Chapter 1
Introduction

1.1 Stem cells

Stem cells are a unique type of cells that have the specialized ability for self-renewal and have the potential to give rise to one or many different cell types. Russian histologist Alexander Maksimov proposed the term stem cell in 1908 however, they were first identified by Leroy Stevens in 1953. After observing different parts from mouse and all the three of its germ layers (endoderm, mesoderm and ectoderm) from muscle, teeth, skin, bone and hair in a its teratoma, he came across the term "pluripotent embryonic stem cell". This gave rise to the modern Stem cell biology. These cells possess the capability of self-renewal through cell division; even after a long period of the dormant stage. They can differentiate into various lineages, a characteristic called plasticity. They can proliferate and replicate indefinitely. Under certain physiologic or experimental conditions, they can be induced to become tissue or organ-specific cells with specific functions such as muscle cells, red blood cells, or brain cells. Stem cell populations are present in both embryos and adults. They are found in almost any multicellular organisms and tissue, such as bone marrow, umbilical cords, and dental pulp.

Stem cells can undergo either symmetric or asymmetric cell division. The symmetrical division results in the amplification of stem cell population by producing two daughter cells identical to their mother cell (cell cloning), as shown in **Figure 1.1**. In contrast, asymmetrical cell division produces one daughter cell that is identical to the mother cell (i.e., still stem cells) and another daughter cell with the potential to differentiate (different from the cell of origin).

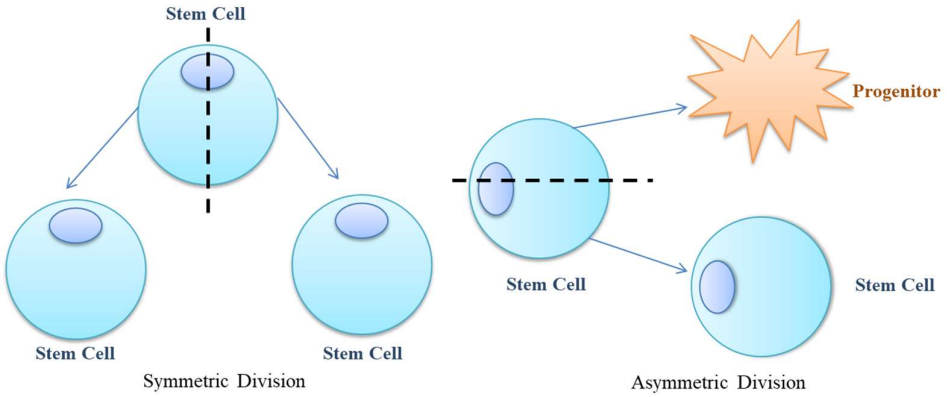

Figure 1.1: Schematic diagram elucidating symmetric vs. asymmetric cell division in a stem cell.

1.1.1 Stem Cells in the History

Table 1 summaries the pioneering discoveries in the history of stem cell research [1-18].

Table 1: *A Brief History of Stem Cell Research*

Year	Name of the scientist and their contribution
1868	Stem Cells is first reported by Ernst Haeckelas "stammzelle" as the ancestor of unicellular organisms. He also used the term stem cell for fertilized egg.
1885	August Weismann's theory suggested germplasm segregation during embryonic development, which was different from the somatic cells.
1892	Boveri traced the cell lineage in a ascaris embryo and proposed the cells between a fertilized egg and committed germ cells as stem cells .
1960	Ernest McCulloch and James were the first scientists to define a stem cell's key properties. They discovered the blood-forming stem cells (hematopoietic stem cells).
1981	Martin, Kufmann and Evans successfully isolated and cultured mouse embryonic stem cells.
1982	Dick and Lapidot discovered that leukemia emerges as a key means for understanding the stem cell's role in cancer.
1984	Civin identified a molecular marker, CD34, specific for a subset of marrow cells containing the blood stem cells.
1987	Bjorklund and Lindwall did the first clinical trials of fetal neural cell grafting in patients with Parkinson's disease.
1988	Broxmeyer first reported clinical use of umbilical cord blood.

1990	Discovery of the Nestin gene, the most commonly used marker for neural stem cells by Lendahl
1992	Bordignon used stem cells as a vector to deliver the genes needed to correct the genetic disorder ADA-SCID.
1994	First separation of cancer stem cells from the majority of cells in cancer.
1998	First human embryonic cell line was derived by the biologist James Alexander Thomson.
2000	Eriksson and Gage demonstrated human brain contains cells with stem-like properties.
2001	The first study on umbilical cord stem cell transplantation in adults was published. Professor Christine Mummery and her team in the Netherlands used stem cells to create beating heart cells outside the body for the first time.
2003	Professor Antonio Beltrami at the University of Udine in Italy described a small population of stem cells in the heart.
2004	First derivation of dopaminergic cells from human embryonic stem cells and Gesine Koegler and colleagues found that in the umbilical cord blood are pluripotent stem cells.
2006	First successful cloning of human embryo to make stem cells was done by Lanza and colleagues. Yamanaka identified four genes as important for reprogramming cells.
2007	De De Coppi and colleagues isolated a new stem cell source in amniotic fluid. Dr. Shinya Yamanaka at Kyoto University found that human skin cells, which are easy to isolate, can be transformed directly into iPS cells.
2009	Induce pluripotent stem cells created with a minimal residual genomic alteration.
2010	Adult stem cells reprogrammed into neurons, cardiac muscle, and blood cells.
2012	John Gurdon and Shinya Yamanaka discovered that mature cells can be reprogrammed to become pluripotent stem cells and were awarded the Nobel Prize for Physiology or Medicine.
2014	Chung and his group cloned the first embryonic stem cell from a man's skin.
2015	First patient in the UK received experimental stem cell treatment for age-related macular degeneration
2018	Rosa and colleagues reprogrammed human and mouse skin cells into immune cells to fight cancer
2019	Ravindra Gupta of University College London reported a second patient free of HIV after receiving stem-cell therapy

The history and continuous advancement and achievement in stem cell biology and its clinical application have clearly indicated the diverse role of stem cell therapy in disease progression, common development and repair and regeneration processes.

1.1.2 Types of Stem Cells

1.1.2.1 The stem cells can be classified according to their developmental potential (plasticity) as totipotent, pluripotent, multipotent, oligopotent, and unipotent.

1.1.2.1.1 (a) Totipotent/omnipotent

They are cells with the most undifferentiated cell form during embryonic development (e.g., the fertilized oocyte (zygote)) up to the stage of the first blastomeres (i.e., three to four days after fertilization). These are single cells with potential to form a new organism with adequate maternal support. These cells have the highest differentiation potential that can rise to all extra-embryonic tissues (placenta), tissues of the body, and the three germ layers.

1.1.2.1.2 (b) Pluripotent

They can differentiate into cell types from the ectoderm, endoderm, and mesoderm, which then produce all cell types for all tissues and organs except then extraembryonic structures.

1.1.2.1.3 (c) Multipotent

These cells have a narrower spectrum of differentiation than pluripotent stem cells, but they can specialize in discrete cells of specific cell lineages.

1.1.2.1.4 (d) Oligopotent

These stem cells can self-renew and differentiate into two or more cells belonging to a specific tissue type. (e.g., hematopoietic stem cells, bronchioalveolar stem cells).

1.1.2.1.5 (e) Unipotent

These cells only produce cells of their own type. They have the property of self-renewal (divide without differentiating and provide everlasting cell supply), e.g., muscle stem cells.

Table 2: *Types of Stem Cells*

	Totipotent (Toti= Whole)	**Pluripotent (Pluri= Many)**	**Multipotent (Multi= Several)**
Relative potency	High	Medium	Low
Cell types capable of generating	Differentiate into any cell type	Differentiate into cells from any of the three germ layers	Differentiate into a limited range of cell types
Examples	Zygote, early morula	Embryonic stem cells, Induced pluripotent stem cells	Hematopoietic stem cells, neural stem cells, mesenchymal stem cells
Found	Early cells of the fertilized egg	Inner mass cells of the blastocyst	In many tissues
Expression of pluripotency genes	+++	++	+
Expression of lineage-specific genes	+	++	+++
Pros of use in research	Highest potency	Easy to isolate and grow, plasticity	Easy to isolate, Less ethical issues, less chance of immune rejection if taken from the same patient
Cons of use in research	Ethical issues, limited source	Ethical issues, teratoma formation	Limited differentiation

1.1.2.2 Stem cells can also be classified based on the source of origin. Scientists are also working on ways to develop stem cells from other cells, using genetic "reprogramming" techniques.

1.1.2.2.1 (a) Adult stem cells

Adult stem cells are undifferentiated cells found in specialized tissues that can self-renew and differentiate into all of the specialized cell types of that tissue. These are also called tissue-specific or somatic stem cells and exist throughout the body from the time an embryo develops in tissues such as the umbilical cord, placenta, bone marrow, muscle, brain, fat tissue, skin, gut, etc. Types of adult stem cells include hematopoietic stem cells, mesenchymal stem cells, neural stem cells, epithelial stem cells, and skin stem cells.

1.1.2.2.2 (b) Embryonic stem cells (ESCs)

These pluripotent stem cells have the potential to become almost any cell type and are only found during the initial stages of development. Embryonic stem cells are derived from human embryos that are three to five days old. They are harvested during a process called *in-vitro* fertilization.

1.1.2.2.3 (c) Induced Pluripotent Stem Cells (iPSCs)

Induced pluripotent stem cells are cells created in the laboratory. They are created by introducing embryonic genes into a somatic cell (a skin cell, for example) that cause it to revert to a stem cell-likestate. These cells, like ESCs are considered pluripotent.

1.1.2.2.4 (d) Cord blood stem cells and amniotic fluid stem cells

Cord blood stem cells are harvested from the umbilical cord after childbirth. Amniotic fluid stem cells are also found in the amniotic fluid surrounding a developing baby inside the mother's womb.

1.2 Why stem cells

Stem cells are the cells which hold unique intrinsic characteristics of differentiating into various types of cells and regenerating functional tissues *in-vivo*. The ability of stem cells to

differentiate has been currently touted to play a pivotal role in treating debilitating diseases and tissue damage. Regenerative therapeutics may provide cures to patients with various ailments by replacing or regenerating human tissues or organs to restore normal physiological functions.

Many degenerative human diseases reflect damaged cells that are not normally repaired or replaced, such as diabetes, Parkinson's disease, hepatic failure, and congestive heart failure. Preliminary studies in animals and humans have suggested that these diseases might be curable through transplantation of healthy cells. Such cells may be obtained by *in-vitro* culture of embryonic stem cells, which are capable of differentiating into many cell types. So, selective differentiation of stem cells into a specific lineage can form the foundation for cellular therapy and regenerative medicine. Embryonic and adult stem cells are potent useful platforms for tissue regeneration, cell-based therapeutics and disease-in-a-dish models for drug screening [19]. Given their unique regenerative abilities, stem cells offer novel potential for treating various diseases such as hematological (e.g., bone marrow transplantation), endocrinological (diabetes), ophthalmology (e.g., age-related macular degeneration), neurodegenerative disorders (Huntington’s disease), retinal disease and genetic disorders. Thus, stem cells have a significant impact on human health and have become a cornerstone for future treatment modalities. However, the major challenge in this field is to control stem cell behavior outside the body reliably [20-24].

The therapeutic value of stem cells is a question of debate in the scientific community. Despite the enormous advantages of stem cells, multiple risk factors are associated with stem cell therapies, summarized in Table 3.

Table 3: *Risk factors in stem cell therapies*

	Risk factors or hazards	Identified risks
Intrinsic factors	Origin of cells	Rejection of cells
Cell characteristics	Tumerogenic potential, proliferation capacity, long term viability, exertion patterns like- growth factors, cytokines etc.	Disease susceptibility, unwanted biological effects, toxicity, neoplasm formation (benign or malignant)
Clinical characteristics	Administration route, therapeutic use, initiation of immune response, irreversibility of the treatment, etc.	Undesired immune response, engraftment at unwanted location, toxicity, lack of efficacy, toxicity, etc.
Extrinsic factors in manufacturing and handling	Lack of donor history, plasma derived materials, pooling or allogenic cell populations, storage conditions, etc.	Disease transmission, reactivation of latent viruses, cell line contamination, etc.

1.3 Stem cell response in different environments

Currently, extensive research is being done to control their self-renewal and differentiation properties. It is well known that stem cells behavior (proliferation/differentiation) is tightly regulated by a combination of physical and chemical factors from their complex extracellular surroundings. Stem cell microenvironment is the general term for the three-dimensional structures and a variety of signaling molecules (growth factors and their receptors, hormones, and signaling molecules) present in the stroma where the stem cells reside and it can regulate the fate of the stem cells, as shown in **Figure 1.2**. Because of its specific three-dimensional structure, it is vividly called niches (niche), which consists of three components: the

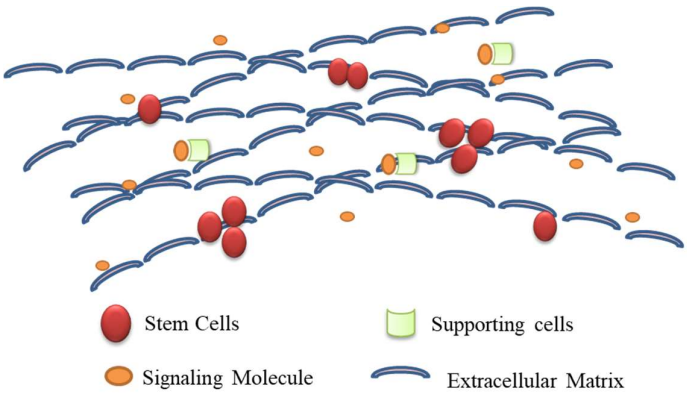

Figure 1.2: Schematic overview of the stem cell microenvironment components.

extracellular matrix (ECM), niche cells (supporting cells, stem cells), and soluble factors derived from the niche cells. The proliferation and differentiation of stem cells are pre-programmed by themselves and are also affected by the microenvironment where they are residing. The stem cell microenvironment can anchor stem cells *in vivo* and regulate the self-renewal and production of their progeny cells through cell-cell, cell-ECM and cytokine-cell interactions. The different macromolecules or cell parameters and ECM interact with each other in a complex and dynamic network [25, 26]. There has been an increase in evidences supporting the fact that the ECM is not only the supportive scaffold but also plays a fundamental role in cell biology in deciphering the development of the cells and regulating their behavior [27] by the production, degradation of its components, remodeling of their structure [28] through hindering their signaling properties directly or indirectly [29]. The polarity, division and migration of the cells can be influenced by the physical properties of the ECM, such as rigidity, porosity, topography and insolubility [30]. Cytokines play an important role in exchanging information from cell-cell and cell-ECM. The changes in the extracellular matrix components also affects the differentiation of the stem cells and the induced differentiation *in vitro* is accomplished by mimicking the cell microenvironment. So,

it is difficult to obtain a long-lasting therapeutic effect in cell-based therapy without the support of a good stem cell microenvironment, even when excellent cells are transplanted. However, rebuilding the stem cell microenvironment becomes the most significant challenge for constructing tissue-engineered tissues and organs.

To employ stem cells for regenerative medicine, it is imperative to understand their *in-vivo* biology and microenvironment. In recent years, the use of *in vitro* models that simulate various components of the niche has helped enriching the understanding of the role of the multiple factors that compose it and even the design of artificial models that recapitulate microenvironment conditions. The conventional cell culture approaches are based purely upon using soluble factors to direct stem cell fate, leading to their limited success. The chemical and physical properties of surrounding microenvironment contribute to the growth and differentiation of stem cells and consequently play crucial roles in the regulation of stem cells' fate. Niche microenvironments can exert tremendous control over a range of functions in stem cell. The ability of the niche to determine the functional spectrum of stem cell activities led us to hypothesize that stem cell niche microenvironments beget stem cell functions [31-33]. Due to their role in maintaining stem cell activity, understanding stem cell-niche interactions may be crucial for their transdifferentiation. Adult stem cells are the main source for developing future new strategies in regenerative medicine, cell- based therapy, and tissue engineering [34,35]. Proliferation and differentiation of stem cells *in vivo* are regulated by their microenvironment, known as niche, which comprises both cellular components and interacting signals between them [32,33]. These niches, in addition to other functions, provide stem cells with physical anchors (by means of adhesion molecules) and regulate the molecular factors that control cell number and fate of these days. Some of these factors are influenced by cell shape, cytoskeletal tension and contractility [36,37]. So, The phenotypic expression and

function of stem cells can be regulated by their integrated response to variable microenvironmental cues, including growth factors and cytokines, matrix- mediated signals, and cell-cell interactions. Recently, growing evidence suggests that matrix-mediated signals include mechanical stimuli such as strain, shear stress, substrate rigidity and topography, and these stimuli have a more profound impact on stem cell phenotypes than that had previously been recognized, e.g. self-renewal and differentiation through the control of gene transcription and signaling pathways. Hence, recreating or simulating this microenvironment may be critical for properly expanding and controlling the differentiation of stem cells outside the body, for both basic biological study and therapeutic translation. So we can comprehend that stem cells receive instructions from their niche environment, which guide their survival and phenotype. Stem cells receive physical and biochemical clues from their extracellular matrix where they reside *in-vivo*. Physicochemical features of a cell nano- environment exert important influence on stem cell behavior and includes the influence of matrix elasticity and topography on their differentiation processes. The presence of growth factors such as TGF-β and BMPs on these matrice provides chemical cues and thus plays vital role in directing eventual stem cell fate. The understanding of the parameters that guide stem cell differentiation is of great interest for the tissue-engineering field. Engineering of functional biomimetic scaffolds that present programmed spatio-temporal physical and chemical signals to stem cells holds great promise in stem cell therapy. Progress in this field requires lucid understanding of the mechanistic aspects of cell-environment nano-interactions, so that they can be manipulated and exploited for the design of sophisticated next generation biomaterials. Therefore, understanding of biomaterial surface topography and elasticity and delivery of chemical and genetic clues via scaffold materials, we can mimic the extracellular matrix to guide stem cell fate.

1.4 Bioengineered platforms for supporting differentiation

Stem cells have great clinical potential because of their capability to differentiate into multiple cell types. Biomaterials have served as artificial extracellular environments to regulate stem cell behavior. Biomaterials with various physical, mechanical and chemical properties can be designed to control stem cell development for regeneration. So, extensive studies are being made at interface of stem cell biology and biomaterials. Biomimetic materials are synthetic (man-made) materials that mimic the natural ones or that follow a design motif derived from nature. These are designed in such a way that they elicit specified cellular responses mediated by interactions of scaffold material with tethered peptides from extracellular matrix.

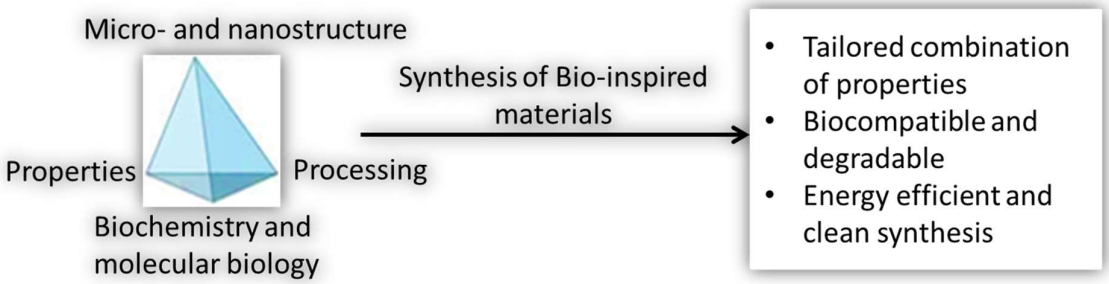

Figure 1.3: Schematic approach for tuning and correlating material properties for fabricating biomimetic materials.

Figure 1.3, presents biomimetic approach whereby interrelations in biological materials between the function of the cells, their properties, their structure at multiple length scales (from the macroscopic scale to nanoscale and biochemistry of building blocks are considered for designing and synthesis of biomimetic materials.

Characteristics of biomimetic materials-

- Intended to interact with a biological system.
- Natural and man-made materials which are constructed of polymers, metals or ceramics.

- Replacement for whole or part of a living structure that performs or replaces a natural function.

So, these biomimetic materials can be engineered in such a way that they tailor stem cells responses. Recent works in field of stem biology and biomaterials science have demonstrated possibility to mimic the natural stem cell microenvironment to control stem cell behavior by considering parameters such as substrate topography, mechanical properties, rheological properties and other material properties.

The extracellular microenvironment plays a significant role in controlling cellular behavior. Identification of appropriate biomaterials that support cellular attachment, proliferation and most importantly, in the case of human embryonic stem cells, lineage-specific differentiation is critical for tissue engineering and cellular therapy. In addition to growth factors and morphogenetic factors known to induce lineage commitment of stem cells, a number of scaffolding materials, including synthetic and naturally-derived biomaterials, have been utilized in tissue engineering approaches to direct differentiation.

Conventional methods for chemically inducing stem cells into specific lineages is being challenged by the advances in biomaterial technology, with evidences for highlighting the fact about material properties being capable of driving stem cell fate. Materials are being designed to mimic the clues stem cells receive in their in vivo stem cell niche including topographical and chemical instructions. Nanotopographical clues that mimic the extracellular matrix (ECM) *in vivo* have shown to regulate stem cell differentiation. The delivery of ECM components on biomaterials in the form of short peptides sequences has also proved successful in directing stem cell lineage. Growth factors responsible for controlling stem cell fate *in vivo* have also been delivered via biomaterials to provide clues to determine stem cell differentiation. An alternative approach to guide stem cells fate is to provide genetic clues

including delivering DNA plasmids and small interfering RNAs via scaffolds. So biomaterial properties such as surface topography and elasticity can also guide fate of stem cells. The understanding of the parameters that guide stem cell differentiation is of great interest for the tissue-engineering field.

Among the biomaterial properties that affect cell behavior, surface topography has great potential to control cell shape and location [38]. Several researchers have observed that microscale and nanoscale topographies in the form of pillars, grooves, pits or pores can induce the differentiation of human stem cells to a certain cell lineage [39-41]. In this regard, the design of biomaterials with architectures that mimic natural cell microenvironments may be a powerful tool to better understand and manipulate cell function as a strategy for future cell-based therapeutics. In this context, surface microstructuring and techniques involving it can play an important role in the field of three-dimensional scaffold fabrication, which may enable *in vitro* cells to mimic in vivo ones, resembling the cellular tridimensional networks and the structural organization of human tissues. So, for tailoring surface properties we can provide different kinds of stimuli to stem cells which can regulate their behavior as well their fate. Following schematic diagram shows how mechanical stimuli such as mechanical strain, substrate stiffness, shear stress and topography can effect stem cell phenotypes in a combinatorial fashion.

To account for the complexities of native stem-cell niches, biomaterials are actively being investigated as artificial extracellular matrices in order to mimic the natural microenvironment. This perspective highlights important areas related to the design of biomaterials to control stem cell behavior, such as cell-responsive ligands, mechanical signals, and delivery of soluble factors. Some important key findings highlighting engineering

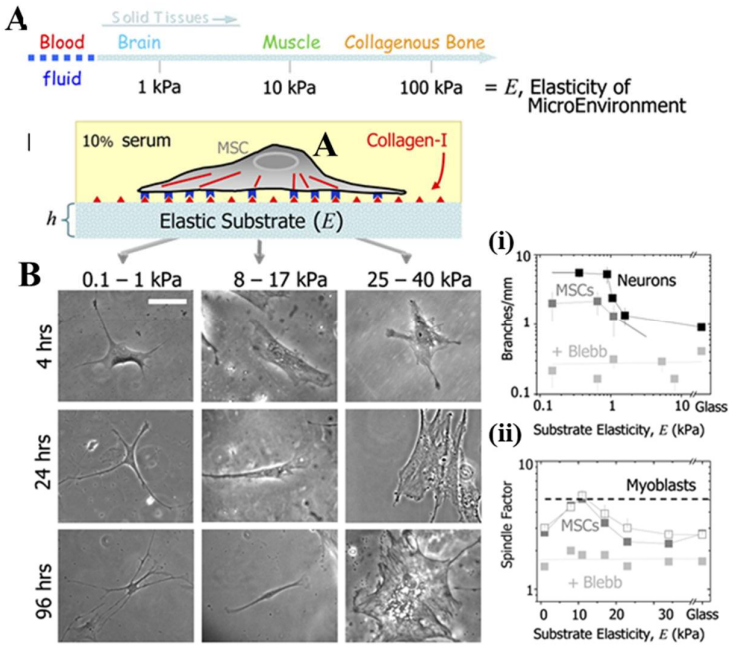

Figure 1.4: Shows substrate elasticity directs the differentiation of MSCs. (A) The range of elastic modulus, E for select tissues. (B) MSCs placed on substrates with varied stiffness are initially small and round but overtime change morphology according to substrate elasticity. (i) cell branching per length of primary mouse neurons, MSCs, and blebbistatin-treated MSCs and (ii) spindle morphology of MSCs, blebbistatin-treated MSCs, and mitomycin-C treated MSCs (open squares) compared to C2C12 myoblasts (dashed line). Figure taken from Engler et al., 2006.

a biomimetic three-dimensional microenvironment enabling a thorough understanding of the mechanisms of governing stem cell fate have been mentioned in the following studies:

In 2006, Engler et al. studied and demonstrated how matrix elasticity directs stem cell lineage specification. They showed that MSCs differentiate into tissues that match closely to the mechanical properties of the polyacrylamide substrate upon which they were cultured (**Figure 1.4**). As a result, MSCs that were cultured on stiff (bone-like) gels differentiated into osteoblasts, those cultured on medium stiffness (muscle-like) gels differentiated into muscle cells and those cultured on compliant gels (neural-like) differentiated into neural cells. Due to the heterogeneity of MSCs, it is unclear if the same cell type responded differently to

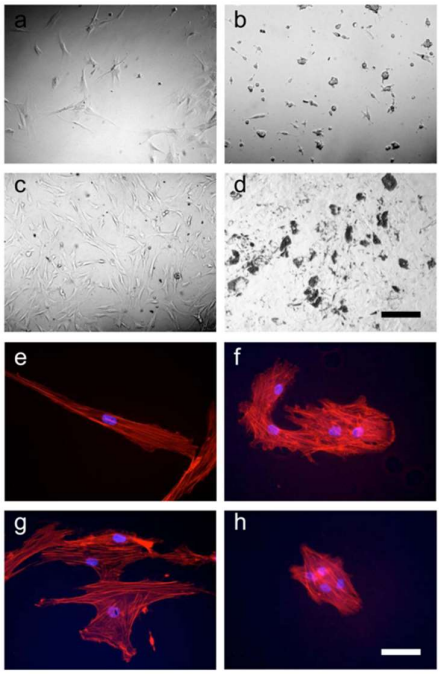

Figure 1.5: Presents hMSC morphology is altered in response to small molecule incorporation into PEG hydrogels. Representative light and fluorescent micrographs of TRITC-phalloidin (red) and DAPI (blue)-stained of hMSCs depicting morphology cultured on PEGDM (a and e), acid (b and f), phosphate (c and g), and t-butyl (d and h)-functionalized surfaces). Figure taken from Benoit et al., 2008.

substrate stiffness or if stiffness resulted in the differential growth of pre-committed progenitor cells [42]. In 2008, Benoit et al. worked and demonstrated that encapsulation can be induced into human mesenchymal stem cells (hMSCs) to differentiate down to osteogenic and adipogenic pathways by controlling their 3D environment using tethered small molecule chemical functional groups. Benoit et modeled the unique chemical environments present in various tissue types and the potential cues these provide to resident stem cells. In their work PEG hydrogels were doped with small amounts of pendant carboxyl groups to mimic glycosaminoglycans in cartilage, phosphate groups for their role in bone mineralization, or tert-butyl groups to mimic the lipid-rich environment in adipose tissue, as shown in **Figure 1.5**. True to their model, these gels were successfully able to direct the differentiation of human MSCs down to chondrogenic, osteogenic or adipogenic pathways, respectively [43].

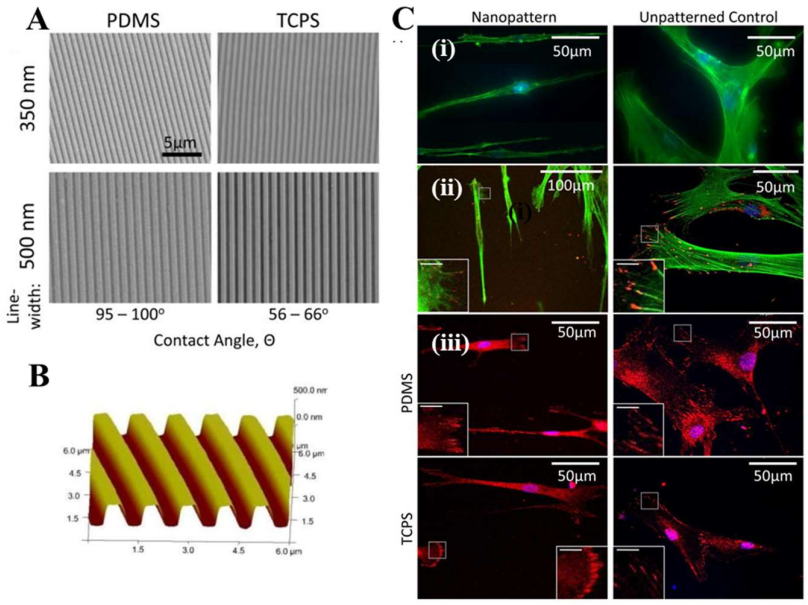

Figure 1.6: (A) Scanning electron micrographs of gratings with 350nm linewidth and 700nm pitch (350nmgratings), and 500nm linewidth and 1µm pitch (500nm gratings) fabricated by soft-lithography and heat embossing on PDMS and tissue culture polystyrene (TCPS), respectively. (B) Atomic force micrograph (AFM) of 350 nm gratings on TCPS. Also, (C) (i)F-actin cytoskeleton visualized by Oregon- green labeled phallodin in hMSC on PDMS with 350nm gratings or unpatterned PDMS, (ii) Distribution of focal adhesions visualized by immunofluorescence staining of tyrosine-397 phosphorylated FAK (pFAK, red) and F-actin (green) and (iii) Distribution of focal adhesions visualized by immunofluorescence staining of vinculin (red). Figure taken from Yim et al., 2010.

In 2010, Yim et al. studied and showed nanotopography-induced changes in focal adhesions, cytoskeletal organization, and mechanical properties of human mesenchymal stem cells. The study elucidated synergestic effect of nanotopography and the mechanical properties of the substrate on communications between stem cells and their enviroment, thereby influencing cellular behavior and alterations in cell viscoelastic properties, possibly via mechanotransduction through an integrin-focal adhesion-cytoskeleton pathway (**Figure 1.6**). This transduction modulated the differentiation process and thus cell fate of hMSCs, suggesting that defined control of these biophysical cues can provide an important tool in

development of “cell-instructive” tissue engineering scaffolds that can influence stem cell behavior in a pre-defined manner [44]. In 2010, Gilbert et al. have shown that substrate stiffness significantly affects the fate of muscle stem cells. They demonstrated that the soft hydrogel substrate mimics physiological elasticity and can induce the propagation of muscle stem cells and thereby muscle regeneration [45].

In 2010, Marklein et al. worked to control stem cell fate with material design. Advancements in understanding stem cell interactions with their environment opens a new door for developing new materials-based strategies to regulate stem cell behavior toward tissue regeneration applications. They hypothesized materials can provide cues based on chemistry, mechanics, structure, and molecule delivery that control stem cell fate decisions and matrix formation. These approaches can help to advance the clinical translation of a range of stem cell types through better expansion techniques and scaffolding for use in tissue engineering approaches for the regeneration of many tissues. With this in mind, they worked considering basic concepts and recent advances in the use of materials for manipulating stem cells. They demonstrated structure, morphology, degradation, and presentation of bioactive sites as important parameters in material design for these applications and may signal the differentiation of stem cells [46].

In 2011, Hu et al. presented the interactions of C2C12 myoblasts and human bone marrow stem cells (hMSCs) with silk- tropoelastin biomaterials and the capacity of each to promote attachment, proliferation and investigate myogenic or osteogenic-differentiation. Temperature-controlled water vapor annealing approach was used to achieve desired architecture with controlled surface roughness, micro/nano-scale topological patterns, elastic modulus, stiffness, yield stress, and tensile strength. The study suggested that low surface roughness with high stiffness of silk-tropoelastin materials aids in high proliferation rate and

myogenic-differentiation of C2C12 cells. Whereas, hMSCs favor a combination of high surface roughness with micro/nano-scale surface patterns. Doping human tropoelastin in the silk tropoelastin materials enhances the proliferation and osteogenic-differentiation of hMSCs. They have concluded about the silk-tropoelastin composition facilitating fine tuning of the growth and differentiation of these cells [47].

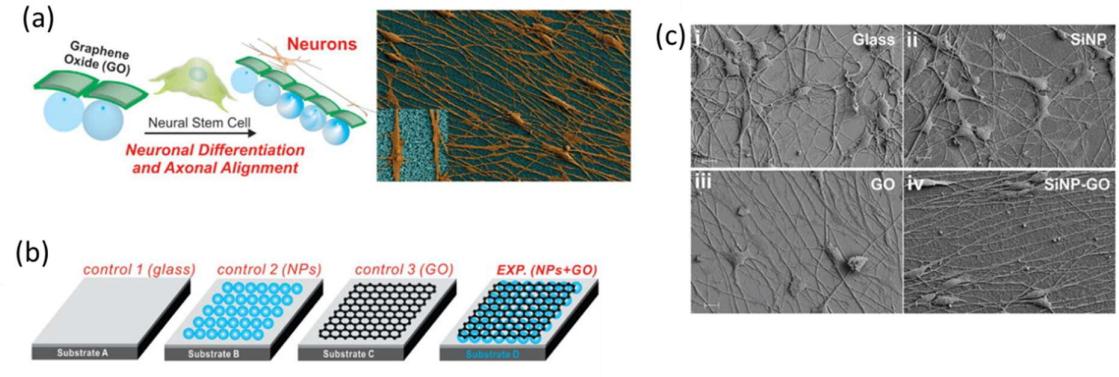

Figure 1.7: (a) Shows strategy adopted for seeding neural stem cell for tuning the differentiation process and other cellular behaviours. (b) Schematic representation of fabricating graphene-nanoparticle hybrid arrays (c)Shows response of neural stem cells and axonal alignment on different substrates. Figure taken from Solanki et al., 2013.

In 2013, Solanki et al. demonstrated axonal alignment and enhanced neuronal differentiation of neural stem cells on graphene-nanoparticle hybrid structures (**Figure 1.7a**). They generated graphene-nanoparticle hybrid arrays (**Figure 1.7b**). with positively charged silica nanoparticles and graphene oxide (GO), with oxygen functional groups attached to the graphene basal plane. This is particularly advantageous as the oxygen functional groups allow the GO nanosheets to attach readily to molecules or surfaces. Here, the GO nanosheets were used to coat the surface of 300 nm silica nanoparticles (SiNPs) to form graphene-silica nanoparticle hybrids (SiNP-GO). They concluded the GO and SiNP-GO substrates significantly enhanced cells survival after 3 weeks of differentiation as compared to the

control SiNP and glass substrates. They also authenicated that the axonal alignment of differentiating neural stem cells (hNSCs) on the SiNP-GO substrates is not dependent on their cellular density but only on the presence of GO (**Figure 1.7c**). This was a remarkable finding as it suggests that the sole factor determining the alignment of axons from differentiating hNSCs is the presence of GO. This result could be very helpful for developing scaffolds to restore neuronal function within damaged regions of the central nervous system [48].

In 2013, Tang et al. worked on enhancement of electrical signaling in neural networks on graphene films, especially in respect to neural excitability and network efficacy. Such knowledge demonstrates possibility of using graphene to regulate the behaviour of neural network in vitro, and would help in designing graphene based neural interfaces for regenerative medicine [49].

In 2015, Park et al. studied 3D-nanoflowers of rutile TiO2 as a film grown on conducting (ITO and FTO) and non-conducting (glass) substrates (**Figure 1.8(i)**) for *in-vitro* biocompatibility studies with mouse MC3T3 osteoblast and human HS-5 cells. They found cell viability and proliferation rate was higher on TiO2 3D-nanoflowers grown on a non-conducting surface as compared to that grown on a conducting substrate (**Figure 1.8(ii)**). Despite the fact that the similar nanostructure and crystallite structures of TiO2 were grown on the conducting and non-conducting surfaces, the improved adhesion and proliferation response of both the cell types on the non-conducting surfaces confirms that the cells prefer a non-conducting surface. This may enable the cells to create and sustain a local surface charge environment, which is induced during the cell– surface interactions, for better cell growth on

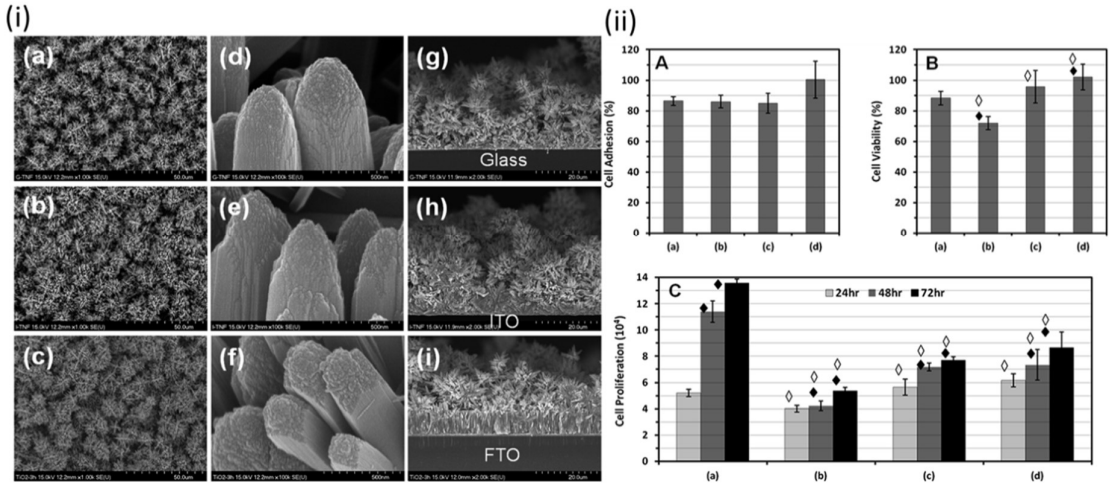

Figure 1.8: (i) FE-SEM images of rutile TiO2 nanostructures grown on glass (a), FTO (b) and ITO (c) substrates with a HCl to water ratio of 50 : 50 and reaction time of 3 h at 180°C. (ii) Osteoblast cell response were observed on (a) tissue culture plastic surfaces and 3D nanoflower-like TiO_2 nanostructures grown on (b)FTO, (c) ITO and (d) glass substrates at 3 h. (A) Adhesion%, (B)Viability%,and (C) Cell proliferation. Figure taken from Park et al., 2015.

non-conducting surfaces; this is not possible on conducting surfaces. Stromal cells had potential to prepare extracellular matrix scaffolds for the ex vivo expansion/differentiation of stem cells. Therefore, the findings could be explored to prepare 3D TiO2 nanostructure supported cellular scaffolds for regenerative medicine in the future. The study demonstrated the role of t surface charge in modulating the cell response and therefore is important parameter for designing biomimetic materials [50].

In 2016, Leijten et al. discussed how from nano to macro: multiscale materials can be used for improved stem cell culturing and analysis. Stem cells respond to nanoscale, microscale, and macroscale cues, such as matrix, growth factors, and niche organization, which are difficult to physiologically recapitulate in culture. They discussed in their work utilize cabout various bioengineering approaches to manipulate and integrate spatiotemporal cues across these discrete length scales can improve traditional methods for controlling cell fate. **Figure 1.9**

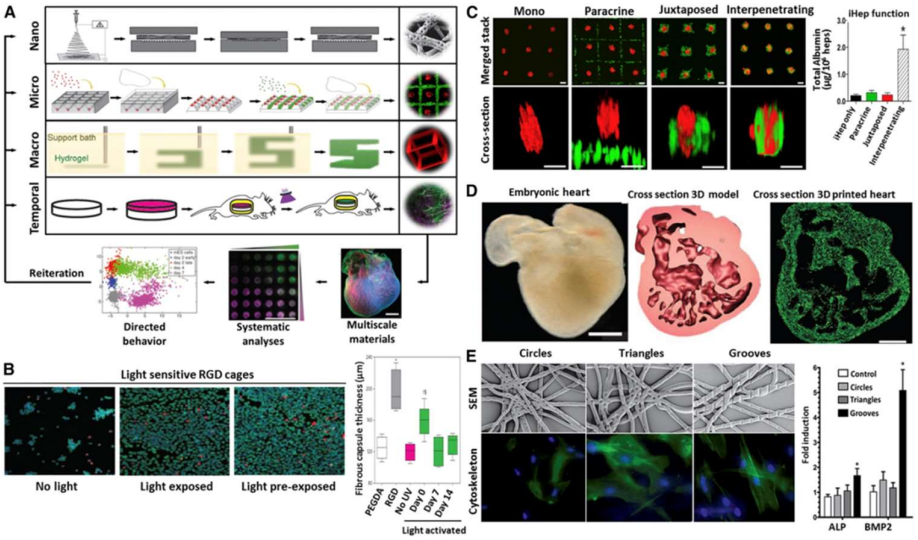

Figure 1.9: (A) Represents technologies and approaches for optimizing desired features across different scales, (B) Demonstrates light-triggered expression of RGD cages, (C) Micropatterning to connect the microtopography and cell placement in co-cultures to enhance albumin production by iPSC-derived hepatocyte-like cells, (D) 3D printing to recreate embryonic hearts with a complex internal trabecular structure, and (E) Nanoscale control to regulate the osteogenic differentiation of human mesenchymal stem cells on grooved electrospun fibers. Figure taken from Leijten et al., 2016.

presents (A) Integrating technologies and approaches for optimizing desired features across a range of length scales, while providing temporal control of selected parameters, will generate robust multiscale materials to improve stem cell culture platforms and facilitate iterative design of biomimetic materials. Specifically, cellular behavior can be guided using (B) temporal control, e.g., light-triggered expression of adhesion moieties, e.g., RGDs, to recruit cell populations such as neutrophils (green) and macrophages (red) in a temporal manner, which can control the formation of the fibrous capsule in vivo; (C) microscale control, e.g.,micropatterning to harness the microtopography and cell placement in co-cultures to enhance the function of assembled microtissues such as albumin production by iPSC-derived hepatocyte-like cells (red) and stromal cells (green); (D) macroscale control, e.g., 3D printing

to recreate organ shape and tissue function such as embryonic hearts with a complex internal trabecular structure; and (E) nanoscale control, e.g., nanotopographies to steer stem cell behavior such as the osteogenic differentiation of human mesenchymal stem cells on grooved electrospun fibers [51].

In 2016, Lin et al. summarized recent progress in stem cell differentiation directed by material and mechanical cues. The study discussed about the advancement of material science and possible strategies to achieve biophysical signals of active nature by designing specific material microstructures for regulating stem cell fate. The study had highlighted significant progress in the design of materials for having different elasticity, micro-structures, coating of extracellular matrix (ECM) materials etc. for achieving material-directed differentiation of adipose-derived stem cells, neural stem/progenitor cells, hematopoietic stem/progenitor cells, mesenchymal stem cells, embryonic stem cells and other cells. Other choice of materials are hydrogel-based, particularly polyacrylamide (PAM) and polydimethylsiloxane (PDMS) materials, for achieving the various combination of mechanical and chemical characteristics by taking a different ratio of crosslinking agents, matrix concentration, and conjugation with other components. Micropatterns of specific designs could be integrated with 3D bioprinting to fabricate 3D structures to control stem cell behavior and thus induce stem cell-based tissue regeneration [52].

A retrospective study by Balikov et al. in 2016 [53] reported the differentiation of human mesenchymal stem cells by decoupling electrical stimulation of 0.3 V for 72 hours at 1 Hz with physical patterning on graphene substrate into the neurogenic lineage. In 2017, Wei et al. discussed how nanomaterials modulate stem cell differentiation: biological interaction and underlying mechanisms. Nanomaterials hold great promise in biological and

biomedical fields owing to their unique properties, such as controllable particle size, facile synthesis, large surface-to-volume ratio, tunable surface chemistry and biocompatibility. They discussed various metal nanoparticles, semiconductor materials and polymeric nanoparticles' roles in the differentiation of stem cells. Nanomaterials modulate the differentiation of stem cells in three ways. Nanomaterials could be used as (a) supplements, (b) 2D matrix or (c) 3D nano-scaffolds that induce differentiation of stem cells [54].

In 2018, Gupte et al. [55] elucidated that the pore size and their interconnectivity regulate capillary ingrowth during bone formation and can modulate chondrogenesis and endochondral ossification of bone marrow stromal cells.

In 2018, Madl et al. studied and worked on engineering hydrogel microenvironments to recapitulate the stem cell niche. They discussed various stem cell applications including regenerative medicine, patient-specific disease modeling, and toxicology screening. However, eliciting the desired behavior from stem cells, such as expansion in a naive state or differentiation into a particular mature lineage, remains challenging. Drawing inspiration from the native stem cell niche, they developed hydrogel platforms to regulate stem cell fate by controlling microenvironmental parameters, including matrix mechanics, degradability, cell-adhesive ligand presentation, local microstructure, and cell-cell interactions. They surveyed techniques for modulating hydrogel properties and review the effects of microenvironmental parameters on maintaining stemness and controlling differentiation for a variety of stem cell types [56].

In 2019, Chen et al. mimicked the nanostructure of natural bone tissue by designing TiO_2 nanotube arrays on titanium substrate to improve osteogenesis. They modified the substrate surface, by conjugating sclerostin antibody via dopamine, and postulated that sclerostin antibody-conjugated TiO2 nanotube arrays (TNTs-scl) could decrease the sclerostin level in

the medium secreted by the adhered osteocytes and induce osteoblast differentiation via the Wnt signaling cascade. The study highlighted a simple and effective approach for surface engineering of titanium to emulate bone repair and healing under osteoporotic conditions [57]. In 2021, Farokhi et al. summarized different conductive materials, including polypyrrole (PPy), poly(3,4-ethylenedioxythiophene) (PEDOT), polyaniline (PANI), multi-walled carbon nanotubes, single-wall carbon nanotubes, graphene, and graphite oxide to induce neural cell activities in terms of growth, differentiation, migration, alignment and synapse formation. The work discusses role of free electrons to provide superior electrical properties

However, a holistic picture of the different cues and their role in controlling cellular behavior and the differentiation process requires further studies on optimizing correlative relations between selective material properties and expected change in cellular functions. For stimulating the proliferation and differentiation of neural stem cells into neuronal or glial cell lineages. The authors brief about the role of electrical stimulation via conductive scaffolds in triggering the signaling pathways involved in neural regeneration [58].

In 2021, Du et al. demonstrated thiol modified glycopolymers for modulating biological responses of stem cells and dictating their fate. The work discussed about behavior about human neural stem cells (hNSCs) and human adipose stem cells (hASCs) by changing the linker length of the N-acyl group of thiol-modified N-acetylmannosamine (ManNAc) analogs, as shown in **Figure 1.10**. Particularly, Ac5ManNTProp and Ac5ManNTBut were reported to modulate Wnt signalling pathway to induce neural differentiation in hNSCs. They elucidated upregulation of Wnt signaling by thio-analogs could trigger adipogenic differentiation in hASCs but did not hinder glial differentiation (e.g., Schwann cell-like differentiation). They

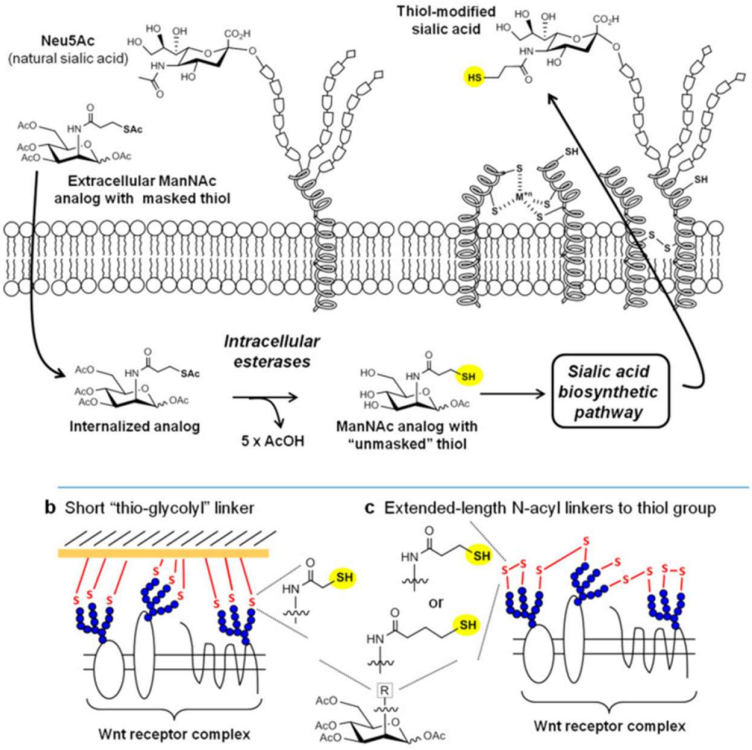

Figure 1.10: Overview of thiol-based metabolic glycoengineering (MGE). (a) The thiol-modified ManNAc analogs intercept the sialic acid biosynthetic pathway and replace naturally-occurring sialic acids in cell surface glycans with their thio-glycosialoside counterparts. (b)Activity of thiol-modified sialic acids with short linker lengths between the thiol group and core sugar (c) Thiol-modified analogs (e.g., Ac5ManNTProp and Ac5ManNTBut with longer linkers between the thiol and core sugar that have enhanced scaffold-independent biological responses. Figure taken from Du et al., 2021.

concluded that amendment of cell-surface glycans via metabolic glycoengineering (MGE) opens a new direction to pleitropic modulating of cellular pathways and thereby elicit desired biological responses in human stem cells, highlighting potential clinical translation of these MGE analogs [59].

In 2022, Cho et al. developed a new platform using a single nanocrystalline metal-organic framework (nMOF) nanoparticle–embedded nanopit arrays (SMENA), nUiO-67, for neuronal cell generation (**Figure 1.11**). Different combinations of metal and ligand were optimized to regulate physical parameters of nanopatterns array, which allowed long-term storage and steadily released retinoic acid, as an essential component for neural differentiation. The study

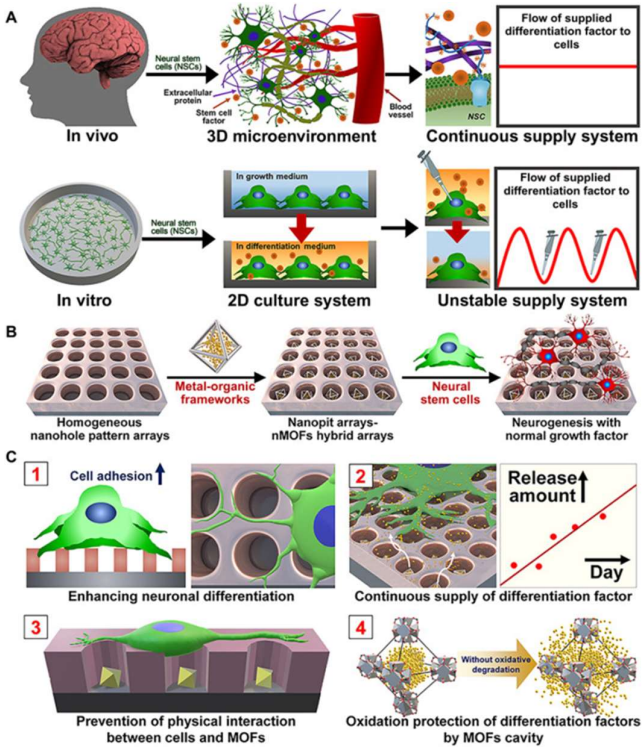

Figure 1.11: Schematic illustration of SMENA. (A) Most critical difference between real in-vivo and in-vitro environments. (B) Single RA-containing metal-organic framework (MOF)–embedded nanopit arrays (RA-SMENA) consist of RA-containing nanocrystalline UiO-67s (RA-nUiO-67) and nanopit arrays with highly homogeneous nanoarrays, which function as a continuous and stable nutrient supply system for enhancing the neurogenesis of the NSCs. (C) RA-SMENA has several advantages for grafting MOFs for eventually accelerating the neurogenesis. (1) Nanopit arrays can guide and support the neuronal differentiation of the NSCs. (2) RA-SMENA can continuously supply RA for a long period (3) Nanopit array prevents the interaction between RA⊂nUiO-67 and the cells. (4) Chemical decomposition of the RA located inside RA⊂nUiO-67 can be prevented. Figure taken from Cho et al., 2022.

highlighted porous nMOFs as potential materials with chemically stability for long periods of time for loading and releasing various types of biomolecules, for instance, caffeic acid and ascorbic acid, by altering pore size, pore volume, and particle size for nurture stem cells to differentiate into a particular lineage [60].

1.5 Role of energy metabolites in stem cell differentiation

During differentiation, intracellular messengers as well as energy metabolites come into the picture. These act as a driving force for the differentiation of stem cells. In recent years,

accumulating evidence has suggested that the regulation of mitochondria dynamics and function is essential for successful differentiation. The mitochondria are maintained at a relatively low activity level in stem cells and upon induction, mtDNA copy number, protein levels of respiratory enzymes, the oxygen consumption rate, mRNA levels of mitochondrial biogenesis-associated genes, and intracellular ATP content are altered. The regulated level of mitochondrial ROS is found not only to influence differentiation but also to contribute to the direct determination of differentiation. Understanding the roles of mitochondrial dynamics during stem cells differentiation will facilitate the optimization of differentiation protocols by adjusting biochemical properties, such as energy production or the redox status of stem cells and ultimately benefit the development of new pharmacologic strategies in regenerative medicine. The role of cellular metabolism during differentiation is not well studied in stem cell biology.

Current evidence has demonstrated that in addition to growth factors and extracellular matrix cues, various metabolic pathways definitively provide important signals for the self-renewal and differentiation potency of stem cells (**Figure 1.12**). The metabolic profile distinguishes the undifferentiated state from the differentiated state of stem cells, with a dynamic mitochondrial morphology and a shift from glycolysis to mitochondrial oxidative phosphorylation (OXPHOS) [61-63]. Glycolysis rapidly fulfills energy requirements by producing pyruvate in the cytosol, which is only accompanied by a net gain of two moles of adenosine triphosphate (ATP) per mole of glucose. However, pyruvate is likely to enter the tricarboxylic acid (TCA) cycle for OXPHOS and generate reducing equivalents to efficiently

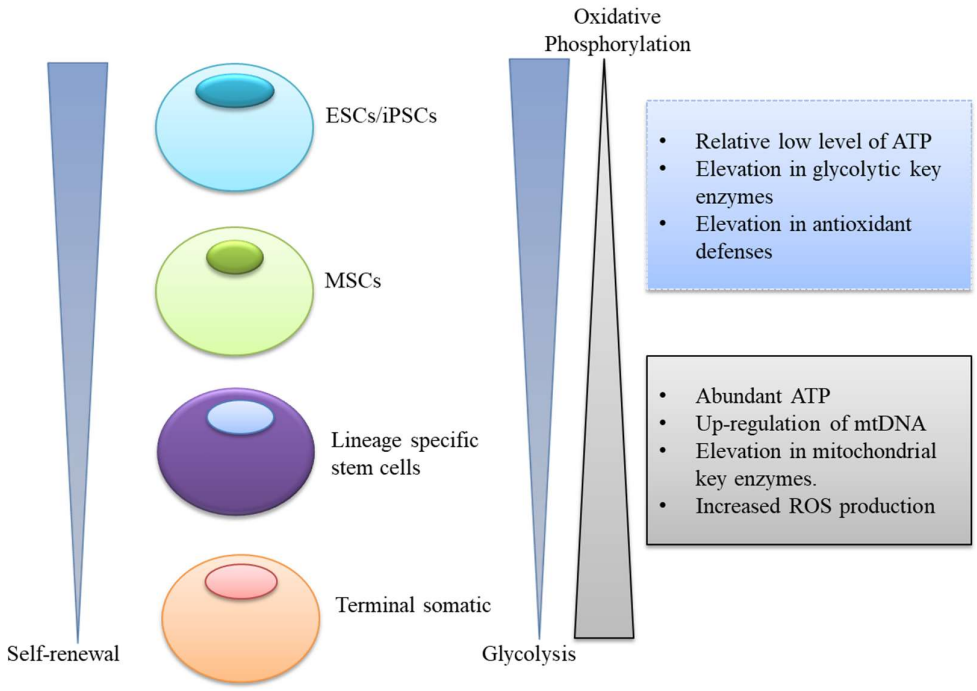

Figure 1.12: Schematic illustration of mitochondrial metabolic pathways that may provide important signals to direct the self-renewal and differentiation potency of stem cells.

produce ATP for a significantly higher energy yield than glycolysis [64]. In fact, the mitochondrial metabolites including ATP, intracellular Ca^{2+} homeostasis, and ROS are crucial for multiple cellular processes. In stem cell metabolic biology, the dynamic balance of each type of stem cell can vary according to the properties of each cell type, and they share some common points. Understanding the mitochondrial properties of stem cells may effectively clarify the stemness and differentiation pathways that control stem cells for regenerative medicine.

The intracellular messenger has a key role in cell signaling pathways in various differentiation stages of stem cells. The increasing interest in stem cell research is linked to the promise of developing treatments for many life-threatening, debilitating diseases and cell replacement therapies. However, performing these therapeutic innovations with safety will only be

possible when accurate knowledge about the molecular signals that promote the desired cell fate is reached. Among these signals are transient changes in intracellular Ca^{2+} concentration [65-67]. The aim of this study is to present a broad overview of various moments in which Ca^{2+}-mediated signaling is essential for the maintenance of stem cells and for promoting their development and differentiation, also focusing on their therapeutic potential.

1.6 Research gaps and objectives of the thesis

Based on ongoing research and current needs, the main gaps identified are:

(1) One of the key challenges for tissue engineering is to exploit supporting materials which can have tunable functionalities towards adjusting it with cell-specific behaviors and to support formation of the functional cellular network.

(2) Another challenge is to provide an engineered microenvironment to stem cells which controls explicitly the selective differentiation of stem cells for the development of more effective treatments. Eliciting the desired behavior from stem cells, such as expansion in a naive state or differentiation into a particular mature lineage, remains challenging.

(3) The third challenge is preventing immunorejection after stem cell transplantation. Immunological rejection is a major barrier to successful stem cell transplantation. A person's immune system may also recognize the transplanted cells as foreign bodies, which can trigger an immune reaction that results in rejection of the transplanted cells. Recipients of the transplant usually have to take strong immunosuppressive drugs to reduce the chances of rejection, but these drugs induce infection by viruses or microbes in the environment.

(4) Most current cell-therapeutic products involved in clinical trials use patients' autologous cells. Autologous therapies pose greater challenges as they are potentially much more expensive to manufacture. Furthermore, patient-specific cell therapies have more significant

inherited variability since the quantity and quality of starting stem cell material varies, as does cell growth and behavior. Autologous therapy is further associated with logistical issues related to the practicalities of delivering cells to patients. Ideally, more suitable off-the-shelf products should be developed.

(5) Reproducibility and scalability.

Based on these research gaps, the objectives of the thesis were defined as below to enhance the understanding of the role of the materials-induced stem cell differentiation process and the role of energy metabolites.

(1) Establishing a suitable culture environment for growing different stem cells.

(2) Fabrication of bioengineered platforms for guiding stem cell differentiation and characterizing their structural and functional properties using SEM, TEM, FTIR, AFM etc.

(3) Optimization of surface parameters of platforms for understanding cellular responses and tuning differentiation process.

(4) Gene and cell surface marker analysis to confirm the lineage of differentiated cells.

(5) Studying the role of mitochondria and intracellular messengers to understand the differentiation mechanism by analyzing mitochondrial membrane potential, ROS, and Ca^{2+}.

(6) Developing cellularized platforms suitable for implantation and evaluating their functional response.