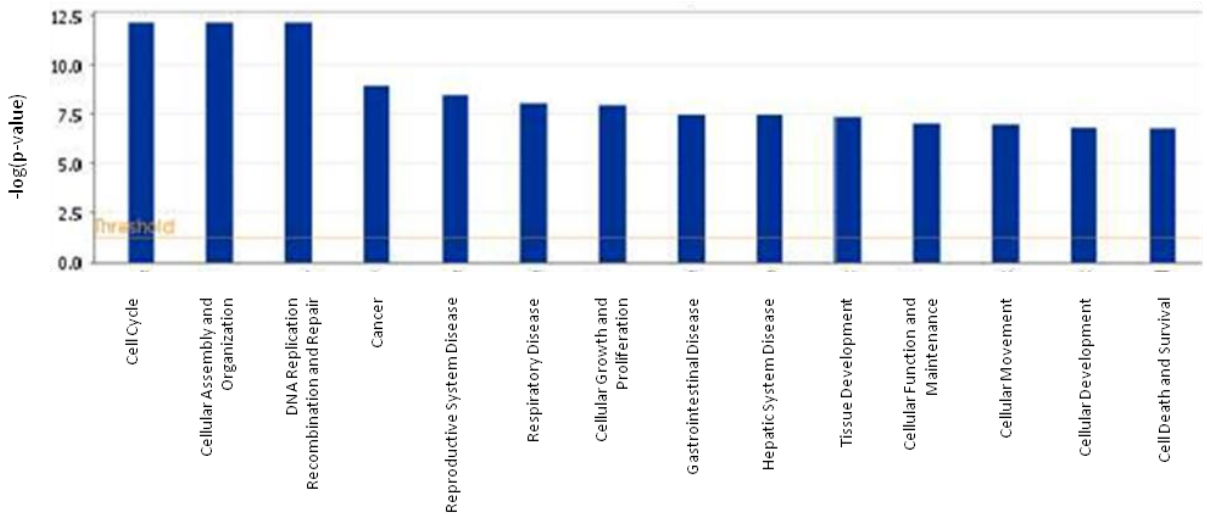


Figure 16: IPA Analysis of Effect of UV Radiation on Cells Cultured in Free-radical 3D System: Differentially expressed genes under 3D R+ (UV+ versus UV-) condition were analyzed using IPA to understand the cellular pathways affected.

6 CONCLUSIONS AND FUTURE DIRECTIONS

We were interested in understanding the effects of various cell culture conditions on the gene expression profile of hMSCs. Our goal was to understand if the currently used conditions for cell culture affect gene expression of cells, since previous studies do not provide that information. We were specifically interested in understanding the effects of UV radiation, 2D versus 3D cell culture system, and free-radicals on the gene expression profile of hMSCs as a model system.

We conclude that, in both 2D and 3D systems, UV radiation alone does not cause significant changes in the gene expression profile. However, when combined with radicals in the 3D radical polymerization system, UV exposure causes relatively higher levels of change in gene expression as compared to the 3D encapsulation system that does not use radicals. We attribute this result to the fact that UV accelerates the production of free-radicals in the system, thus placing the cells under greater oxidative stress.

Another important observation is that the microenvironment of the cell plays a very important role in controlling its genetic expression. Our results showed that culture systems play an integral role in regulating the gene expression of hMSCs. 5164 genes had significant changes in their expression when we compared 2D system with the 3D systems (without radicals). We also saw 3030 genes with altered expression levels in between the two 3D systems- with and without radicals. These observations provide direct evidence that radicals in the system affect cells at the gene level. Across these multiple sample sets we were able to generate an enormous amount of data in the form of changes in gene expression levels of

thousands of genes. Our data will help us better understand the changes in functional pathways with differential gene expression.

Our aim for the scope of this project was to understand if these experimental conditions caused any changes in the gene expression of hMSCs. We were successful in concluding that the UV radiation used in tissue engineering applications does not cause any significant change in the gene expression of hMSCs under typical use. Additionally, culturing hMSCs in 2D compared to 3D systems leads to a significant change in the gene expression levels of hMSCs. We were also successful in demonstrating that, at the genetic level, radicals present in the system in combination with UV exposure causes changes in the expression levels of genes associated with immune response, apoptosis and cancer pathways.

Our future goals are to analyze the genes changed with these experimental conditions and understand their role in different functional pathways of cellular functions with assays such as reverse transcription PCR and western blots. The data generated by our experiments can be used in the future to understand the consequences of these conditions on particular pathways and also various other downstream effects caused by the gene expression change. Within the generated data set, there is a need to further analyze the data in depth and understand the exact consequences of the different conditions-whether they have a positive effect or a deleterious effect. It would also be interesting to understand the consequences of culturing cells in dynamic (degrading) systems and analyze cell recovery after the fabrication process.

Finally we conclude that the cell culture systems need to be designed keeping in mind the changes that may occur in the genetic makeup due to those processes. Additionally, UV

radiation used for experiments around cells does not play a significant role in changing the gene expression but a more thorough analysis of the microarray data is necessary.

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