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Effect of Dimensionality on In Vitro Growth Environment and Mesenchymal Stem Cell Function

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EFFECT OF DIMENSIONALITY ON IN VITRO GROWTH ENVIRONMENT AND MESENCHYMAL STEM CELL FUNCTION

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biomedical Engineering

By

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B.S. in Textile Technology, Bangladesh University of Textiles, 2011

2018

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Fatema Tuj Zohora ENTITLED Effect of Dimensionality on In Vitro Growth Environment and Mesenchymal Stem Cell Function BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science in Biomedical Engineering.

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The use of the standard two dimensional (2D) cell culture has laid down the fundamentals of molecular and cell biology. However, recent advances in cell-based regenerative medicine raises the concern on deconstructing cellular behaviors in more physiologically relevant three dimensional (3D) microenvironments. Differences in cell response in 2D versus 3D systems arise from the perturbations in gene expression patterns that stem from how cells sense their underlying 2D or surrounding 3D matrices and adjust their phenotypes accordingly. Thus, cells are no longer considered as a solitary entity of genome but a context arises from a combinatorial interactions of cell-ECM, cell-cell, and cell-biomolecules that constitutively organize the tissues and ultimate functional organs. In this regard, this project was intended to identify cell behaviors and gene expression pattern in 2D versus 3D culture systems. To construct 2D versus 3D cultures of human adipose tissue-derived mesenchymal stem cells (hASCs) were seeded on the top of plastic substrates or encapsulated in the self-assembled polypeptide-based hydrogel PuraMatrix™, respectively. Adipogenic, osteogenic, and chondrogenic differentiation in these two different culture systems was induced with the corresponding lineage-specific biochemical induction medium. The effect on growth and differentiation of hASCs under these two culture conditions was assessed through changes in cellular morphology,
survivability, proliferation, and gene expression pattern during lineage-specific differentiation.

According to the cytotoxicity assay, PuraMatrix™ provides a suitable 3D microenvironment for hASCs proliferation and differentiation without the need of any exogeneous cell adhesion motifs, however an influence of initial cell seeding density was seen in maintaining high initial cell viability and subsequent functionality. Gene microarray analysis revealed that, compared to 2D culture, 3D culture shows more of the expected lineage-specific gene expression and supports tissue specific developmental characteristics.
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CHAPTER I
INTRODUCTION

For over a century, cell culture has been a valuable tool in maintaining viable cells outside the body and supporting a myriad of research studies for deconstructing cellular behaviors, tissue physiology, and developing cell based therapeutics. The standard plastic/glass-based cell culture techniques has defined our basic understanding of key molecular and biophysical mechanisms at the single cell level and helped in the development of cellular and molecular biology field as a whole. However, this strategy has failed to fully represent the natural *in vivo* microenvironment, to make meaningful associations between tissue specific cellular architecture and function, such as cell proliferation, migration, differentiation, or immunomodulation, due to the lack of cell-cell, cell-matrix interactions, and spatial distribution of signaling molecules that cells experience in natural microenvironments. This natural microenvironment constitutes a complex fibrous network known as the extracellular matrix (ECM), which involves fibers with a wide distribution of diameters and intrafibrillar spacings, and provides the basis for distributing biochemical and mechanical cues to regulate cell functions\(^1\). This is very different from 2D culture systems, where cells grow on top of hard substrate, with good access to oxygen, growth factors, nutrients, and the removal of waste metabolites\(^2\). Switching cultured cells from a flat 2D environment to a 3D matrix-based systems one not only stresses cells physically, but also alters their spatial distribution and expression of adhesion molecules through which they interact with their environment\(^3\). This event
triggers a rearrangement of their cytoskeleton and activation of mechanotransduction pathways to control their proliferation, polarity, fate, gene/protein expression, cellular metabolism, motility, and survivability.

The pioneering work by Mina Bissell showed how 3D culture systems changed the malignant behavior of breast cancer cells, just by altering the way cells interact within 3D microenvironments: the cells reversed their aberrant phenotype and growth patterns, and seemed to become non-malignant, things previously unnoticeable in 2D cultures\(^4\). When similar type of tumor cells were injected into nude mice, they restored their form and functionality and reduced the malignancy\(^4\). Chondrocytes are another notable example, which require to be cultured in 3D spheroids or encapsulated in gels to retain their functionality\(^5\). Also, mesenchymal stem cells (MSCs) grown in chondrogenic induction media show minimal lineage-specific marker expression on 2D flat surfaces, which significantly increases when grown as 3D spheroids\(^6\). These are just few examples of how in vitro 3D cultures manipulate cellular behavior relative to native microenvironments. Furthermore, 3D culture possesses key biological features, such as collective cell migration, force generation and tissue folding, which occurs during gastrulation, angiogenesis, and migration of cancerous cells in metastasis; all of these are associated with high-order cell processes, inherent in 3D but unnoticeable in 2D culture\(^7\). Therefore, considering physiologically relevant cell or tissue specific responses, 3D culture strategies show a promising potential to quantitatively model biological systems, from cells to organisms, along with improved prediction to drug, cytotoxicity screening which may potentially reduce the use of laboratory animals\(^8\) and consequent immunogenic disparity. However, defining a 3D model to model tissue-specific responses is still challenging, since
it requires consideration of biomaterial properties and its interaction with diverse signaling domains and cell types. Since, researchers have demonstrated that hydrogels are a better model of the \textit{in vivo} microenvironments, the proposed research comprises a synthetic polypeptide hydrogel named PuraMatrix™ for 3D modeling of MSCs.

MSCs are an attractive cell source for developing cell therapeutics or tissue engineering because of its multipotency, low immunogenicity, free of ethical concerns, and easy accessibility due to the variety of tissue sources\textsuperscript{9}. Moreover, there is no evidence that, like embryonic stem cells or induced pluripotent stem cells, MSCs express cancer genes under any conditions\textsuperscript{10}. However, re-organizing the niche microenvironment \textit{in vitro} is a key challenge, as MSC functionality varies not only with its tissues of origin\textsuperscript{11}, but also with the species\textsuperscript{12} and even within strains of same species\textsuperscript{13}. Another fact is that, native MSC niche is still not well characterized, probably due to the lack of having defined the appropriate biomarkers\textsuperscript{14}. Culture dimensionality is another crucial aspect that have been shown to directly affect their phenotype, mechanotransduction pathway activation, and ultimate functionality. We hypothesized that, MSCs in a 3D microenvironment will behave differently than their 2D counterparts in lineage specific differentiation. Therefore, the primary purpose of this study was to decipher MSCs behavior and gene expression pattern in lineage specific differentiation pathway to adipogenesis, osteogenesis, and chondrogenesis in 2D versus 3D models.
EXPERIMENTAL AIMS

The present research evaluated:

i) Viability and proliferation of 3D hydrogel encapsulated human adipose tissue-derived mesenchymal stem cells (hASCs) compared to their 2D counterparts.

ii) Effects of cell seeding density on hASCs viability and proliferation in 2D versus 3D culture systems.

iii) PCR array-based gene expression analysis on hASCs to their tri-lineage differentiation pathway to adipogenesis, osteogenesis, and chondrogenesis in 2D versus 3D microenvironment.
CHAPTER II
LITERATURE REVIEW

1. Mesenchymal Stem/Stromal Cells

In 1970, Friedenstein, et al. first isolated fibroblasts like non-hematopoietic cells from Guinea pig bone marrow that were adherent to glass substrates and were capable to form colonies during 9 days of culture. Grown in monolayers, when the initial cell seeding density was high enough (i.e., $1 \times 10^7$), these cells showed bone tissue formation without osteogenic induction medium. Later, additional studies revealed their multi-differentiation potential to various mesenchymal lineages, including osteoblasts, adipocytes, chondrocytes, tenocytes, and smooth muscle cells, as well as to non-mesenchymal lineages, such as neuronal cells, endothelial cells, hepatocytes, and cardiomyocytes either in vitro or in vivo within various animal models. Since Friedenstein’s seminal work, the gold standard to identify these cells was the colony forming unit fibroblast assay, which defines fibroblasts like spindle shaped adherent cells that proliferate to form distinct colonies from single precursor cells under appropriate culture conditions. These fibroblasts-like adherent cells are currently referred to as either ‘mesenchymal stem cells’ due to their ability to differentiate into mesenchymal lineages, or ‘mesenchymal stromal cells’ as they coexist with the stroma and provide suitable microenvironment to support the proliferation and differentiation of hematopoietic stem cells. However, to elucidate the apparent discrepancy between nomenclature and biological properties, The
International Society for Cellular Therapy (ISCT) proposed the terminology ‘multipotent mesenchymal stromal cells’, irrespective of their tissue source, while the term ‘mesenchymal stem cells’ is specifically limited for those who meet the stem cell criteria. Meanwhile, based on existing data, the ISCT proposed three criteria in defining human MSCs: adherence to plastic substrates maintained under standard culture conditions; expression of specific surface antigens CD105, CD73, and CD90, and lack of expression of CD34, CD45, CD14 or CD11b, CD79α or CD19, and HLA-DR; and in vitro differentiation to three mesenchymal lineages, i.e., osteoblasts, adipocytes, and chondroblasts.

Bone marrow (BM) is the widely-studied and well-accepted source of MSCs, which has been used for several therapeutic applications, including the treatment of acute myocardial infarction, graft versus host disease, and osteogenesis imperfecta. However, researchers have isolated MSCs from a variety of tissues including compact bone, articular cartilage, synovial membrane, synovial fluid, skeletal muscle, deciduous teeth, fat, blood vessels, lung, heart, tonsil, skin, and several birth related tissues (umbilical cord/Wharton’s jelly, cord blood, placenta, and amnion). Regardless of tissue sources, MSCs share similar type of morphology and functional features, although, finer differences exist probably due to singularities in tissue-specific niches. Moreover, the frequency of MSCs from each tissue types vary significantly. Mechanical separation, enzymatic degradation, or gradient centrifugation yields around 0.0001% of MSCs from bone marrow (BM) and almost 0.1% from adipose tissue (AT). Yoshimura, et al. found that, among rat MSCs isolated from five mesenchymal tissues (i.e., BM, synovium, periosteum, adipose, and muscle), synovium possessed the highest proliferation and colony forming
capability, while BM had the lowest. In contrast, with the similar tissue sources from human, Sakaguchi, et al.\textsuperscript{11} found the greatest cell number per colony in BM-derived cells. However, in both cases, synovium-derived MSCs possessed the highest capacity for chondrogenesis. Therefore, beyond separation methods, experimental settings, or donor specific issues (such as age, healthy or diseased state), MSCs possess distinct proliferation and differentiation capability depending on tissue source and species. Also, there are some marked differences between adult and neo-natal tissue-derived MSCs. There is mounting evidence suggesting the superior performance of neo-natal over adult MSCs in terms of non-invasiveness, greater yields, survivability, proliferation, differentiation, immunosuppression, and immune-regulation capabilities\textsuperscript{30,32}. However, Kern and coworkers\textsuperscript{25} found similar immune phenotypes in BM, AT, and umbilical cord blood (UCB)-derived MSCs taken from several human donors. In addition, they observed significant difference in the success rate of MSCs isolation: BM and AT (100%) > UCB (63%). The colony forming frequency was greater in AT and lower in UCB, and strikingly, UCB MSCs did not show adipogenic differentiation capacity. Nevertheless, UCB MSCs showed the longest and highest proliferation capacity over other cell types. These findings demonstrate that, both UCB and AT MSCs could be attractive alternatives to BM MSCs in clinical applications.

2. In vivo MSCs niche

The so called ‘stem cell niche’ is a 3D microenvironment, which provides stem cells with an anatomical and functional structure\textsuperscript{33}. The native stem cell niche is an
organized structural unit, which consist of stem cells surrounded by a number of diverse non-stem cells, ECM, and other soluble and insoluble molecules that help stem cells either to maintain their quiescent state, self-renew, or differentiate into specific lineages\textsuperscript{34}. The cells interact with ECM molecules via adhesion receptors known as integrins, whereas cell-cell interaction occurs mainly through cadherin-based adherens junction\textsuperscript{34} and integrins\textsuperscript{35,36}. These transmembrane adhesion proteins act as physical anchors, through which the niche microenvironments regulate stem cell behavior by activating cellular signaling programs or mechanotransduction pathways\textsuperscript{33}. The niches’ unique microenvironments, complex biochemistry, and architecture provides a combinatorial array of intrinsic (e.g., cytoskeletal proteins, transcription factors) and extrinsic stimulatory factors (e.g., secreted factors by other cell types like TGF-beta, bone morphogenetic proteins and Wnts; anchorage proteins such as integrins and cadherins) in a spatiotemporal manner\textsuperscript{37}.

MSCs occupy a prevalent and tissue-specific stem cell niches owing to their existence in a large number of tissue types\textsuperscript{38}. However, the niche microenvironments of MSCs within their respective tissues are still undefined, mostly attributed to the absence of unique, reliable \textit{in situ} identification markers in their different developmental stages\textsuperscript{14}. For example, although there is growing evidence suggesting the perivascular location of MSCs\textsuperscript{39}, thus far, no direct evidence has been found, showing pericytes’ ability to proliferate and differentiate into mature cell phenotypes in response to injury \textit{in vivo}, a key characteristics of stem cells including MSCs\textsuperscript{12}. 
ECM is a key regulatory component of stem cell niche. Beyond just providing anchorage support to hold the cells in their niche, ECM proteins act as a reservoir that binds and releases growth factors and signaling molecules in a controlled manner\(^{40}\). Moreover, the ECM helps cells maintain their natural phenotype, coordinate cellular communications, and provides architectural support to direct tissue morphogenesis\(^{9,41}\). Research studies suggest that, the presence of ECM is enough to manipulate MSC fate\(^{42}\). However, no specific ECM components have been identified, which have been shown to induce changes from their undifferentiated state\(^{43}\).

Oxygen concentration in the niche environment is an important soluble factor, which regulates cell viability and lineage commitment\(^{33}\). MSCs in the bone marrow niche experiences hypoxia\(^{43}\), which experimental data suggests, is necessary to maintain the pliability and proliferative capacity of MSCs\(^{43}\). Moreover, hypoxia has been found to
enhance the expression of MT1-MMP by BM-derived MSCs, which is essential to promote cell migration and capillary-tube formation\textsuperscript{44}.

3. In vitro culture microenvironments

In 3D culture systems, cells are generally grown as 3D aggregates, either by seeding them onto solid 3D extracellular matrices, or by dispersing cells into liquid gel followed by solidification\textsuperscript{3}. A wide range of natural and synthetic materials have been investigated as matrix materials, including purified molecules such as collagen I, Matrigel, and even the decellularized matrices\textsuperscript{45}. On the other hand, in traditional 2D monolayers, cell culture is performed on plastic or glass substrates coated with ECM proteins (for adherent cells) and the cells are immersed in a soluble culture media. The monolayer is generally comprised of proliferating cells, as the dead cells are usually detached from the substrate and removed during the exchange of culture medium\textsuperscript{3}. To mimic the physiologic environment, researchers started to use flexible 2D gels forming thin films or coatings on plastic or glass substrates, made up of ECM molecules. However, the mechanics of synthetic gels used to create these pseudo 3D microenvironments vary substantially from that of natural ECM\textsuperscript{46}. Such synthetic gels exhibit linear elastic behavior, whereas the fibrillar nature of natural ECM shows complex non-linear elastic behavior with appreciable viscous components\textsuperscript{46}. Contrary to 2D culture, a 3D matrix requires additional control over internal microstructures such as pore size and shape, porosity, pore interconnectivity, and matrix degradability, for efficient cell seeding density, cell migration, and effector transports, which is far more challenging than in 2D cultures\textsuperscript{47-51}. In brief, the cell behavior can be regulated by incorporating biochemical (i.e., ECM/scaffold composition), and molecular
(i.e., growth factors, cytokines) factors, by manipulating the geometry and physical properties of the matrix, or by applying external mechanical forces, which may induce different cellular responses, depending on how they are presented in 2D or 3D.

3.1 Cell adhesion control:

The MSC adhesion process in cell culture involves protein adsorption, cell interaction with the adsorbed proteins, cell attachment and spreading on the implant surface. In cell culture, protein adsorption on flat surfaces (e.g., tissue culture polystyrene) occurs nearly instantaneously forming a 2–5 nm layer through molecular-scale interactions with the substrate. Such proteins are part of the serum which is used to supplement the cell culture media and include ECM proteins such as collagen, thrombospondin, fibronectin, vitronectin, and osteopontin. The adhesion strength of anchorage dependent cells, including MSCs, relies not only on surface topography, surface charge, and hydrophilicity of physical substrate, but also on the presence of proteins, peptides, and other ECM molecules on the surfaces, added exogenously or secreted by them. A range of cell adhesion molecules have been explored, including integrins, cadherins, proteoglycan (e.g., syndecans), selectins, and the immunoglobulin (IgCAMs). Among these molecules, integrins are the most-studied and major transmembrane receptors for ECM proteins, expressed on the cell surfaces via an endocytic-exocytic transportation mechanism, and can recognize the specific amino acid motifs for binding. A minimum density of integrin ligation is critical for cell adhesion. On the other hand, overexpression of adhesion receptor may diminish cell motility.

Adherent cells express integrin heterodimers towards specific motifs found in various ECM proteins, such as, fibronectin (α5β1 and αvβ3), collagen (α2β1 and α1β1),
vitronectin (αvβ3), and laminin (α6β4)\textsuperscript{56,59,60}. MSCs are known to express α1-5, v, and β1,3,4, as well as α6, 11, x, and β2,7,8 integrins\textsuperscript{36}. However, integrin expression has demonstrated to be differentially regulated in 2D versus 3D culture\textsuperscript{61}. Cell adhesion to the ECM components causes integrin clustering and the formation of focal adhesion complexes which in time, grow in size and complexity, and recruit numerous cell signaling proteins such as FAK, vinculin, tensin, paxillin, c-src, p130Cas, and others\textsuperscript{62}, which regulate all subsequent cellular events such as strengthening of cell adhesion, cell survival, growth, differentiation\textsuperscript{63}, immune responses, and hemostasis\textsuperscript{64}.

Cell-matrix interactions can also be manipulated by tethering synthetic moieties, on 2D substrates or 3D hydrogels, even without adhesive proteins or in serum-free medium\textsuperscript{65,66}. In protein-free conditions, positive functional group manipulate integrin ligation via electrostatic interactions\textsuperscript{65}. Moreover, serum proteins have been shown to absorb non-specifically onto substrates in a poorly controlled manner, which may influence cell-matrix interactions\textsuperscript{6}. Although, direct comparisons on how surface functionalization correlates with culture dimensionality during cellular adhesion is scarce, it is obvious that cell adhesion in 3D is highly variable owing to their structural diversity. For example, substrate stiffness or specific topographies may recruit many structural proteins and organize differently in 3D than in 2D, or the specific pore sizes which may encourage the cells to form mature focal adhesions\textsuperscript{7,66}.

3.2 Initial Cell Seeding Density

Optimization of cell seeding density is particularly important in 3D cultures, where high density and spatially uniform distribution of cells within a construct is required to maintain their viability and subsequent functionality\textsuperscript{67,68}. Regarding 3D culture, gel types
and properties, concentration of gel macromers, and initial cell seeding density are crucial factors in defining an optimum formulation\textsuperscript{69}. For example, cartilage tissue constructs based on an agarose hydrogels are independent of cell seeding density, as agarose does not provide natural cell adhesion motifs, which preclude cell-mediated tissue remodeling\textsuperscript{69}. Conversely, in a hydroxyapatite hydrogel, which support direct MSCs contact via CD44, showed enhanced biomechanical properties of a mature cartilage construct in low macromer concentration with high initial seeding density\textsuperscript{69}. Also, a recent study on how MSC seeding density affects cell morphology and proliferation showed that cells seeded on soft (i.e., 500 Pa) polyacrylamide 3D hydrogels at higher densities, exposed to cell-cell interactions, were less sensible to matrix stiffness, increased cell coverage area, than when seeded at low densities\textsuperscript{70}. In addition, they formed mature focal adhesions and prominent stress fibers similar to cells cultured on stiff substrates. This higher density seeding also induced an increase in cell proliferation by decreasing the intercellular spacing and allowing cells to interact with each other by contracting the hydrogel fibers. This dependency on cell-cell mechanical contact via contractibility for cell proliferation has been previously demonstrated\textsuperscript{71}. The hydrogel contraction also induces an increase in matrix stiffness, which has been shown to dictate MSC lineage commitment during differentiation\textsuperscript{72}. These effects will be further discussed below.

\textbf{3.3 Matrix mechanics:}

\textit{Matrix Stiffness}

Substrate stiffness is one of the major physical factors that has been demonstrated to manipulate a large number of cellular behaviors, including cell adhesion\textsuperscript{73}, directional migration\textsuperscript{73}, cell spreading\textsuperscript{33}, proliferation\textsuperscript{74}, apoptosis\textsuperscript{75}, and differentiation\textsuperscript{76}. In native
environments, the cells encounter a range of stiffness values, from soft to hard tissues, loosely to tightly packed connective tissues, or from early to late stages of wound healing\textsuperscript{45}. Cells employ stress on their matrix during normal physiological functioning, differentiation, morphogenesis, and tissue remodeling\textsuperscript{50}. Alternatively, matrix elasticity directs intracellular rheological properties, i.e., a stiffer matrix promotes stiffer cells and vice versa\textsuperscript{77}, inducing cells to differentiate into specific lineages. For instance, cells on stiffer substrates promote osteogenesis, intermediate myogenesis, and soft substrates induce adipogenesis or neurogenesis\textsuperscript{78,79}. Therefore, to get the appropriate cellular responses, cells must be placed in a substrate where they can feel their native tissue specific mechanical properties.

As the anchoring points of adhesion molecules vary with covalent crosslinks and consequently the pore sizes (longer on greater pore sizes and vice versa), the adhesion strength and mechanical feedback from cells decreases rapidly with increasing anchoring distance\textsuperscript{80}. Therefore, except on the softest gels, cells do not directly sense the underlying bulk stiffness\textsuperscript{80}, but instead they can sense the ECM anchoring distance through which they anchor and pull against to deform it, translating this force into mechanotransduction signals\textsuperscript{45,78}. Furthermore, by varying the density of a covalently attached collagen layer on a polyacrylamide hydrogel of constant stiffness affected MSC differentiation capacity. Lower anchoring points induce cells to behave in a way typically found on softer substrates\textsuperscript{80}. At the same time, ECM molecules have a minimal effect on softer matrices, as cells get easily dislodged from their anchoring points and cannot form stable focal adhesion complexes to generate enough traction forces.
For both 2D and 3D cultures, cell-mediated traction forces for deforming the ECM determines the structure and dynamics of cell-matrix adhesions, and subsequent cell signaling and behavior\textsuperscript{46}. The sensing of ECM stiffness and ultimate cell fate may vary from 2D to 3D cultures depending on how ECM is presented in respective system\textsuperscript{46}. For example, in 3D, ECM spacing may alter during matrix remodeling, deposition of cell secreted ECM proteins over culture periods, matrix degradation, and so forth\textsuperscript{45}.

*Topography:*

Similar to tissues *in vivo*, cells may encounter a range of surface topographies from macro-, to micro-, and nanoscale\textsuperscript{81}. Each of these features has a great potential to directly modulate cellular morphology and function by physically confining or aligning the cell body according to the topographies, via changes in available surface area for protein adsorption, and ECM deposition\textsuperscript{81}.

In spite of a large number of studies demonstrating that microtopography can precisely manipulate MSCs behavior *in vitro*, stem cells in native tissues *in vivo* interact with their surroundings through nanoscale features\textsuperscript{10}. Also, in native microenvironments cells reside within hierarchically organized nanofibrillar networks of ECM\textsuperscript{82}. In case of 3D scaffolds with fiber mesh, both nano and microfiber possesses some disadvantages associated with cell adhesion, mass transport and cellular infiltration through the pores (the pore size increases with the fiber diameter). For example, large pores in microfiber mesh may inhibit cells from creating bridges between them, or high flow rate of effector molecules may wash them away, thus compromising efficient cell adhesion and seeding density\textsuperscript{83}. On the other hand, cells on nanofiber scaffolds may create a monolayer due to the hindrance of cellular infiltration\textsuperscript{84}. 
3.4 Biochemical cues:

In vivo, the stem cell niche microenvironment is finely tuned with spatiotemporal distribution of biochemical and physical factors that direct their stemness or lineage specification. However, several studies with 2D cultures demonstrated that physical cues, either internal or external, are enough to regulate stem cell fate, including MSCs.

In a 2D experiment, Park, et al.\textsuperscript{85}, showed that, matrix stiffness alone is not always enough to elicit specific or terminal cell differentiation, such as in the case of chondrogenic vs. adipogenic differentiation, as the same matrix stiffness showed the capacity to differentiate into multiple lineages depending on the presence of a specific cytokine. Furthermore, while several 2D and 3D experiments demonstrate that external strain alone can induce MSCs differentiation to bone cells, smooth muscle cells, or cardiac muscle cells\textsuperscript{86,87}, others suggest that the synergy between mechanical and biochemical stimulation is required\textsuperscript{88}, being able to significantly enhance bone cell formation and matrix mineralization by adding a small amount of induction factors, than by mechanical stimulation alone\textsuperscript{89}.

3.5. Effector biomolecule delivery:

In the native microenvironment, an interplay exists among cells, soluble bioactive agents, and ECM proteins that provides biochemical and mechanical cues to direct stem cell fate\textsuperscript{33}. In addition to serving as adhesive supports to the cells, the ECM plays crucial roles in manipulating the spatiotemporal distribution of oxygen, nutrients, and soluble effector molecules such as growth factors, morphogens, cytokines, hormones, and
bioactive peptides. These molecules usually act together in directing the growth, migration and differentiation of cells in a dynamic, 3D microenvironment. These bioactive agents remain matrix-bound forming concentration gradients, which diffuse through or stay sequestered by the ECM, activating cellular signaling pathways upon binding cell surface receptors. These gradients of signaling molecules are crucial for maintaining a wide range of biological functions, including development, inflammation, tissue homeostasis, angiogenesis, wound healing, and cancer metastasis.

In 2D monolayer cultures, biomolecules, either secreted by cells or exogenously added, can mix via convection or undergo free diffusion through the medium, leading to rapid equilibration. In contrast, in 3D matrices convection-based mixing is reduced, limiting the diffusion of large biomolecules or nutrients. Stable gradients or sustained release of soluble biomolecules are necessary to reveal the long-term morphogenetic events which are typically in the area of 3D cultures where ECM supports the sustainable gradients. Even in 3D spheroids, aggregates on pallets, or as suspensions in media can effectively capture the diffusion-mediated sustainable gradients.

4. Effects of culture microenvironments on MSC behavior

The structural differences between 2D and 3D microenvironments influences cells, making them behave in different ways. One of the major differences observed in 2D versus 3D, is their effect on the cell shape or morphology. On 2D substrates cells attach to the underlying substrate with only one side, while in 3D matrices cell adhesion occurs via entire surfaces. This is how they recognize their substrate geometry. Another crucial aspect is, on restrain-free 2D planar surfaces, cell spreading occurs within minutes, while it may
take hours, or even days for this to occur within 3D matrices, as the cell spreading occurs via compromising with the surrounding matrices\textsuperscript{94}. Furthermore, 3D cultures sustain cell-cell communication in a physiological manner, which is crucial for collective cell migration\textsuperscript{95}. Moreover, some cells, such as epithelial cells show epical-basal polarity which is found to be lost on 2D planer surfaces, but which is regained when cultured in 3D\textsuperscript{7}. This epical-basal polarity is especially important for tissue organization and cell viability\textsuperscript{7}. However, according to researchers, MSCs does not show this kind of polarity. Briefly, culture dimensionality along with the matrix properties guide cell shape, degree of cell spreading, the way cells interact with the neighboring cells and matrices which ultimately control the cell proliferation, migration, viability, gene expression, differentiation, and mechanotransduction pathways.

4.1 Cell shape/geometry:

Cell shape, a key regulator of MSCs differentiation\textsuperscript{96} appears to be manipulated by the substrate stiffness and topography. Similar to other anchorage-dependent cells, the morphology of MSCs is determined by the adhesive interactions with adjacent cells, ECM, and internal configuration of the cytoskeleton\textsuperscript{97}. At the molecular level, cell shape is regulated by the polymerization of actin microfilaments affiliated with myosin filaments, resulting in an assembly of actomyosin filament bundles (known as stress fibers) and consequent propagation of traction forces that cells exert on their ECM via focal adhesion contacts\textsuperscript{98}. Evidence suggests that cell shape and cytoskeletal organization changes, occur mainly on soft substrates, and not on rigid substrates, due to the presence of highly tensed stress fibers\textsuperscript{98}. Several \textit{in vitro} studies show that MSCs can respond to matrix stiffness by differentiating into specific lineages related to tissues with a similar stiffness, changing
their shape accordingly. In particular, 2D culture substrates shown to directly manipulate cell shape and subsequent functionality via alternations in stiffness-mediated integrin binding, adhesion strength, stress fiber formation, and cell contractility\textsuperscript{98}. Moreover, an interplay exists between geometric shape and cell population, where the cytoskeleton dynamics of cell, due to sensing of the pattern edges, influences adipogenic differentiation\textsuperscript{99}. Tang, et al.\textsuperscript{99} found that the manipulation of cell-cell contacts affected the choice between adipogenic and osteogenic differentiation of MSCs on micropatterned surfaces via gap junctional communication. However, research studies on 3D MSCs suggests that neither the cell nor nuclear morphology are particularly required to direct the MSCs fate in response to 3D substrate stiffness, but rather that the matrix rigidity and adhesion ligand density seemed to act conjointly by directly regulating the formation of integrin-ligand bonds at the cell-matrix interface. Hence, opposed to 2D culture systems, 3D cultures requires the controlling of various parameters including ligand density, matrix elasticity (or crosslinking density), and crosslink type or degradation mechanics of the hydrogel, may act cooperatively to regulate the MSCs fate in 3D.

4.2 Survivability:

For anchorage dependent cells, apoptosis is typical in those that dissociate from their ECM or attach through the wrong molecules\textsuperscript{100}. Apoptosis via impairment of integrin-dependent cell-matrix contact is termed anoikis. Activation of focal adhesion kinase via specific integrin ligation can effectively suppress anoikis\textsuperscript{100}. Not only integrin occupancy, but also minimal changes in cell shape and cytoskeleton organization are necessary to completely inhibit cell anoikis or to promote long-lasting cell survival. It has been found that, endothelial cells attached on RGD-coated plastic substrates in which they could only
spread, effectively rescued them from anoikis, whereas if the adhesion area was too small, with round shape either on fibronectin, vitronectin, or RGD, led them to apoptosis. This phenomenon can simply be explained as cell number regulation via competition for available adhesive interactions. Like cell shape, cell-cell interactions are also sensitive to anoikis. Disruption of cell aggregation via an αv integrin antibody was found to induce apoptosis. In vivo, local cell viability is particularly dependent on the local oxygen concentration. Core cells in a 3D constructs or monolayered cells on 2D substrates, both may experience depleted oxygen and poor viability if cultured under static conditions. On the other hand, hyperoxic conditions (21% O₂), show detrimental effects on cell growth via enhancing reactive oxygen species, and subsequent chromosomal aberrations.

4.3 Gene/Marker expression

In 2D versus 3D microenvironments, MSCs often show distinct differentiation and gene expression patterns, depending on the adhesion molecules expression, initial cell seeding density or cell-cell contact, matrix stiffness, topography, external physical forces, or soluble and insoluble factors.

Initial cell seeding density is one of the major factors that can strongly influence the expression of specific genes as a function of time in certain cell types. This is seen in MSCs via changes in cellular morphology or by altering the auto/paracrine signaling distances. According to the several research works, low initial seeding density induces the expression of genes associated with cell proliferation. This is due to the facts that proliferation is manipulated by the available surface area for attachment, as well as the contact-inhibition mechanism between adjacent cells. It has been demonstrated that, in 2D cultures, MSCs seeded at a high density tend to promote adipogenesis, whereas those
seeded at low cell density differentiate towards bone, cultured in a mixed or respective differentiation media\textsuperscript{106}. Likewise, substrate stiffness and topography effects MSC gene expression via changes in cell shape or morphology. Although, several studies demonstrate that it is actually the role of cell-matrix interactions rather than cell morphology. Approaching 3D culture systems an experimental analysis manifested that, in place of cell density and associated cell-cell interactions or morphology, cell-mediated traction forces via cell-matrix interactions determine the lineage specific gene expression in MSCs encapsulated in a covalently crosslinked hyaluronic acid hydrogel\textsuperscript{94}. Moreover, gene expression patterns in 3D matrices is highly variable, depending on the several controlling parameters.

5. ECM mimetics

The infrastructure of native ECM contributes to the complex network of fibrous structural proteins including fibronectin, collagen, laminin, and vitronectin varies with tissue types\textsuperscript{107}. These proteins provide the binding sites for integrins. These are a type of transmembrane protein expressed on the cell surfaces through which the cells find its structural support and senses the mechanical properties of ECM. Hydrated proteoglycans occupy the inter-fiber free spaces and sequester a range of soluble biomolecules, such as growth factors, cytokines, hormones, and other nutrients. Cells continuously reorganize this microstructure via integrin ligation with ECM motifs, acting in a bidirectional manner that elicits intracellular signaling cascades to secrete ECM-cleaving metalloproteinases (MMPs) and at the same time deposit matrix proteins. A finely tuned event which plays a
crucial role in cell migrations, tissue homeostasis, pathogenesis, morphogenesis, and regenerating stages\textsuperscript{11}.

A range of biomaterials have been widely investigated for their suitability as scaffold structures. Natural polymers include collagen, fibrin, chitosan, alginate, agarose, and hyaluronic acid; whereas common synthetic polymers include poly(ethylene oxide), poly(acrylic acid) and e.t.c\textsuperscript{108}. Hydrogel, a form of crosslinked networks of either natural or synthetic polymers with a high water-retention capacity have been demonstrated to effectively capture physiological microenvironments. The crosslinking method constitutes temperature, UV light, or incorporation of chemical chelators\textsuperscript{108}. Some important properties of hydrogels that regulate their physical, mechanical and diffusive properties, and potentially help to maintain cell physiology are: porosity, wettability, adhesive ligand presentation, gelling condition, degradation kinetics, and non-toxic degradation products\textsuperscript{108}. Synthetic hydrogels are often restricted by not being capable of producing natural ECM-type fibrous networks, which can be overcome by coupling self-assembly or nanofabrication strategies with degradable hydrogels\textsuperscript{107}.

Moreover, promoting cellular functionalities by incorporating biological motifs in synthetic hydrogels sometimes possess serious limitations, such as, denaturation of entrapped proteins, heterogeneous dispersal of motifs throughout the hydrogels, or agglomeration due to multiple binding sites in a single space, resulting in disconcert in normal cell binding mechanisms\textsuperscript{107}. This requires the use of a unique hydrogel that supports natural cell binding phenomena by stimulating the cells to deposit their own ECM.
6. PuraMatrix™ Hydrogel

PuraMatrix, also called RAD16-I/RADA16-I is named after its purity in molecular specificity compared to other biologically-derived biomaterials, such as Matrigel, and collagen which is contaminated with other unspecified components\textsuperscript{109}.

PuraMatrix is a type of synthetic, molecularly designed ionic self-complementary oligopeptides, consisting of alternative hydrophilic and hydrophobic motifs, 16 amino acid in length\textsuperscript{110}. In water, they assemble to form a stable β-sheet structures with two surfaces; one non-polar alanine residues and the other polar surfaces with ionic complementary side chains of positively charged arginine and negatively charged aspartate, respectively. When these oligopeptides come across monovalent salts or physiological solutions, they spontaneously associate into stable macroscopic membranes composed of interweaving individual filaments of about 10-20nm in diameter\textsuperscript{110,111} with interstitial voids of approximately 50-200nm\textsuperscript{112}. The porosity, high water content, and nano-fibrillar network they form, closely mimics the gross extracellular environments of native tissues. Although, the resulting hydrogel is very fragile, its mechanical properties dramatically increase with cell-secreted ECM deposition\textsuperscript{112}. This hydrogel is stable under a wide range of pH values, high temperatures, denaturation agents (like SDS, guanidine hydrochloride, and urea), and is resistant to proteolytic enzyme degradation\textsuperscript{111,113}. The fiber density, and consequently the mechanical properties, are proportional to the concentration of peptides used to form hydrogels\textsuperscript{114}. Since, the amino acids used are L-isomers, their degradation yields normal amino acids\textsuperscript{110}, which can be reused by the body\textsuperscript{115}. 
Figure 2: Molecular modeling and amino acid sequence of RADA16-I\textsuperscript{116}.

Several studies suggest that a large variety of mammalian cells can attach and proliferate on these hydrogel surfaces and even help to differentiate in a controlled manner\textsuperscript{117,118}. \textit{In vivo} studies with various xenograft models demonstrated their biodegradability, non-cytotoxicity, lack of immunogenicity or tissue inflammation\textsuperscript{110,117}. Although, the RAD motif simulates the well-characterized RGD integrin binding motif, the cell adhesion mechanism is independent of integrin ligation\textsuperscript{110}. An alternative theory for initial attachment is ionic interactions since RAD16 contains complementary charged residues which may interact with cell surface molecules\textsuperscript{110}. However, with time cell outgrowth may occur via interactions with absorbed ECM proteins secreted by them\textsuperscript{110}.

7. Role of chemical induction factors on lineage specific differentiation of MSCs

The current protocol to induce adipogenesis is to supplement MSC growth medium with dexamethasone (Dex), isobutylmethylxanthine (IBMX), indomethacin, and insulin. For osteogenic differentiation, the cocktail comprises Dex, ascorbic acid, and beta-glycerophosphate (β-GP). Chondrogenesis can be initiated with insulin, ascorbic acid, and
a growth factor TGF-β3. These biochemicals work cooperatively in a concentration and time-dependent manner to direct lineage specific differentiation of MSCs.

Ascorbic acid (L-ascorbate 2-phosphate, derivative of ascorbic acid) is reported to enhance proliferation of diverse cell types, either by direct modulation of growth-regulated signaling pathways or possibly indirectly via deposition of collagen matrices\textsuperscript{119,120}. L-ascorbate 2-phosphate is an effective osteo-inducer for hASCs. Dexamethasone controls MSC proliferation in a concentration-dependent manner\textsuperscript{121}. Where 10nM encourages MSCs proliferation\textsuperscript{122}, 100nM is enough to restrain their expansion. Depending on the induction time, concentration, cell type, and ECM type, dexamethasone can induce all three lineages\textsuperscript{123,124}. In an \textit{in vitro} analysis with human BM MSCs isolated from 30 normal patients, the optimal concentration of Dex for mineralized bone nodule formation was 10nM, which is equivalent to the physiological glucocorticoid level\textsuperscript{125}. For osteogenic induction, Dex works together with L-ascorbate 2-phosphate. A research study with hASCs showed that, lower Dex (5~10nM) and higher L-ascorbate 2-phosphate (250~150µM) promote cell proliferation, expression of Runx2 and higher ALP activity\textsuperscript{122}. In contrast, higher concentration or prolonged exposure of Dex favor adipogenesis over osteogenesis in MSCs\textsuperscript{124}. IBMX is a phosphodiesterase inhibitor which works in conjunction with Dex to regulate PPARG. IBMX elevates intracellular cAMP and activates the protein kinase A (PKA) signaling pathway, essential for transcriptional regulation of PPARG. Moreover, both IBMX and Dex induce C/EBPβ and C/EBPδ, key transcription factors for preadipocytes\textsuperscript{124}. Furthermore, in adipogenesis-induced MSCs, while IBMX induce Pref-1 expression and maintains the preadipocyte stage, Dex suppresses Pref-1 overexpression in a time-dependent manner, promoting adipocyte differentiation\textsuperscript{126}. Indomethacin acts as
a cyclooxygenases (COX) inhibitors and accelerates adipocyte differentiation via activation of PPARG\textsuperscript{127}.

Beta -glycerophosphate (β-GP) appears as a phosphate reservoir. Inorganic phosphate (Pi) serves as an intracellular signaling molecule which enters into the cells and activates ERK signaling pathways to promote Pi-induced osteopontin and BMP-2 expression\textsuperscript{125}. Insulin promotes cell proliferation and absolutely necessary for in vitro chondrogenesis of MSCs\textsuperscript{128}. It has been suggested that, this substance structurally conforms to IGF-I, resulting in IGF-I receptor activated MAPK signaling to control MSCs adipo- or chondrogenesis\textsuperscript{124,128}. Moreover, in adipogenesis, insulin stimulates the cells to accelerate glucose uptake from the microenvironment, which restored as triglycerides in adipocytes\textsuperscript{129}.

In this study, we hypothesized that the combination of abovementioned protocol with appropriate 3D culture conditions could improve the lineage-specific differentiation of MSCs.
CHAPTER III
MATERIALS AND METHODS

1. Experimental design: The two major experimental groups were: hASCs seeded on 2D plates or encapsulated hASCs in 3D hydrogel. Each experimental group consisted of three different biochemical stimulation groups, namely adipogenic, osteogenic, and chondrogenic differentiation. The entire experiments were divided into two sections. The first part contains: (i) hASCs viability and proliferation in 2D versus 3D as a function of time, (ii) Effect of seeding density on viability and proliferation in 2D versus 3D as a function of time, and (iii) Cell growth with the progression of lineage specific differentiation in 2D vs 3D. The second section consists of comparative study of gene expressions in 2D vs 3D of hASCs biochemically-induced to adipogenic, osteogenic, and chondrogenic differentiation.

2. hASCs culture/subculture: Passage 1 human hASCs were purchased from Lonza. According to the manufacturer, these cells were isolated from a non-diabetic adult lipoaspirates via liposuction and express CD13, CD29, CD44, CD73, CD90, CD105, and CD166 and do not express CD14, CD31, and CD45 until passage 5. Passage 1 cells were subcultured until passage 4 and maintained in human MSCs expansion media (ScienCell, USA) at 37ºC in 5% CO₂. The media was changed every two to four days based on cell confluency. Upon full confluency at passage 4, the cells were cultured in 2D and 3D systems for experimental analysis.
3. **2D versus 3D culture:** In 2D culture, ADSCs were seeded in tissue culture treated plastic well plates. For mimicking 3D microenvironments, cells were encapsulated within the Puramatrix™ hydrogel and then placed in non-treated plastic well plates. The seeded cells were maintained either in normal ADSCs growth media for control or treated with biochemical induction factors for adipogenic, osteogenic, or chondrogenic differentiation. For 3D culture, two-third media was changed every two days, whereas in 2D culture, almost all media was changed every three days. The initial cell seeding density was 100,000 cells/1.9 cm² (2D) or 100,000 cells/100 µl of total gel (3D) for all analysis, except for low density cultures where seeding density was reduced to half of the total amount per above mentioned area.

4. **Biochemical induction to tri-lineage differentiation:** For adipogenic induction, proprietary hMSCs (ScienCell, USA) growth media was supplemented with 10⁻⁷M dexamethasone (Acros Organics), 0.5mM IBMX (Sigma), 60µM indomethacin (Alfa Aesar), and 10µg/ml insulin (Santa Cruz). This recipe was used in cells analyzed for adipostimulant cell proliferation, actin staining, and adipogenic differentiation staining. However, for adipogenic gene expression analysis the above-mentioned recipe was revised with the addition of 250µM L-ascorbate-2- phosphate (Sigma)¹³⁰ and a slight increase in dexamethasone concentration, from 10⁻⁷M to 10⁻⁶M. Osteogenesis was induced with 10⁻⁷M dexamethasone (Acros Organics), 2mM beta-glycerophosphate (Santa Cruz), and 50µM L-ascorbate-2- phosphate (Sigma) in hMSCs growth medium. Chondrogenic induction media consisted of 10ng/ml TGF-β3 (Sigma), 6.25µg/ml insulin (Santa Cruz), and 50nM L-ascorbate-2-phosphate (Sigma). Cells were cultured in normal hMSCs growth media (ScienCell, USA) as control.
5. Cell viability and proliferation: Lactate dehydrogenase (LDH) (CytoTox 96® Non-Radioactive Cytotoxicity Assay kit, Promega, USA) colorimetric cytotoxicity assay was used to quantify cell viability and proliferation. LDH is a cytosolic enzyme released from damaged cells and indicates cellular cytotoxicity. The enzymatic reaction converts the tetrazolium salt INT into a red formazan which shows strong absorbance at 490-520nm. For high initial seeding density, cytotoxicity assay was performed at 7 h (day 0), 48h (day 2), 168h (day 7), and 288h (day 12) of culture. The same time points were selected for the low-density culture, but with the exclusion of 7h. For cell growth during differentiation, the seeded/encapsulated cells were induced at the onset of day 3 and the growth observed at 168h and 288h of initial culture. For proliferation analysis at each time point, cultured cells were first washed with Dulbecco’s Phosphate Buffered Saline (PBS) and then incubated at 37°C with 50µl (4mg/ml) type-I collagenase per well for 30mins. After incubation, the samples were lysed with 500 µl 1× TritonX-100, followed by bath sonication for 1 hr. Then, lysed samples were centrifuged at high speed for five minutes. Next, 50µl of supernatant and 50µl of LDH substrate was added in a flat bottom clear 96 well plate and kept on an orbital shaker for 30 minutes. The reaction was stopped immediately after 30 minutes with stop solution. Finally, absorbance or optical density was recorded at 490nm wavelength by using BioTek™ Synergy™ H1 Plate Reader and cell counting was performed with calibration curve from standard well plate.

6. Differentiation staining: For differentiation staining, all samples were first washed with PBS, then fixed in 4% cold (4°C) paraformaldehyde for 10 and 15min for 2D and 3D cells, respectively. Subsequently, the fixed samples were washed three times with Milli-Q water. For adipogenic staining, the samples were incubated with 60% Oil Red O working
solutions at room temperature in the dark for 30 mins. After removal of dyes, the samples were washed with Milli-Q water for several times. For chondrogenic differentiation, cells were stained with Alcian blue 8GX (1% dye, made with 3% acetic acid), and kept in dark at room temperature for around 16 hours. Then the sample was rinsed with 0.1M HCl for 1 time and subsequently washed with PBS and Milli-Q water for several times.

For osteogenesis assessment the culture was stained for Alkaline Phosphatase with Stemgent® AP Staining Kit II, according to manufacturer’s protocol. Briefly, osteogenic samples were washed with PBS containing 0.05% Twin 20. Then cell fixation with fixative (provided in the kit) solution: 5min for 2D and 10min for 3D. The samples were washed two times with DPBS, stained with staining solution for 15 min, followed by washing in DPBS for twice and fixed in mounting medium for microscopic observations.

7. Actin staining: The cytoskeleton was stained with F-actin Visualization Biochem Kit™ (Rhodamine-Phalloidin based) following manufacturer’s instructions. Briefly, the samples were first washed with wash buffer, then fixed with provided fixatives for 10 min. After fixation, the cells were washed two times with wash buffer, permeabilized with permeabilization buffer at room temperature for 5 min, followed by washing twice again in wash buffer. Next, F-actin and DAPI was added concurrently and kept for 30min in the dark at room temperature. After staining, the samples were washed twice with the same washing buffer and mounted with mounting medium. The stained samples were visualized by using Cytoviva Hyperspectral Microscope System with triple-pass emission filter for DAPI/FITC/TEXAS RED.

8. RNA isolation, cDNA preparation, and qPCR assays: RNA was isolated at day 9 of induction with RNeasy plus mini kit (Qiagen, USA). Initially the samples were washed
and incubated with type-I collagenase, similar to the cell viability assay procedure. After incubation, samples were lysed with 350 µl buffer RLT plus and subsequent vortexing (for 2D) or homogenization (for 3D) with TissuRuptor II (Qiagen, USA). The rest of the procedure was proceeded with the manufacturer’s protocol. Isolated RNA was quantified spectroscopically with NanoDrop 1000. cDNA was prepared from 500ng total RNA using the RT² First Strand Kit from Qiagen. Gene expression analysis was performed with RT² Profiler PCR array for human mesenchymal stem cells (Qiagen, USA), which contains 5 housekeeping genes (i.e., ACTB, B2M, GAPDH, HPRT1, and RPL13A), genomic DNA control, reverse transcriptase control, positive PCR control, as well as different 84 genes for expression analysis. All the genes were simultaneously amplified with 96-well Applied Biosystems StepOnePlus™ Real-Time PCR. The real-time amplifications were observed with StepOnePlus™ software version 2.3. The baseline and threshold value were set in agreement with manufacturer’s instructions and kept constant across all samples analyzed. The Ct value for each gene was extracted from StepOnePlus™ software to analyze the gene expressions data with PCR array online data analysis software from Qiagen. To be mentioned, all three cell types in 2D models were normalized against B2M, whereas 3D adipogenesis were normalized with RPLP01 and 3D Osteo and chondrogenesis with HPRT1. All the genes from each differentiated group were subsequently normalized with the expression level of genes in control group from the same time point. The pathway enrichment analysis was performed by using Kyoto Encyclopedia of Genes and Genomes (KEGG) database.
9. Statistical analysis: The statistical analysis for cell viability and proliferation was accomplished with ANOVA in JMP Pro13. Residual plots and normality assumptions were investigated, and data transformation was performed as well wherever necessary. PCR array online data analysis software from Qiagen provided p-values built on student’s t-test of the replicate $2^{(-\Delta CT)}$ values of each gene in the control and treatment groups. Except clustergrams, all other graphs for data visualizations were constructed with Python coding, version 3.6.
1(a). Cell proliferation and effects of initial seeding density

hASCs viability and proliferation was measured at different time points: 7h (day 0), 48h (day 2), 168h (day 7), and 288h (day 12), with initial seeding density of 100,000 cells/1.9 cm$^2$ (2D) or 100,000 cells/100 µl of total gel volume (3D) (Fig 3A). 7-hour post-seeding, 2D showed higher cell number than the initial value, indicating that some cells might have proliferated in the meantime, whereas 3D compromised their viability with 27.9% reduction in number (Fig 3A). After 48 hours, 2D became full confluent or overconfluent, while 3D regained their initial seeding number. This scenario dramatically changed after 168 hours: 2D proliferated slightly, but cells in 3D became 7.87-fold higher than the cell number found at day 2. The progression of proliferation became slow after 168 hours and 3D possessed higher cell number than 2D constructs.

To assess how seeding density effects on hASCs growth in 2D versus 3D microenvironments, the same experiment was repeated with half seeding density, i.e., 50,000 cells/1.9 cm$^2$ (2D) or 50,000 cells/100 µl of total gel volume (3D). Cell proliferation was assessed in three-time points: 48h (day 2), 168h (day 7), and 288h (day 12) (Fig 3B). Only 66.4% cells survived at day 2 post-encapsulation, whereas 2D cell number was 3.9 times larger than the initial seeding value. At day 7, 3D showed significant proliferation, a phenomenon also observed in high density 3D culture. At the same time, 2D exhibited
significant increase in number than day 2 2D cell number, and 1.4-fold higher than the corresponding high-density culture. The 2D proliferation rate decreased after day 7, but the cell number is higher than high density 2D culture at day 12. As for 3D model at day 12, the proliferation rate significantly reduced than day 7, and the total cell number is lower than the complementary day 12 3D culture with high initial seeding density.

Figure 3: hASCs growth in 2D versus 3D microenvironments in low vs high seeding density (A and B). Effects of seeding density on ADSC growth by fold change (C and D). Fold change represents the ratio of cell number between two time points.

It was assumed that, high initial seeding density would help to maintain high hASCs viability encapsulated in hydrogel. After 48 hours of encapsulation, while the high-density culture induced a 5% increase in cell number, the low-density culture had a 33.6% reduction. It seems that hASCs encapsulated in PuraMatrix hydrogel takes approximately 2 days to adapt to the microenvironment before starting to proliferate. Since PuraMatrix
provides only ionic interactions\textsuperscript{110}, it is plausible that these peptides lack necessary binding motifs to induce the cells to express specific integrin receptors for proper cell-matrix interaction. Minimal changes in cell shape, cytoskeleton organization, and integrin occupancy is essential to inhibit cell ‘aniokis’, i.e., the apoptosis via impairment of integrin-dependent cell-matrix contact\textsuperscript{100,101}. Moreover, in the initial period of encapsulation, diffusion limited access to media and gases in the nanoporous structures and consequent low pH of the microenvironments might be a prominent cause of initial cell death in 3D constructs. However, serum absorption with time or cell secreted ECM deposition likely stimulated the cells to express specific integrins for strong adhesions, which helped them to regain inherent fibroblastic morphology, rearrangement of matrix molecules and intracellular machinery to actuate cellular functionalities. Furthermore, the high-density culture promotes high cell-cell contact, induces paracrine signaling, and changes in many local environmental cues\textsuperscript{96}, which might explain the initial high cell viability in the high-density culture. Nonetheless, due to contact-inhibited growth arrest, the proliferation rate was much lower in high density 3D culture. An interesting outcome is that, in both high and low-density 3D models, the cell proliferation was significantly higher between days 2 and 7, where they attained full confluency and after which proliferation proceeded to slow down.

Contrary to 3D encapsulation, the 2D culture maintained a high cell viability during all time points. In high density culture, cells on 2D substrate proliferated faster, reached confluency earlier, resulting in a constant and slower proliferation at later time points. In contrast, low density 2D model showed steady and high cell proliferation for a longer time, from day 0 to 7, after which the proliferation decreased significantly. To be noted, despite
the number of cells seeded in both 2D and 3D constructs, the growth area is different and larger in 3D hydrogel, which explains higher cell proliferation or larger cell numbers in 3D before they reach confluency. In all, comparing high- versus low-density culture in 2D and 3D model, high initial seeding density stimulates high initial cell proliferation, maintains greater cell viability, and vice versa.

1(b). Cell growth during lineage specific differentiations

Next, we look at cell proliferation biochemically stimulated using induction media for adipogenesis, osteogenesis and chondrogenesis (Fig 4).

Figure 4: hASC proliferation under adipo, osteo and chondrogenic media as a function of time in 2D(left) vs 3D(right).

Adipocyte differentiation proceeds in several stages: pre-confluent preadipocyte starts to proliferate and continue until they stop proliferation due to contact inhibition. Then, the confluent preadipocytes enter into cell cycle again and go through several mitotic divisions, called mitotic clonal expansion. When cell division arrests at confluency, preadipocyte starts to differentiate into adipocyte\textsuperscript{131}. In both 2D and 3D adipogenic system,
cell number is approximately constant after day 7, suggesting growth arrest of preadipocytes to initiate cellular differentiation into adipocytes. Regardless of similar trend in cell growth in 2D versus 3D adipogenic microenvironment, the gene expression analysis shows different scenario in stage specific maturation during adipogenesis, as the recipe for adipogenic media was different for proliferation and gene expression analysis. Introducing the revised induction media acted on higher cell growth (data not shown). In 2D model, lipid droplets started to accumulate at day 5 and 98% cells had intracellular lipids by day 9, whereas in the same period, most cells in 3D adipogenic constructs still showed fibroblastic morphology. The possible explanation here is that due to the large growth area in hydrogel, cells in 3D proliferated for a longer time until contact inhibition allowing the start of adipogenesis. However, this was not a focal area of this paper and no data is shown. Further investigation is necessary to identify whether ascorbate plays any role in adipocyte differentiation via enhancing cell proliferation and direct cell to cell contact. Contrary to adipogenesis, osteogenic models are representing linear increase in cell growth, probably supporting the growth of osteoprogenitors. The osteogenic differentiation proceeds with rapid cell proliferation and formation of tightly packed cell colonies which promote dense cell patches. In the case of chondrogenesis, the 3D chondrogenic model demonstrates exit from cell cycle, suggesting the onset of prehypertrophic zone, whereas cells in 2D chondrogenic model is still proliferating, but at a slower rate.
2. Nuclear Shape and Actin Cytoskeleton Organization

![Fluorescently stained images of F-actin cytoskeleton (left) and nucleus (right) of hASCs following 9-day induction with adipogenic, osteogenic, chondrogenic, or normal growth medium. The stained samples were visualized by using Cytoviva Hyperspectral Microscope System. Scale bar: 60 µm.](image)

Figure 5: Fluorescently stained images of F-actin cytoskeleton (left) and nucleus (right) of hASCs following 9-day induction with adipogenic, osteogenic, chondrogenic, or normal growth medium. The stained samples were visualized by using Cytoviva Hyperspectral Microscope System. Scale bar: 60 µm.
Images representing stress fiber organization and changes in nuclear shape for 2D versus 3D constructs; 2D adipogenesis with disrupted actin cytoskeleton and round shaped nucleus, suggesting a switch from fibroblastic to adipocytic morphology. 3D adipogenesis exhibits 3D fibrous network with a long-distance interconnection, indicating lower cell density in 3D. Although, the network consists of bundles of stress fibers, the nearly rounded nuclei is substantiating the stress formation at the basal level, whereas tension in perinuclear actin cap is probably not strong enough to elongate the nuclei (Fig 5.a,b). Osteogenic models feature cell and cytoskeleton alignment possibly because of high cell confluency following induction. This cytoskeleton alignment and elongated nuclei is expected during osteogenesis. The strong actin filaments and elongated cell nuclei are more prominent in 3D than 2D osteogenic model (Fig 5.c,d). The chondrogenic system appears to have a large variation in nuclear shape and cytoskeleton organization in 2D versus 3D. 3D chondrogenic constructs show thick actin fibers and highly elongated nuclei consistent with cellular condensation during chondrogenesis, while 2D depicts less tensed and nearly rounded nuclei (Fig 5.e,f). hASCs on 2D substrate and in hydrogel encapsulation sustained fibroblastic morphology in normal growth medium (Fig 5.g,h).
3. Differentiation Staining

Figure 6: Tri-lineage differentiation of hASCs in 2D versus 3D culture microenvironments assessed with oil red O for lipid, alkaline phosphatase staining kit for alkaline phosphatase, and Alcian Blue staining to confirm proteoglycan components of the ECM associated with adipocytes, osteoblasts, and chondrocytes, respectively. The original magnification is 100X for all images. Adipogenesis and chondrogenesis was captured at day 21 of differentiation induction, whereas osteogenesis represents alkaline phosphatase activity at day 14 of osteogenic induction. The stained samples were visualized by using Olympus Epi Fluorescence Spot Scope.
4. Gene expression

The predefined 6 experimental groups of hASCs for the biochemical induction of three lineage differentiation in 2D and 3D are: 2D (2AM) vs 3D (3AM) adipogenesis, 2D (2OM) vs 3D (3OM) osteogenesis, and 2D (2CM) vs 3D (3CM) chondrogenesis. These were analyzed for gene expression profiles using the RT² profiler PCR array for human mesenchymal stem cells. A total 84 genes were divided into several categories. Besides lineage specific genes, including adipogenesis, osteogenesis, chondrogenesis, myogenesis, and tenogenesis the array comprises stemness markers, MSCs specific genes, and some other genes MSCs can express. In all figures, bar graphs representing the data from PCR array analysis as fold change of total 84 genes in 6 defined models relative to controls (horizontal dotted lines). Error bars denote 95% confidence interval and * depicts p-value of <0.05.

(i) Adipogenesis-related genes

PPARG is the key transcription factor of adipogenesis, whereas RHOA and RUNX2 are negative regulators of adipogenesis. 2D adipogenic group shows PPARG as being up-regulated, while RUNX2 and RHOA being down-regulated (Fig 7A). 3AM shows no changes in PPARG expression, but at the same time significantly downregulated RHOA, and RUNX2 expressions (Fig 7B). 2D osteogenic representative shows downregulation of PPARG and RHOA, but upregulation of RUNX2 (Fig 7C). In contrast, 3D osteogenesis upregulated both PPARG, and RHOA, but shows no changes in RUNX2 expression (Fig 7D). 2D and 3D chondrogenic model exhibited similar expression pattern-downregulated PPARG expression significantly, but upregulated RHOA and RUNX2 (Fig 7E and F).
Figure 7: Adipogenic genes in 2D vs 3D adipo, osteo, and chondrogenesis model. Error bars denote 95% confidence interval and * depicts p-value of <0.05.
(ii) Osteogenesis-related genes

Osteogenic category comprises 14 genes of which 2AM overexpressed BMP6, FGF10, PTK2, SMURF1, and TGFb1, and minimally expressed or downregulated all other osteo-associated genes (Fig 8A). Compared to 2D adipogenic model, 3D adipogenesis induced significant downregulation of PTK2, SMURF1, and TGFb1, but overexpressed HNF1A, and TBX5 (Fig 8B). However, both adipogenic model significantly downregulated RUNX2 expressions. 2D osteogenic model upregulated three genes- BMP4, RUNX2, and BGLAP, and downregulated all other genes including SMURF1 and SMURF2, known as negative regulators of osteogenesis (Fig 8C). In contrast, 3D osteogenesis didn’t show RUNX2 expression, however roughly expressed other osteo-associated genes, such as BMP2, KDR, PTK2, TBX5, and differentially expressed TGFb1, and TGFb3 along with downregulation of SMURF1, and SMURF2 (Fig 8D). 2D chondrogenic model overexpressed most of the osteo-related genes, including COL1A1, RUNX2, BGLAP, BMP2, BMP6, HDAC1, KDR, PTK2, SMURF1, and TGFb1 (Fig 8E). Compared to 2D model, 3D chondrogenesis downregulated BGLAP, and HDAC1, however induced significant upregulation of BMP2, BMP6, FGF10, HNF1A, KDR, PTK2, SMURF1, TBX5, and TGFb1 (Fig 8F).
Figure 8: Osteogenic genes in 2D vs 3D adipo, osteo, and chondrogenesis model. Error bars denote 95% confidence interval and * depicts p-value of <0.05.
(iii) Chondrogenesis

Adipogenic model shows some similarities in expression pattern with corresponding chondrogenic model. For instance, HAT1 significantly upregulated in 2AM (Fig 9A) and 2CM, but underexpressed in both 3AM (Fig 9B) and 3CM. BMP6, and SOX9 is overexpressed in all four models. Moreover, TGFb1 is overexpressed in 2AM as well 2CM, whereas ABCB1, GDF7, and ITGAX is significantly expressed in both 3AM, and 3CM. In contrast, 3D osteo model represents upregulation of BMP2, GDF6, ITGAX, KAT2B, and TGFb1 (Fig 9D). Except BMP4, 2D osteogenic model downregulated all chondrogenic genes (Fig 9C). 2D as well as predefined 3D chondrogenic model overexpressed BMP2, BMP6, GDF6, ITGAX, SOX9, and TGFb1. Moreover, 3CM showed additional overexpression of ABCB1, GDF7, whereas 2CM showed no changes of these genes. Furthermore, HAT1 overexpressed in 2CM (Fig 9E), but underexpressed in 3D counterparts (Fig 9F).
Figure 9: Chondrogenic genes in 2D vs 3D adipo, osteo, and chondrogenesis model. Error bars denote 95% confidence interval and * depicts p-value of <0.05.

(iv) Myogenesis

Adipogenic model expressed *JAG1*, and *NOTCH1* in both 2D and 3D microenvironments. However, 2AM stimulated significant upregulation of *ACTA2* (Fig
10A), which is underexpressed in 3AM (Fig 10B). It is noticeable that, all three types of 2D model upregulated ACTA2 with significant level in adipo- and chondrogenesis, but is downregulated in all 3D models. Although, NOTCH1 is upregulated in all models, only significantly upregulated in 2AM and 2CM. Moreover, JAG1 is overexpressed in both adipo models, but underexpressed in chondrogenic models.
Figure 10: Myogenic genes in 2D vs 3D adipo, osteo, and chondrogenesis model.

(v) Tenogenesis

Akin to ACTA2, the most interesting thing here to observe is, GDF15 is overexpressed in all 2D models, but at the same time downregulated in all 3D models.
SMAD4, and TGFb1 is overexpressed in 2AM (Fig 11A), but underexpressed in 3AM (Fig 11B). Another interesting thing is, all tenogenesis classified genes showed inverse relationship in expression pattern in 2D versus 3D osteogenic model. While BMP2, SMAD4, and TGFb1 is downregulated in 2OM (Fig 11C), all three of them upregulated in 3OM (Fig 11D).

Figure 11: Tenogenesis genes in 2D vs 3D adipo, osteo, and chondrogenesis model.
(vi) Stemness markers

This group comprises eight genes, including *FGF2, INS, LIF, POU5F1, SOX2, TERT, WNT3A*, and *ZFP42*. *FGF2, LIF, POU5F1*, and *WNT3A* is overexpressed in 2AM (Fig 12A) but shows underexpression or no significant expression in 3AM (Fig 12B). Although, *INS, SOX2, TERT, and ZFP42* is upregulated in 2AM, these genes are overexpressed in 3AM. Both osteogenic model upregulated *WNT3A*. However, only 2OM showed significant upregulation of *POU5F1* (Fig 12C), which is downregulated by 3OM (Fig 12D). FGF2 overexpressed in both 2D and 3D chondrogenic model. Moreover, except *LIF*, all stemness-related genes overexpressed in 3CM (Fig 12D and F), which is found with opposite scenarios in 2CM (Fig 12E).
Figure 12: Stemness genes in 2D vs 3D adipogenic, osteogenic, and chondrogenic models. Error bars denote 95% confidence interval and * depicts p-value of <0.05.

(vii) MSCs specific genes
Among MSCs specific genes, *MCAM* is overexpressed in all systems, except 2OM. CD44, *ENG* is significantly upregulated in 2AM (Fig 13A) but downregulated in 3OM (Fig 13B). Both 2AM and 3AM represents similar expression pattern for *ALCAM, ANPEP, NGFR, and THY1*. Moreover, *ITGA6* and *FZD9* is significantly upregulated in 2AM (Fig 13A), and 3AM (Fig 13B), respectively. 2OM and 3OM represents no overlap in any significant gene expression. Only *ENG* is underexpressed in 3OM (Fig 13D), whereas 2OM (Fig 13C) induced underexpressed genes are- *CASP3, ITGAV, and NT5E*. In chondrogenic models, *ALCAM, CD44, NGFR, NT5E, and VCAM1* expression is similar in two microenvironments. *ITGAV* is overexpressed in 2CM (Fig 13E) but downregulated in 3CM (Fig 13F). Furthermore, *PROM1*, and *FZD9* is overexpressed in 3CM, but didn’t express in 2CM. 3CM, and 3AM share similar expression pattern of *ANPEP, CASP3, ENG, ERBB2, FGD9, ITGA6, ITGAV, NGFR, PROM1, THY1*. However, *ALCAM, CD44, NT5E* is overexpressed in 3CM and underexpressed in 3AM. On the other hand, *VCAM1* is overexpressed in 3AM, but underexpressed in 3CM. In contrast with 3D models, 2D adipo and chondro shares similar pattern of expression of only two genes: *CD44, and NGFR*. 
Figure 13: MSCs-specific genes in 2D vs 3D adipo, osteo, an chondrogenesis model. Error bars denote 95% confidence interval and * depicts p-value of <0.05.
(viii) Other MSCs related genes

Other genes corresponding to MSCs include 27 genes. Cells possessed dissimilar behavior in expression pattern in 2D versus 3D microenvironments in the same induction group. 2D adipogenic model (Fig 14A) overexpressed \textit{CTNNB1, GTF3A, IL1B, ITGB1, KITLG, MMP2, NUDT6, PIGS}, and \textit{VEGFA}. Among these genes, 3D adipogenesis (Fig 14B) activated only \textit{NUDT6} and minimally upregulated \textit{IL1B} and downregulated all other genes. Conversely, 3AM overexpressed \textit{ENG, FUT1, IFNG, IGF1, IL10, PTPRC}, and \textit{TNF}. 3OM (Fig 14D) overexpressed only \textit{VIM}, and underexpressed \textit{CSF2, IL1B, and IL6}, whereas 2OM (Fig 14C) overexpressed \textit{ICAM1}, and significantly downregulated \textit{SLC17A5}. Moreover, \textit{MMP2, NES, VEGFA, and NUDT6} were upregulated by both 2D and 3D osteogenic model. Some other genes upregulated by 3OM are \textit{PTPRC, SLC17A5, TNF, and VWF}. 2OM upregulated \textit{CSF3, HGF, IGF1, PIGS}, and \textit{VIM}. The expression of \textit{CSF3, MMP2, VEGFA, MMP2, and VIM} is significant in both 2D and 3D chondrogenic models (Fig 14E and F). Besides these genes, 3CM overexpressed \textit{CSF2, FUT1, ICAM1, IFNG, IL6, MMP2, PTPRC, SLC17A5, and TNF}, whereas 2CM overexpressed \textit{CTNNB1, GTF3A, and NES}. 
Figure 14: Other MSCs related genes in 2D vs 3D adipo, osteo, and chondrogenesis model. Error bars denote 95% confidence interval and * depicts p-value of <0.05.
Figure 15: Hierarchical clustergram showing co-regulation of genes from entire datasets across groups or individual samples in 2D culture systems.
Figure 16: Hierarchical clustergram signifying the co-regulation of genes from entire datasets across groups or individual samples in 3D adipogenic model.
Figure 17: Hierarchical clustergram showing co-regulation of genes from entire datasets across groups or individual samples in 3D osteo and chondrogenic models.
Gene expression profiling was initiated with the identification of genes known to contribute lineage-specific hASCs differentiation. Although, the microarray analysis used in this study is not intended to identify differentiation stages, since the plate doesn’t provide stage specific differentiation markers. Moreover, gene expression was not analyzed as a function of time. Yet, to discern hASCs progression with lineage specific differentiation in 2D versus 3D microenvironments, an estimation was performed based on the available differentiation related genes included in the array plates.

To begin with 2D adipogenic model, overexpression of \textit{PPARG} and significant downregulation of \textit{RUNX2} supports that the cells entered into adipocyte differentiation stages, since \textit{PPARG} transcript differentially expressed during preadipocyte to adipocyte differentiation\textsuperscript{134}. However, unlike 2D model where it exhibits the formation of immature adipocytes at day 9, cells in 3D are behind the 2D model regarding their progression to adipocyte differentiation. 3AM induced significant downregulation of \textit{RHOA} and \textit{RUNX2}, known as negative regulators of adipogenesis, however no changes in \textit{PPARG} expression is observed. \textit{BMP7}, a marker of progenitor cells is differentially expressed only in 3AM. Another gene overexpressed in 3D construct is, \textit{FGF10}, which is a potent inducer of adipocyte differentiation and adipose tissue development. Overexpression of \textit{FGF10} indirectly substantiates that the cells are possibly in the clonal expansion stage of pre-adipogenesis, since \textit{FGF10} maximally expressed in clonal expansion stage and exerts its function via stimulating \textit{C/EBP}\textbeta{} expression which is one of the major inducer of \textit{PPARG} transcripts during adipocyte differentiation\textsuperscript{135}.

The osteogenic development can be categorized as three distinct phases: cell growth, matrix maturation, and mineralization\textsuperscript{136}. The cell proliferation and osteoblasts
maturation exhibit reciprocal relationships. According to the cell proliferation data measured at day 10 of osteogenic induction, both 2D and 3D models show nearly linear increase of cell growth, indicating the presence of proliferative state in both culture models. In 2OM, no differentially expressed gene is observed, apart from BGLAP. Upregulation of RUNX2, and BMP4 is detected, but with no statistical significance. BGLAP can express in proliferative stages and reaches its maximum level during matrix mineralization\(^\text{137}\). On the other hand, RUNX2 is an early transcript of osteogenesis, plays pivotal role in the osteogenic commitment of MSCs, but inhibits osteoblasts maturation\(^\text{138}\). In the context of gene expression, it is conceivable that, cells in 2D model is representing the onset of matrix maturation.

Contrary to 2D model, 3D osteogenic model perhaps indicating the accumulation of osteoprogenitors, since no changes in RUNX2 expression, but induced significant upregulation of TGF\(\beta1\) and TGF\(\beta3\) along with BMP2. TGF\(\beta1\)/BMP2 targets RUNX2 expression, which activates matrix regulatory genes, including BGLAP, and COLIA1\(^\text{139,140}\). Research evidence suggests that, TGF\(\beta\) superfamily members promote osteogenesis primarily via enriching the pool of osteoprogenitors\(^\text{141,142}\).

On the other hand, both chondrogenic model induced overexpression of SOX9, a primary transcript of chondrogenesis. Runx2 is a key regulatory factor of hypertrophic chondrocyte differentiation during endochondral bone formation\(^\text{143}\). 3D features upregulation of RUNX2, which is overexpressed in 2D chondrogenic constructs. PPARG, a negative regulator of RUNX2 signaling and osteogenesis is significantly downregulated in both culture systems. It is acknowledged that, SOX9 initiates early chondrogenesis of MSCs, but inhibits chondrocytes to become matured into hypertrophic chondrocytes,
whereas activation of *RUNX2* helps to promote chondrocyte maturation$^{144}$. *RUNX2* starts to express in columnar or prehypertrophic chondrocytes, and exerts its function upon downregulation of *SOX9* in hypertrophic zone$^{144,145}$. *BMP6*, *VEGFA*, and *KDR* is differentially expressed in both chondrogenic model. Expression of VEGF ligand A stimulates upregulation of receptor tyrosine kinase *KDR* to promote angiogenic sprouting and proliferation during skeletal development$^{146,147}$. Furthermore, upregulation of *BMP2*, and *BMP6* precedes chondrogenic hypertrophy$^{147,148}$. Overall, the gene expression form is relatively similar in both culture dimension. However, overexpression of *RUNX2*, *BGLAP* and other osteo-associated genes as well as downregulation of stemness markers in 2D system indicates that, the cells are already in prehypertrophic zone. On the other hand, minimal upregulation of *RUNX2* and overexpression of *BMP2*, *BMP6* implying the inception of prehypertrophic zone in 3D chondrogenic system.

The above discussions substantiate that, the lineage commitment or differentiation progression is more advanced in 2D compared to 3D constructs. A presumption is that, these discrepancies primarily emerged from inefficient cell-cell/cell-matrix interactions at the initial stage of chemical induction. After seeding in corresponding microenvironments, hASCs exposed to lineage specific chemical induction at day 3 at which time cells on 2D substrates showed overconfluency, whereas encapsulated cells possessed sub-confluency, observed under microscopy. The cell proliferation data in 2D versus 3D microenvironments with high initial seeding density also supports this possible scenario. Two days post-seeding, encapsulated hASCs regained their fibroblastic morphology with nearly sparse distribution throughout the matrices, since cell number is low for a larger growth area. McBeath et al showed, regardless of cell growth, hMSCs with lower initial
seeding density (1000 cells/cm²) failed to accumulate cytoplasmic triglycerides in adipogenic induction medium. Contrarily, in osteogenic induction, lower cell density (1000 cells/cm²) showed higher ALP activity than the cells seeded at higher density (25,000 cells/cm²)⁹⁶, suggesting that, cell density in the first few hours of induction is crucial for lineage specific MSCs commitment. High cell density decreases RH OA activity, increases cell to cell communications, and paracrine signaling. Accordingly, we can assume that, 2D possessed earlier lipid accumulation owing to high cell confluency which encouraged cells to sense the softness of neighboring cells rather than the hardness of underlying substrates¹⁴⁹. Regarding osteogenesis, confluent cells on 2D substrate also supported osteogenesis. Since, density dependent osteogenic differentiation was excluded from this experiment, it is impossible to describe how high cell confluency altered osteogenic behavior on 2D substrate, however we observed BGLAP, RUNX2 upregulation and concurrent downregulation of PPARG expression. In contrast to McBeath’s findings, lower cell density in 3D hydrogel didn’t endorse earlier or enhanced osteogenesis, implying that some other factors might play with the cell density to induce lineage commitment in 3D matrices. Puramatrix hydrogel used to encapsulate cells doesn’t provide RGD-dependent integrin ligation. It is assumed that, cells initially interact with matrix molecules via electrostatic interactions and with time progression the cells deposit their own ECM. Owing to this scenario, it is probable that, along with negligible cell to cell adhesions, encapsulated cells also possess weaker interactions with the surrounding matrices and reduced actomyosin contraction, an aspect that is sufficient to reduce osteogenic commitment.
Additionally, there might be an interplay between cell density and substrate stiffness. The present experiment comprised only one type of gel concentration (0.5% w/v) for all three lineages. Perhaps, this concentration is not optimal for all tri-lineage differentiation of hASCs. The Young’s modulus of 0.5% Puramatrix is 2.5KPa, 2D plastic plate is 9GPa\textsuperscript{150}, whereas the modulus of secreted thin matrix in osteoblasts culture is 20-40KPa. Osteogenic commitment predominates in MSCs cultured in matrices that falls within this range of stiffness\textsuperscript{151}. However, the mechanical inadequacy of Puramatrix for osteogenic commitment can possibly be compensated by incorporating ECM proteins. Studies showed, due to highly hydrophilic nature, biomacromolecule composites enhance mechanical stability of fibrils via accumulating matrix bound water as well as releasing free water\textsuperscript{152}. On the other hand, chondrogenic progression seems closer in 2D vs. 3D microenvironments. Chondrogenic commitment depends on the efficiency of cell aggregation, rounded cell nuclei, few focal adhesions, and less tensed or loosely organized actin cytoskeleton\textsuperscript{153}. In conjunction with chondrogenic induction factors, weak mechanical properties of encapsulated cells perhaps fostered 3D chondrogenesis, whereas high cell confluency on stiffer substrates promoted cellular aggregation, spheroid formation, and ultimately chondrogenesis.

Next, we tried to identify expression of genes regulating cellular mechanical properties and mechanotransduction. The mechanical properties of MSCs depend on intracellular actin fiber rearrangement which varies in differentiation pathway\textsuperscript{153}. \textit{RHOA} is a prominent intracellular regulatory gene associated with stress fiber assembly, actomyosin contraction, and consequent activation of many signaling cascades regulating cell functionalities\textsuperscript{154}. Where constitutively active RhoA is essential for MSCs osteogenesis,
inhibiting endogenous $RHOA$ activity promotes adipogenesis or chondrogenesis, regardless of biochemical induction factors$^{153}$. This scenario can be presented in reverse way, where chemical induction media can disrupt or activate actin cytoskeleton to promote adipo/chondrogenesis or osteogenic lineage, respectively. Correspondingly, in this experiment, $RHOA$ is downregulated in both 2D and 3D adipogenic model. Similarly, in osteogenesis, 3D exhibits upregulation of $RHOA$, although at a minimal level. However, $RHOA$ expression is not detected in 2OM, which is in more advanced stages of differentiation than 3OM. Because of high cell growth and consequent densely packed cellular organization in 2D osteogenesis, it is possible that the soluble factors didn’t reach to the single cell level. Serum starvation has been demonstrated to significantly reduce $RHOA$ activity in confluent hMSCs culture$^96$. The same rationale can be applied to 3D chondrogenesis, where excessive gel contraction probably induced the similar effect to express minimal mRNA level of $RHOA$. Moreover, MSCs withstand several shape, cytoskeleton, and matrix reorientation to become hypertrophic chondrocytes which controls $RHOA$ activity. In contrast to 3CM constructs, upregulation of $RHOA$ is higher and significant in 2CM. Research studies suggests that, $RHOA$ signaling is essential to promote columnar chondrocytes proliferation, but it inhibits or delays chondrocyte maturation to hypertrophy$^{155}$. Additionally, two other cytoskeletal gene, $VIM$ and $NES$, encoding intermediate filament protein called Vimentin and Nestin, respectively has differentially expressed in 2CM model. Vimentin is also significantly upregulated in 3OM constructs, shown to play essential role in maintaining cell integrity and Rho or ERK1/2 pathway dependent osteogenesis$^{156}$. The role of Nestin in bone or cartilage formation is underexplored, however Nestin has been demonstrated to express in hypoxic conditions to
impede cellular apoptosis\textsuperscript{157}. In the present experiment, \textit{NES} is downregulated in adipogenic models, upregulated in osteogenic models, and overexpressed only in 2D chondrogenic model.

\textit{PTK2} has a leading role in cell-matrix adhesion. \textit{PTK2} gene encodes focal adhesion kinase (FAK), a component of focal adhesions (FAs). FAs are dynamic structural protein complexes assembled at the cell surfaces which connects intracellular cytoskeleton proteins to the extracellular matrix molecules, hence acts as a signal transducer in a bidirectional manner\textsuperscript{158}. In both chondrogenic model, \textit{PTK2} is overexpressed, perhaps due to the deposition of cartilage matrix molecules. Large assemblage of focal adhesions is particularly important for MSCs undergoing osteogenesis. Nonetheless, in 2D osteogenic construct, \textit{PTK2} is significantly downregulated. This effects probably emerged from high cell proliferation in osteogenic stimulation which dominates cell to cell communications instead of cell-substrate interactions, whilst in hydrogel, \textit{PTK2} might have induced from three-dimensional adhesion of cells with the surrounding matrices. Since, integrin binding mediates focal adhesions, the 3D matrix adhesion is also supported by the upregulation of alpha-6 integrin subunit coding gene, \textit{ITGA6} in 3OM. In contrast to osteogenesis, formation of few focal adhesion encourages adipogenesis. In consonance, 3AM produced significant downregulation of \textit{PTK2}. Yet, 2D adipogenic form which is ahead of 3AM in lineage progression, overexpressed both \textit{ITGA6} and \textit{PTK2}. Enhanced cell-substrate interactions in 2AM probably emerged from protein secretions with the progression of lineage commitment. To be noted, actin disruption may not have any effects on FAs assemblage, which resolves concurrent presence of strong focal adhesions with disrupted actin cytoskeleton in 2D adipogenesis. \textit{ACTA2} (encoding cytoskeleton protein alpha-SMA)
involves in FAs maturation and force generation\textsuperscript{159}, has been overexpressed in 2D chondrogenic microenvironments. *ACTA2* can express in chondrocytes possessing contractile behavior\textsuperscript{160}. This gene is also upregulated in 2D adipogenesis. However, the expression of *ACTA2* is non-detectable in all 3D models.

The above discussion suggests dissimilar behavior of gene expressions in 2D versus 3D microenvironments. Some of them might be attributed to the succession of differentiation stages in 2D than 3D counterparts. To investigate this issue extensively, we looked at the overall gene expression patterns, especially the genes associated with lineage specific differentiation or tissue development. The first noticeable entity is, 3D represents multifarious gene expression in a single system, which is required to function in an *in vivo* like multi-unit complexes to foster complete functional tissues. For instance, the cells in 3D osteogenic model which is assumed to be osteoprogenitors exposed some important genes associated with osteogenic regulations or bone tissue development. These genes include *BMP2, VEGFA, KDR, PTK2, and TBX5*. *BMP2* has a pivotal role in transcriptional and post-transcriptional regulation of *RUNX2* expression\textsuperscript{148}. *PTK2* plays active role in mechanotransduction and particularly important for osteogenesis and bone modeling\textsuperscript{161}. *VEGFA* is known as a key regulator of angiogenesis, expresses during mesenchymal condensations and play essential role in intramembranous and endochondral bone formation\textsuperscript{162}. Overexpression of *VEGFA* during MSCs osteogenesis has been claimed to stimulate matrix mineralization via autocrine mechanisms\textsuperscript{163}. *VEGFA* receptor *KDR* has been found to express in bone cells, such as osteoblasts, osteoclasts, and hypertrophic chondrocytes\textsuperscript{164}. mRNA level of *VEGFA/KDR* increases with the progression of osteoblasts differentiation\textsuperscript{165}, while activation of NOTCH1 signaling is strongly correlated
with VEGFA/KDR-mediated developmental processes\textsuperscript{162}. Besides, VEGFA and NOTCH1 all other genes are downregulated in 2D osteogenic model.

In another example, 3D chondrogenic model additionally induced overexpression of skeletal development markers- $TBX5$, and $FGF10$, which is non-detectable in 2CM. $TBX5$ is essential in activation and maintenance of $FGF10$ expression, which in turn, regulate $TBX5$ expression to initiate and support forelimb outgrowth\textsuperscript{166}. $TBX5$ knockout newborn mice failed to form all skeletal components of the forelimbs\textsuperscript{167}. Hence, although 2D model is ahead in lineage progression, it lacks expression of some important genes regulating tissue development. The expression of few genes may unable to arise complete complexes for functional tissues. Time dependent gene expression analysis is necessary to ensure that the differences in gene expression pattern is a consequence of culture dimension and not resulting from stage specific lineage progression.

Some erratic behaviors in gene expressions have also been observed with 2D model. For instance, $TGFb1$, $Wnt3A$, as well as $CTNNB1$(β-catenin) is significantly upregulated in 2AM. TGFb signaling is acknowledged as a negative regulator of adipogenic differentiation, fostering expansion of preadipocytes, whilst concurrently suppressing preadipocyte to adipocyte differentiation. Additionally, TGFb indirectly inhibits adipogenesis via upregulating WNT signaling pathways\textsuperscript{131}. Likewise, $WNT3A$ and $CTNNB1$, key components of Wnt/beta-catenin signaling pathway inhibits MSCs adipogenic potentiality via suppressing $C/EBP\alpha$ and $PPARG$ expressions\textsuperscript{168}. Although, statistically significant, mRNA levels of these genes are not very high and mRNA expression does not ensure their stable or functional protein synthesis. Probably this is the reason, despite these negative scenarios, the same 2D adipogenic model differentially
induced *PPARG* expression. On the other hand, in 3AM, while *WNT3A* is upregulated, TGFβ and β-catenin is significantly downregulated.

Furthermore, cells in 2D models induced some genes that are not related to specified lineage specific. *ACTA2*, classified as a myogenic gene is differentially expressed in 2D chondro- and adipogenic model with concurrent overexpression of TGFβ1. *ACTA2* is a TGFβ1 responsive gene, encoding α-SMA which promotes contractile activity. α-SMA is a known marker of myogenesis and for contraction of myofibroblasts, and smooth muscle cells, but it can also express in contractile chondrocytes\(^{160}\). However, α-SMA acts as a negative regulator of MSCs adipogenesis and may induced from stiffer underlying 2D substrates\(^{169}\). Nonetheless, 2D adipogenesis showed overexpression of *PPARG* and the image at day 21 exhibits formation of nearly matured adipocytes. Another gene, *GDF15* included in tenogenic differentiation, is differentially expressed in all 2D culture microenvironments. Besides tenogenic regulation, it is also known as stress-stimulated cytokines which protects cells from diverse cellular stresses in culture microenvironments\(^{170}\). It is speculated that, metabolic perturbations due to overconfluency of cells on 2D substrates might have contributed to the emergence of physiologic stresses and consequent overexpression of *GDF15*. Apparently, 2D culture promotes aberrant behaviors in gene expressions in lineage specific differentiation pathway. However, the gene expression analysis used in this experiment is not sole enough to confirm that 2D culture induces non-specific gene expressions. Additional pathway specific analysis is necessary to confirm whether the upregulation of genes from other categories have any meaningful role in the intended lineage specific pathways, since there may have some unidentified roles of genes or crosstalk among signaling pathways, not yet fully discovered.
The only concern with 3D models is upregulation of PPARG (although at a negligible level) in 3D osteogenic constructs, while no expression of RNUX2. PPARG transcript levels found to increase during osteogenesis of hMSCs when the cells were stimulated with mineralized matrix induced hormone DEX, which can directly stimulate PPARG expression\textsuperscript{171}. Study revealed that, 100nM Dex (hBMSCs) can parallely induce adipogenesis and osteogenesis. An optimal condition was found with 10nm Dex with 500ng/mL BMP2 for osteogenic mineralization without upregulating adipo related markers\textsuperscript{172}. Although, the present study was conducted with 100nm Dex for both 2D and 3D osteogenesis, PPARG upregulated only in 3D model. Presumably, 100nM dex along with weak mechanical properties of hydrogel and encapsulated cells may have induced PPARG upregulation. Since, the cells in hydrogel are still in proliferative stage, studies need to clarify whether PPARG downregulates as a function of time with the progression of osteogenic differentiation.

Finally, this study also helps to identify the coregulation of key transcription factors in the progressive pathway of tri-lineage differentiation. To simplify, SOX9 is a key transcription factor of chondrogenic induction, however SOX9 also interacts with RUNX2 and PPARG transcripts to regulate cell fate and differentiation into adipo, osteo, and chondrogenesis. A high ratio (~2) of RUNX2/SOX9 is a key marker of osteogenic potentiality of hMSCs\textsuperscript{173}. Activation of SOX9 induces MSCs commitment to chondrogenesis, but inhibits chondrocytes, as well as osteoblasts maturation\textsuperscript{174}. Although, the role of SOX9 in MSCs transition to osteo- and chondrogenesis is well-studied, but its mechanism in MSCs to adipogenic pathway probably needs more exploration. At the very initial stage of adipogenic differentiation, Pref-1 (preadipocyte marker) upregulates SOX9
gene that directly binds and interacts with \( C/EBP\beta \) and \( C/EBP\delta \) promoters and suppresses their expressions. The expressions of these early markers are crucial to activate adipogenic transcripts: \( PPAR\gamma \), and \( C/EBP\alpha \). Thus, \( SOX9 \) maintains the cells in preadipocyte stages and inhibits preadipocyte differentiation to form adipocyte. In other words, repression of Pref-1 mediated \( SOX9 \) overexpression is essential before induction of key transcription factors of adipogenesis\(^{144,174}\). The 3D adipogenic constructs in this experiment appears to confer with this conclusion. However, deviating from this finding, 2D adipogenesis simultaneously overexpressed \( PPAR\gamma \) and \( SOX9 \) transcription factors. Sabine Stockl et al observed repression of \( C/EBP\beta \) expression, after silencing \( SOX9 \) in a 2D model of rat BMSCs. The researchers concluded that, reduced activity of \( SOX9 \) indirectly affects \( C/EBP\beta \) protein level by post-transcriptional regulation of \( C/EBP\beta \) mRNA stability via p38 MAPK signaling pathway\(^{175}\). However, the researchers didn’t show any direct link between \( SOX9 \) and \( PPAR\gamma \) expression, yet from the above discussion we can assume that, although \( SOX9 \) promotes \( C/EBP\beta \) stability and activity, but \( SOX9 \) expression decreases gradually with the expression of key transcription factors of adipogenesis. Therefore, the gene expression pattern in 2D adipogenic model does not seem to fit in the above concepts. Before justifying whether 2D model is showing false signal or any artificial behavior, we need to identify how cells in 3D model behaves when they are in more advanced stages of adipogenic differentiation.
5. Pathway analysis

The pathway enrichment analysis was performed by using PANTHER and Kyoto Encyclopedia of Genes and Genomes (KEGG) database based on highly expressed genes. The analysis did not show any pathways for osteogenesis, since there was no high or differentially expressed lineage specific genes which could reveal the osteogenesis. For MSCs differentiation to adipogenesis five pathways have been predicted, including TGF-beta, TNF, PI3K-AKT, RAP1, and Adherens Junctions pathways. TGF-beta, and PI3K-AKT pathways have also been predicted in hASCs differentiation to chondrogenesis. In addition, chondrogenic differentiation also induced HIPPO signaling pathway.

![Figure 18: TGF-beta signaling pathway for adipogenesis](image_url)
Figure 19: TGF-beta signaling pathway for chondrogenesis

The transforming growth factor beta (TGF-beta) superfamily includes TGF-betas, bone morphogenic proteins (BMPs), and activins. BMP signaling promotes adipogenesis, however TGF-beta negatively regulates adipogenesis via inhibiting preadipocyte to adipocyte differentiation. Therefore, TGF-beta signaling must have to be shut down for lipid accumulation. The gene expression data shows no differential expression of \textit{TGFB1} and \textit{TGFB3}, which is conforms to the pathway analysis where TGF-beta have been similarly affected in both 2D and 3D adipogenesis. Akin to adipogenesis, chondrogenic differentiation in both constructs have similar TGF-beta activity. \textit{TGFB1} is differentially expressed in both 2D and 3D chondrogenic model, whereas \textit{TGFB3} is underexpressed in both dimensions.
Figure 20: PI3K-AKT signaling pathway for adipogenesis

Figure 21: PI3K-AKT signaling pathway for chondrogenesis
The phosphatidylinositol 3’ kinase (PI3K)-AKT pathway in adipogenesis and chondrogenesis have also been similarly affected in both culture conditions. This pathway involves in regulating fundamental cellular properties, such as transcription, proliferation, and cell survival. Enhanced phosphorylation of AKT activates transcription of adipocyte regulatory genes\textsuperscript{176}. On the other hand, PI3K-AKT signaling is a key pathway in regulating terminal differentiation of chondrogenesis. AKT signaling has been demonstrated to activate in proliferative zone to promote chondrocyte proliferation, however repressed in hypertrophy zone and inhibited terminal differentiation to hypertrophic chondrocytes\textsuperscript{177}. The corresponding activation of this pathway in adipogenesis and chondrogenesis in both constructs corroborate our stage specification in lineage progression, where 2D versus 3D differentiation to adipogenesis and chondrogenesis are representing clonal expansion/early adipocytes and proliferating chondrocytes/prehypertrophy, respectively.

Figure 22: HIPPO signaling pathway for chondrogenesis
HIPPO pathway regulates chondrogenesis in a similar manner as PI3K-AKT signaling. Research study shows that, Yap1, an effector of HIPPO signaling promotes chondrocytes proliferation, but inhibits chondrocytes maturation via suppressing COL10A1 expression. HIPPO signaling has been similarly affected in 2D and 3D model.

Figure 23: TNF signaling pathway in adipogenesis

The pathway analysis also detected TNF signaling similarly affected in 2D and 3D adipogenesis model. TNFα signaling decreases PPARG expression in adipocytes. Moreover, two other pathways in adipogenesis, RAP1, and Adherens Junctions pathways affected differently in 2D versus 3D adipogenesis model.
CONCLUSION:

The expression profiling in artificial versus physiological microenvironment demonstrates that, cells in 3D behaves differently than monolayered cells. A time-dependent study is necessary to truly reveal the genes associated with lineage progression in 2D vs 3D microenvironments. Regarding osteogenesis, both 2D and 3D model shows no differentially expressed genes related to osteogenesis. Since, the sample number is only three and intra-data variability is high for most genes, it is probable that the data and corresponding discussion about osteogenesis may not be reliable. Moreover, the major limitation of this experiment i.e., lack of cell-cell interactions in 3D constructs in the introductory period of chemical induction needs to resolve before drawing any conclusions in 3D cell behaviors compared to 2D correspondents.
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