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Nanotechnology in stem cells research: advances and applications

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1. ABSTRACT

Human beings suffer from a myriad of disorders caused by biochemical or biophysical alteration of physiological systems leading to organ failure. For a number of these conditions, stem cells and their enormous reparative potential may be the last hope for restoring function to these failing organ or tissue systems. To harness the potential of stem cells for biotherapeutic applications, we need to work at the size scale of molecules and processes that govern stem cells fate. Nanotechnology provides us with such capacity. Therefore, effective amalgamation of nanotechnology and stem cells – medical nanoscience or nanomedicine - offers immense benefits to the human race. The aim of this paper is to discuss the role and importance of nanotechnology in stem cell research by focusing on several important areas such as stem cell visualization and imaging, genetic modifications and reprogramming by gene delivery systems, creating stem cell niche, and similar therapeutic applications.

2. INTRODUCTION

Nanotechnology and stem cell research are two of the most exciting fields of modern research that promise significant contributions toward improving human health. Let us start with a question, in 1900, the average life expectancy used to be 35 years, which today stands more than doubled at 80 years, will 2100 see a man living for 150 years? And if yes, then do we have the resources to manage the needs, luxuries, and health of this aging population? Will the 150-year-old man live the same quality of life as 40-year-old lives today? The answer may lie in nanotechnology and stem cells. Amalgamation of both is expected to revolutionize our understanding of nature, and gives us innumerable tools to surmount its vagaries. A review of current literature shows varying similarities and differences between these two fields of science.

Table 1. Some typical size ranges of objects around us

Object	Size (nanometer)
Ions	0.1-0.5
Proteins	1-5
Quantum Dots	10-20
Largest Virus (Poxvirus) Smallest Bacteria (Mycoplasma)	300
Smallest Virus (parvovirus) Largest Proteins (Hemocyanin)	20
Herpes Virus (Enveloped) Herpes Virus (Naked)	200 100
Adenovirus	70-75
Adeno Associated Virus	20-25
Retrovirus	100
Metallic Nanoparticles	18-25
Liposomes/ polymeric nanoparticles	40-500
Dendrimers	1-5
Polymeric Micelles	10-100
Carbon Nanotubes	100
Collagen Bundle Collagen fibril Collagen filament	10,000-20,000 200,000 20,000
Hydroxyapetite Bioceramics	60-70
RBC, Bacteria, Most cells	1,000-5,000
DNA	2
ATPase molecular motor	12
Stem cells	Debatable but can vary in size from 5,000 to 50,000

Nanotechnology in simplest terms is defined as the science and technology of nanoscale (one billionth of a meter), typically in the range 1-100 nm. In other words, it has to do more with the size rather than the field of work. Table 1 lists some typical size ranges of objects around us. Genesis of nanotechnology date to the famous lecture by Nobel laureate Professor Richard Feynman in 1960, wherein he said, "The principles of physics, as far as I can see, do not speak against the possibility of maneuvering atom by atom (1). The essence of this statement lies in the fact that nanotechnology works at a depth wherein individual atoms and molecules are manipulated to obtain the desired goals. It allows us to reach the most fundamental units that make materials around us and determine their behaviors (a typical atom is of the size of around 0.1 nm and protein is about 5 nm). The impact of nanotechnology has already started percolating the common person. This is felt in improved and sustainable sources of energy, miniaturized data storage disks, materials with super magnetic and optical properties, automated manufacturing assemblies, and smart fabrics to name a few. Nanotechnology in healthcare, also called nanomedicine or bio-nanotechnology is manifested in efficacious nanobiomaterials, drug delivery systems, gene therapies, robotic-based diagnostics and surgical aids, nano biomedical devices, biological microelectromechanical systems (BioMEMS), amongst others. A recent study, that analyzes a $34.2 billion US nanotechnology-based medical product industry, suggests that US demand for nano-sized medical products will grow 17.1 percent annually through 2014 (2). Stem cells research, also started drawing interest of biologists from the 1960's. Stem cells are distinguished from other cell types by their ability of indefinite proliferation and multi-lineage differentiation. Recent state of the art is driving biologists to translate their findings into clinical therapeutics. Efforts are made to prompt stem cells to grow into all body tissues under defined and reproducible conditions and assume characteristic 3-D morphologies. Stem cells breed optimism to find cures for degenerative disorders, cancer, and congenital abnormalities and radically transform management of these disorders. However, delivering on these expectations require lot more extensive work at present.

Applying nanotechnology along with genetic engineering and gene silencing- the other two greatest scientific assets we inherited from 20th century- is more of a necessity and less of exploration now to derive the therapeutic benefits out of stem cells. Nanotechnology greatly magnifies the visualization of stem cells, helps to monitor their *in vivo* fate, allows us to engineer the genetic features of cells, and create an environment suitable for their morphogenesis into clinically useful entities.

The interaction of nano-scale surface topography with the activity and vitality of stem cells has become one of the main topics in both nanotechnology and stem cell research. There are many studies suggesting nano-size effects on stem cells activities and magnifying the importance of further investigations at the interface of both disciplines. The separate influence of topographical and chemical cues on cell attachment and spreading are well documented, however, Berry *et al.*, 2007 (3), studied the effect of graft copolymers that sterically stabilized biological surfaces alongside nanotopographical features fabricated by colloidal lithography, on cell adhesion and spreading. The study indicated an enhancement of cell response because of modifications in material topography, and demonstrated that topography modulated the effects of the chemical environment.

Bauer *et al.*, (2009) (4) reported that nanoscale surface topography of materials may be more important than their chemistry for stem cells (Fig.1). They looked at how stem cells behave on zirconium and titanium oxide nanotube surfaces and found the size-specific reaction to the nano-patterns. They created patterns of ZrO2 and TiO2 of differing diameters. They then seeded the nanotubes

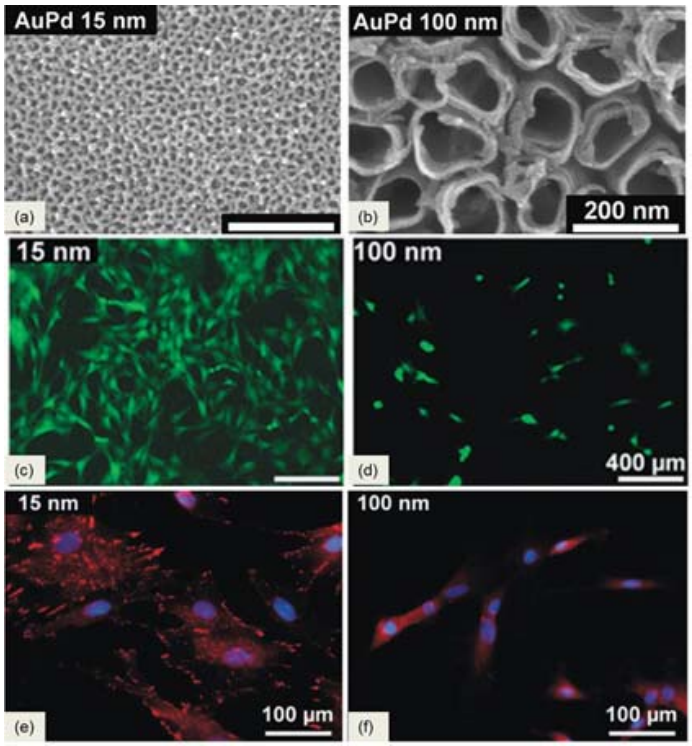

Figure 1. Size matters to stem cells: SEM images of self-organized layers of TiO2 nanotubes with diameters of 15 and 100 nm (a, b), fluorescence images of GFP-labeled mesenchymal stem cells after 24 h adhesion and 3 d proliferation onto TiO2 nanotubes with diameters of 15 and 100 nm (c,d), focal contact formation of mesenchymal stem cells stained for anti-paxillin observed under fluorescence microscopy on AuPd coated TiO2 nanotubes with diameters of 15 and 100 nm after 3 d proliferation (e, f). Adapted with permission from (4).

with mesenchymal stem cells that express a green fluorescent protein. For both nanotube materials, TiO2 and ZrO2, cell adhesion and spreading were enhanced for nanotube diameters of 15–30 nm, while a strong decay in cell activity was observed for diameters larger than 50 nm. They also found that the cell density on the surface depended on the nanotubes' diameter but not their length or chemistry.

Several studies have now demonstrated the size dependent effect of niche on stem cell differentiation. A recent study by Shanmugasundaram *et al.*, (2010) (5) evaluated the effect of scaffold designed over a range of nano to micron-sized fibers and resulting pore size and mechanical properties, on human mesenchymal stem cells (MSCs) derived from the adult bone marrow during chondrogenesis. They showed that micron-sized fibers with large pore structures and mechanical properties comparable to the cartilage ECM enhanced chondrogenesis. All these studies clearly demonstrate that architectural features as well as mechanical properties of nanomaterial-based scaffolds regulate stem cell differentiation. In this work, we provide an overview of some applications of nanotechnology in stem cell research and development. Despite major differences between the two fields, they have one thing in common that needs to be addressed before their widespread application, both nanotechnology and stem cells have to overcome clinical hurdles, e.g. the ethical use of some types of stem cells (6) and critical regulatory issues regarding toxicological concerns over nanomaterials (7).

3. NANOTECHNOLOGY FOR STEM CELLS IMAGING AND VISUALIZATION

Some mammalian tissues can be restored to their original state after injury; however, most adult tissues can be repaired but not completely restored, as a natural process. For tissue regeneration to occur correctly (usually a lineage progenitor) sufficient number of cells needs to be produced and delivered to the right location at the right time. The potential for long lasting regeneration of the tissues remain within the stem cells and their ability to divide symmetrically and asymmetrically, determining cell fate. Determining how cell fates are controlled at the molecular level is crucial for us to understand how regeneration happened and how stem cells can be employed to regenerate tissues *in vivo*. It is therefore imperative that any manipulation or study on stem cells must first enable us to visualize stem cells and track changes associated with their morphogenesis and *in vivo* delivery. The existing imaging techniques can be divided into two major categories: electron microscopy imaging techniques and continuous imaging techniques that will be explained in the following sections.

3.1. Electron microscopy imaging of stem cells

Electron microscopes are more powerful than the traditional light microscopes. They allow the user to see the fine details of the specimens, which are normally not seen in microscopes of a lower power. The phenomenon of microscopy revealing nanostructures and their interactions with complex three-dimensional cellular structures started with the invention of the Transmission Electron Microscopy (TEM), but soon flourished with the Scanning Electron Microscopy (SEM), Scanning Probe Microscopy (SPM), Atomic Force Microscopy (AFM), and Scanning Tunneling Microscopy (STM). Imaging technologies have thus been in the forefront in biomedical research as evidenced by the number of Nobel prizes awarded in this area of development: for Phase contrast microscopy (in 1953), electron microscopy (1986), scanning tunneling microscopy (1986), nuclear magnetic resonance spectroscopy (2002) and magnetic resonance imaging (MRI) (2003). Besides studying cellular structures at nano levels, these tools are also widely used to characterize and visualize nano-biomaterials and nanoparticles. Below we present a synopsis of some of the microscopes and their application in stem cells and nanomaterial research.

3.1.1. Transmission electron microscopy (TEM)

Conventional light microscopes can at best provide a magnification of 1000X and resolution of 0.2 micrometers. This is inadequate to image nanometer range structures and led to development of transmission electron microscopy (TEM). In this, and all other electron microscopes, an electron gun is used instead of light to pass through the specimen. Combination of condensers focuses the electron beam onto the sample and the objective lens then projects transmitted electrons on to a phosphor screen for the user to see it. Magnifications up to 15 million times can be obtained by TEM, which is much more than that of SEM (up to 100000 times the normal size of sample). In TEM, density of sample determines the brightness of image produce (dense areas in sample produce darker areas in image i.e. through which less number of electrons have been transmitted).

TEM observations have helped in determining the role of migration of stem cells to the site of injury after intravenous administration. Such an application has been validated in a myocardial infarction animal model. The ultrastructure of cardiac cells at the site of ischemia was observed and this established the phenomenon of stem cell migrating to injured sites after *in vivo* administration (8). TEM also allows us to investigate the mechanisms that are responsible for cell migration. This has been achieved by following the alignment and polarity of actin filaments in the cells and integrating time-lapse video microscopy (9). This microscopic technique also allows to distinctly identify cells of particular phenotype in a milieu of other cells types. For example, it is believed that all adult tissues contain a specific population of progenitor cells or stem cells. Isolation of these cells for tissue engineering requires their correct identification. TEM allows observers to define ultrastructure configurations for identification. This has been explored for interstitial cajal like cells recently (10).

3.1.2. Scanning electron microscopy (SEM)

When a high voltage is applied, tungsten filaments emit electrons. The electrons are allowed to pass through a hole, which focuses them into a beam. The beam then strikes the sample and some of these striking electrons are reflected back. Analysis of the specimen by collecting the reflected (secondary electrons) electrons forms the basis of scanning electron microscopy (SEM) imaging. SEM produces images of greater clarity and three dimensional qualities and requires less sample preparation compared to TEM. The intensity of image is determined by the number of secondary particles, which in turn depends upon the scattering angle of secondary electrons. Thus, SEM can be used for topographical analysis also (surface analysis). Initially SEM could not be used for wet specimens as the vapors produced interacted with the electrons. However, controlled vacuum chambers and gold coupling have now enable SEM imaging of wet biological samples. SEM is a quintessential part of all cell culture experiments and allows us to understand ultrasturcture and intracellular localization of various particles. These images also reveal if the cellular structures are intact or have they undergone any damage during processing. SEM images can also be used to visualize the process of coating cells with membranes for bioencapsulation (11).

3.1.3. Atomic force microscopy (AFM)

AFM produces a topological image of the sample under study by motion of a tip of a cantilever. The tip is 10-20 nm in diameter and about 1000 nm long. As the tip is moved over the surface, forces between the tip and specimen (e.g. hydrogen bonding, van der Waals forces etc.) causes the cantilever to bend, bending being proportional to the strength of force between the atoms of tip and atoms of specimen. The deflection in cantilever is measured by a laser beam, which is emitted from the gold-coated cantilever after bending. A positional sensitive detector collects the signals and generates the image. The detector can distinguish the changes in position of incident laser beam as small as 1 nm and thus generates sub-nanometer resolution. The tip can be coated with different materials for specific applications. AFM operates in two modes- contact and non-contact modes. In contact mode, the tip actually goes through the surface of the specimen. In non-contact mode the cantilever is oscillated and a distance of around 5-10 nm is maintained between the specimen and tip. The tip senses the "intermittent" van der Waals forces. Non-contact modes AFM does not cause any damage to soft samples but could not be used in liquid environments. The principal merit of the AFM is as a nonintrusive local probe of live cells and their dynamics in the biofluid environment. For example, spermatozoa are increasing receiving attention as a rich of stem cells for banking (12). Therefore, it is necessary to follow the events in development and maturation. Dynamic force AFM has helped scientists to follow the events that mark the maturation of such cells and allows them to segregate cells belonging to different stages of maturation. Offering high spatial resolution imaging in one or more operational modes, the AFM can also deliver characterization of mechanical properties of biological samples and local

chemistry through operation in a force-vs-distance mode. AFM aids in understanding of intracellular biochemical and biophysical processes that may direct cell's commitment to a particular lineage. This method can be used to image live cells and probe their mechanical properties in physiological conditions in a non-destructive manner. It also allows elastic data acquisition with a high spatial resolution in real time to follow the fast changes in the cell mechanics. In these experiments, AFM cantilever serves as a microindenter to poke the cell, and further analysis of force-indentation data yields the local Young's modulus. In addition, AFM indentation technique can be used to characterize the viscoelastic behavior of the cell cytoskeleton, including viscosity, loss and storage moduli, and stress relaxation times. A study has characterized the distinct biomechanical properties of mesenchymal stem cells, including the average Young's modulus determined with AFM (3.2 +/- 1.4 kPa for hMSC vs. 1.7 +/- 1.0 kPa for fully differentiated osteoblasts), and the average membrane tether length measured with laser optical tweezers (10.6 +/- 1.1 micron for stem cells, and 4.0 +/- 1.1 micron for osteoblasts) (13). Another study reports the effective Young's modulus of a supported section of the plasma membrane in the range 1–10 kPa, whereas the corresponding value for a membrane more strongly supported by the cytoskeletal structure in the range 15–50 kPa (14). In a recent study, it is found that, before and after adding CD34, CD44 or CD29 antibodies, the AFM images of mouse adipose tissue derived mesenchymal stem cells (ADMSCs) had significant changes in both cell morphous (shape) and the average roughness of cell membrane. However, there was no significant difference in the cell shape and the average roughness of cell membrane, after adding a different set (CD45 or CD144) of antibodies. Thus, we can also use the AFM to scan cell shape or to calculate the average roughness changes of cell membrane to analyze the existence of cell membrane antigen qualitatively (15).

3.1.4. Scanning tunneling microscopy (STM)

Very similar to the AFM in operation, STMs use a sharpened, conducting tip with a bias voltage applied between the tip and the sample. STM exploit the wave properties of electrons to scan the surface. The tip is brought to about 1 nm distance of the cells and electrons are allowed to pass through the 1 nm gap, the electrons generated cause a tunneling current to be set in and are used to form the image. Obviously, for STM both the tip and the sample have to be conductive in nature. Scanning ion conductance microscopy can be used to identify individual contracting cardiomyocytes among different cell types. This has been performed in embryonic stem cell derived cardiomyocytes by measuring the amplitude and rhythm of these cells. In a single experiment, thus an assessment of the number and position of ESCM within the layer of mixed cell types was made (16).

AFM and STM are the two types of SPM and their numerous applications in stem cells is continuously being explored. As evident, high spatial resolution achieved in SPM's is not the only advantage of these methods. Even more important is the possibility to study biological molecules in various environments including air, water, and physiologically relevant solutions, buffers, and organic solvents. External factors such as temperature, pressure, humidity, and salt concentration can be varied during measurements. This gives a unique opportunity to study conformational changes of biomolecules such as proteins and DNA *in situ* and reflect on the developmental status of stem cells (17). It is obvious the impact of microscopic techniques to reveal nanostructures and ultrastructure of cells have huge implication for identification, validation of cells as also allow monitoring their morphogenesis. We just attempted to provide list of such microscopic techniques while their application for stem cells research is as wide as imagination.

3.2. Continuous imaging of stem cells

To fully understand the complex continuous process of regeneration of a tissue from stem cells, it is important to observe the biological process continuously, instead of at discrete time points (18). Discontinuous analyses have led to several misinterpretations and decade long debates in this field. At present there are several controversies, including the identity of the stem cells in adult organs, the exact lineage potential of these cells, their proliferating ability, the reason for their incomplete reconstitution potential following a transplant, their ability to divide asymmetrically, their transdifferentiaton potential, and the contribution of organized cellular movements leading to organ or cellular regeneration. So far, no single imaging approach has been developed for the continuous observation of stem cell driven regeneration deep inside an organ or living tissue.

In addition, the number of resident and often quiescent adult stem cell population in a tissue is very less and rare, and is surrounded by many other cells (19-21). The detection of such low numbers of stem cells requires an imaging modality that has single cell resolution. Another drawback in stem cell research is that, except for a few exceptions a defined identity of adult stem cells, based on specific molecular markers is lagging (19, 21) .Very often stem cells are identified and characterized by a set or combination of molecular markers. Therefore, an imaging modality that allows multicolour labeling (a different colour for each marker) and detection must be used. Also labels that allow genetic marking are also used, usually in live cell tracking, and to study differentiation into progenitor populations. Many of the stem cell progenitors, following a tissue injury or transplantation travel long distances and are highly migratory. Imaging such events requires large volume imaging of the whole organ in a high temporal resolution (that is with only very short intervals between each frame of the movie being constructed) (22). Many nanoparticle based imaging techniques have been developed for continuous monitoring of cell growth and migration. However, the current technologies also have several limitations. For example, several of these particles do not differentiate between live and dead cells, e.g., iron oxide particles will continue to generate signal even after the stem cell is dead and has fallen apart. Thus, the future belongs to stem cell tracers that are sensitive to differences between the intra- and extra- cellular milieu, e.g., pH

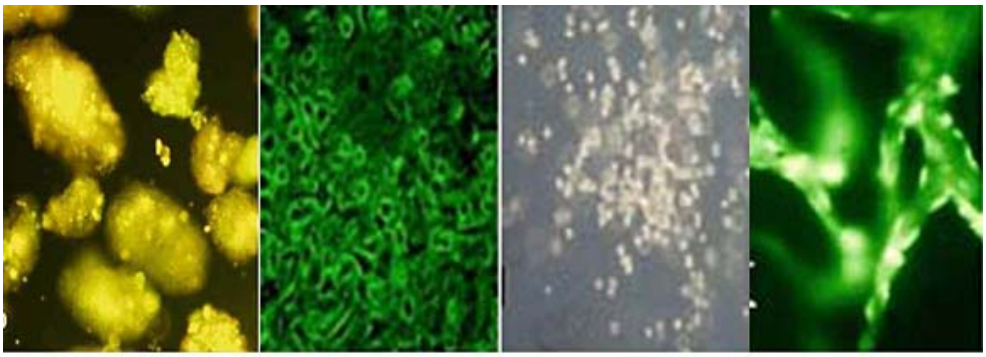

Figure 2. *In vitro* Quantum Dots (SiO2 wrapped CdTe nanoparticles) labeling of murine embryonic stem cells: (a) prior to differentiation, and after differentiation into (b) neurons (c) hematopoietic cells, and (d) endothelial cells. Adapted with permission from (27).

sensitive tracers. We discuss various imaging techniques including nanoparticle-based technologies available for studying stem cell tracing in the following sections.

In this section, we briefly talk about the major imaging modalities for regeneration research: planar and tomographic fluorescence imaging (in this case wide field fluorescence microscopy, confocal fluorescence microscopy, multiphoton fluorescence microscopy and fluorescence molecular tomography), bioluminescence imaging, single photon emission tomography (SPECT), positron emission tomography (PET), and magnetic resonance imaging (MRI). MRI is among the least invasive of available imaging technologies (23). Expensive new experimental machines have almost enough spatial and temporal resolution for continuous single-cell imaging. A major current limitation however, is the lack of genetically encodable labels with high signal strengths.

3.2.1. Nanodiagnostics

One nano-based technology of interest that offers great advantages for stem cell studies is the real time imaging or bioluminescence imaging. Most of the imaging technologies that allow non-invasive visualization of the body are also a great aid for stem cells therapies. These techniques are based on different forms of energy interacting with tissues. Some methods, such as computed tomography and MRI, rely on energy–tissue interactions, whereas SPECT and PET require the administration of reporter probes. However, in both computed tomography and MRI, exogenous contrast agents can be exploited to enhance contrast or follow biological markers (24). Contrast agents are chemical substances introduced into the anatomical or functional regions to be imaged to increase the contrast between intact and abnormal tissues by altering relaxation times. A host of nanotechnology based diagnostic and contrast agents for stem cell imaging are now developed.

3.2.2. Quantum dots (QD)

Quantum dots are semiconductor nanocrystals (single crystals few nanometers in diameter) and are generally made of the Cd, Se, CdTe, InP, InAs etc. The size range of QD from 2 to 9.5 nm correspond to the emission wavelengths from 400 to 1350 nm. Their role in bio-imaging is attributed to their broad excitation spectrum for fluorescence, size-tunable and narrow emission spectrum, and resistance against photobleaching. Thus they offer to overcome almost all the practical disadvantages associated with traditional fluoroprobes like organic dyes. QD are hydrophobic in nature so are solubilized by attaching polar groups such as thiol, carboxyl acid; block copolymers, peptides and polysaccharides, and functionalized with oligonucleotides, DNA and proteins for recognition to specific cells. Adsorption of functional groups also make them non-toxic and suitable for live cell imaging (25, 26).

In stem cell therapy, monitoring of cell survival and location after transplantation is important for determining their efficacy. However, the absorption and scattering of light in native biological tissue can be considerable, optical signals transmitted from deep tissues to the surface tends to diminish in strength. Thus, QD offer attractive propositions for stem cells imaging. Lin and coworkers have recently shown the ability of QD for multiplex imaging i.e. tracking different cell populations with different QDs using different emission wavelengths at the same time. QD are delivered to the cell by non-specific endocytosis, microinjection, liposome mediated uptake, electroporation, and peptide-based reagents.

Wang *et al.* (27) observed that SiO2 wrapped CdTe nanoparticles (QD) can enter into murine stem cells, and stay in the induced-differentiated neurons cells, hematopoietic cells and endothelia cells, with no cytotoxicity effects within the used concentration (see Fig.2). Utility of existing QD for *in vivo* imaging is limited by the requirements to have excitation from external sources resulting in autofluorescent background and paucity of source light at non-superficial locations. This can be overcome by coupling QD with luciferase proteins. This is called as bioconjugation of QD and the phenomenon of imaging Bioluminescent resonance energy transfer. Self-illuminating QD have shown to increase sensitivity in small animals with a signal to background

ratio of 103 to 5 picomoles of conjugates (28). Upon further improvements (e.g., near-infrared QDs, better serum stability, and improved cell retention), QDs will have greater potential for tracking of stem cells within deep tissues (29).

3.2.3. Iron nanoparticles

Different iron oxide nanoparticles usually consisting of a crystalline iron oxide core, typically Fe3O4 or maghemite, surrounded by a dextran polymer, polyethylene glycol, or ionic substances such as citrates, or biotinylated iron oxides, have proven efficiency in biomedical imaging. Ultra-small paramagnetic iron oxide (USPIO) particles have a hydrodynamic diameter of less than 50 nm, and in super-paramagnetic iron oxide (SPIO) particles, the diameter exceeds 50 nm (30). As the internalization of particles hold key to their imaging properties, several approaches have been described to optimize the internalization process, including linking nanoparticles to the highly cationic cell penetrating peptides such as HIV tat peptide or the use of an anti-transferrin receptor monoclonal antibody covalently linked to nanoparticles (MION-46L) (30)

Various reports are available in literature describing the use of iron oxide nanoparticles for stem cell imaging. For example, Jendelova *et al.* have followed the fate of embryonic stem cells and mesenchymal stem cells labeled with dextran coated SPIO nanoparticles in rats with cortical lesions and tracked their migratory activities (31). Other paramagnetic contrast agents include gadolinium nanoparticles, which can be internalized into cells by coupling with cell penetrating peptides and does not affect cell viability (32).

Gold nanoparticles and gold nanoshells provide great sensitivity for the detection of DNA and proteins. They can be used to label DNA or protein molecules (including antibodies), which can then bind to their respective targets. Surface plasmon resonance is an optical technique that measures the refractive index of very thin layers of material adsorbed on a metal. It offers real-time *in situ* analysis of dynamic surface events and is capable of defining rates of adsorption and desorption for surface interactions. Plasmon–plasmon resonance, resulting from the interaction of locally adjacent gold nanoparticle labels that have bound to a target, produces changes in optical properties that can be used for detection. It is known that the characteristic red color of gold colloid changes to a bluish-purple color on colloid aggregation because of this effect (33).

Gold nanoparticles have been developed as intracellular molecular imaging platform using multifunctional moieties for both cytosolic delivery and targeting. The utility of these intracellular sensors was demonstrated by monitoring actin rearrangement in live fibroblasts (34). These multifunctional nanosensors can be adapted to target various intracellular processes in stem cells based drug delivery screening especially where transfection or cytotoxic labels are not feasible.

For molecular imaging, PerFluroCarbon nanoparticles can carry very large payloads of gadolinium to detect pathological biomarkers with magnetic resonance imaging. A variety of different epitopes, including alpha (v)beta (3)-integrin, tissue factor and fibrin, have been imaged using nanoparticles formulated with appropriate antibodies or peptidomimentics as targeting ligands (35).

A new class of nanostructured magnetizable materials that can function as magnetic switches has been created with a femtosecond laser surface etching technique. These nanomaterials function as multiplexed magnetic field gradient concentrators. Combined with magnetic beads coated with cell adhesion ligands, these materials form microarrays of 'virtual' adhesive islands that support cell adhesion and maintain cell viability. By removing the external magnetic field, cells can be caused to round up and die. This simple technology thus acts as molecular switches and guides for stem cells and depends upon nanomaterials that can be well incorporated inside the stem cells (36).

4. NANOTECHNOLOGY FOR GENETIC ENGINEERING AND STEM CELL THERAPIES

To completely understand stem cell biology, and make them suitable for tissue engineering and organ regeneration, it is necessary to genetically engineer (or manipulate or reprogram) them. This may be done with a view to generate patient-specific stem cells, make them pluripotent, for differentiation, proliferation, transdifferentiation or dedifferentiation, induce expression of signaling factors, produce transgenic animals as models of diseases, upregulate or downregulate receptor molecules on surface, amongst others. For genetic manipulation, somatic cell nuclear transfer, cell fusion, and forced expression of genes are some of the techniques available. With the mapping of the human genome project, considerable amount of information on the genetic basis of disorders has become available. With the help of this database and along with genes identified through experimental and clinical studies for particular diseases, current stem cell researchers are trying to produce therapeutic options based on target genes. Above purpose is achieved only when stem cells can be efficiently transduced to express or repress genes of interest. Systems that carry genes of interest into the target site inside the cell (and nucleus of cell), prevent denaturation of genetic elements before they reach the target site are known as gene delivery carriers or vectors. Vectors are required since genetic elements like (DNA peptide nucleic acid or more importantly the siRNA) have to overcome many barriers before they can be integrated into gene expression machinery of the cell. Naked genetic elements have suboptimal permeability across membranes and can be degraded by the cytosolic enzymes. A variety of nanovectors has been constructed which and help gene transfection with their properties to be selectively internalized into target cells and high efficiencies. Nano-vectors can be used both for *in vivo* and *in vitro* gene transfection are of two types- Viral vectors and non-viral vectors.

4.1. Viral nanovectors

Viruses are particles consisting of nucleic acid surrounded by a proteinaceous capsid and occasionally a lipoprotein envelope. Viruses adsorb on the cell surfaces through interactions between their envelope proteins and receptor molecules on the target cells. Viruses were discovered as infectious agents causing many diseases, however, their ability to genetically transduce cells have led them as suitable nano-manipulators. Segments of genes responsible for the pathogenicity of viruses is removed and followed by insertion of gene of interest for genetic engineering. Some of the viruses widely used to transducer stem cells are mentioned below.

4.1.1. Retroviruses

Retroviruses are unique in the sense that they infect only rapidly dividing cells and are the most common type of virus used in clinical trials for gene therapy. The single stranded RNA is reverse transcribed into double stranded DNA after the virus gains entry into the cell. The major advantage to retroviral vectors is integration into the host cell chromosome, offering stable transformation and sustained gene expression. Retroviridae, however, can cause insertional mutagenesis through non-specific integration into host genome. Other drawbacks include a small size of possible DNA insert up to 8.5 kb and a lower yield of infectious viral titers, particularly in comparison to adenovirus. The most frequently used recombinant retroviral vectors are derived from murine leukaemia virus (37) ,where replication deficiency is achieved by replacing genes encoding essential viral proteins such as gag, pol and env with therapeutic genes (38).).

The retroviral-mediated transfer of a suicide gene into donor T cells has been proposed as a method to control alloreactivity after hematopoietic stem cell (HSC) transplantation. Gene-modified cells (GMC) may be infused into the patient either at the time of transplantation, together with a T-cell depleted HSC graft, or after transplantation, as a donor lymphocyte infusion. Administration of a so-called pro-drug activating the "suicide" mechanism only after occurrence of GvHD should selectively destroy the alloreactive GMC *in vivo*, eventually leading to GvHD abrogation. Phase I and II clinical trials have already proved the concept while modifications continued to be made to make the approach more successful (39). In another study, we can see lentivirus are used for transgene expression of GFP in hepatocytes derived from hamatopoeitic stem cells and transplanted in animals deficient of such cells. This method was used to indicate the topologically and functionally proper integration of transplanted cells into the host liver parenchyma (40).

4.1.2. Adenoviruses

In a significant advantage over retroviruses, adenoviruses-the second most popular viral vector in clinical trials for gene therapy- can transduce both proliferating and quiescent cells, and the viral genetic material remains episomal (i.e. does not get integrated into the infected cell genome). Adenoviruses are linear double-stranded DNA viruses and are rendered replication-defective by substitution of the essential E1 gene and the deletion of the E3 gene maximizes the target gene payload (up to 7.5 kb) without affecting viral growth (38). They can also be produced to very high titers and therefore attain high levels of transgene expression. Disadvantage of adenoviruses lies in rather short-lived transgene expression obtained in vivo, which is attributed to a strong immune reaction that these viruses induce in the host (41). Helper dependent adenoviral vectors can further accommodate about 37kb of transgene load (42).

4.1.3. Herpes simplex nanovector

Epithelial cells and neural stem cells are the major target of the Herpes Simplex Virus, though many other cells such as cardiomyocytes and fibroblasts can also be infected. HSV genome stays episomal while the viral infection can remain latent for a long time in cell bodies of sensory ganglia. Moreover, because of its large genome, 30–50 kb of transgene can be introduced into recombinant HSV-1 vectors (43) .Herpes simplex virus type 1 based amplicon-mediated transfer of Fibroblast Growth Factor-2 into ES cell-derived CNS progenitors has been shown to lead to the amplification and subsequent differentiation of these precursors into neurons (44). Similarly, data suggest that the FGF-2 gene-modified MSCs with the HSV-1 vector can contribute to remarkable functional recovery after stroke compared with MSCs transplantation alone (45).

An earlier study demonstrated that adenoviral vector mediated cotransfection of MSCs with the combination of two genes- CTLA4Ig for immunosuppresion and the BMP2 stimulates osteoblastic cell differentiation in vitro (46). Adenoviral mediated transduction of MSC has also increases VEGF expression and results in significant improvement in cardiac function (47).

4.1.4. Adeno-associated viral particles

These viral particles are non-pathogenic to human cells and need the presence of a helper virus such as adenovirus or herpes virus to grow. AAV have small genome size (less than 5 kb), thereby only small transgenes up to 4.5 kb can be inserted. Wild-type adeno-associated viruses are integrated into the host genome, display a unique tropism for chromosome 19q13.3 and can infect both dividing and non-dividing cells. These advantages make it a promising viral vector for long-term transgene expression (48). Stender et al., have examined the efficiency and kinetics of in vitro transduction using AAV serotype 2 in human MSC and assessed whether such as transduction leads to MSC multipotentiality. They found MSC transduction by AAV2 with 65% efficiencies at high viral titres and showed transduced MSC to differentiate into osteogenic, adipogenic and chondriogenic lineages (49). However, applications of viral vectors also pose a safety concern as it can lead to mutations and cancer.

4.2. Non-viral nanocarriers (e.g. nanoparticles) for gene delivery, cell therapy, and regenerative medicine

4.2.1. DNA nano-carriers

A host of non-viral DNA carriers (both natural and synthetic) have been designed that formulate DNA into discrete particles in the nano- to sub-micron size range. This size range is ideal as it permits efficient uptake into the cell via the process of endocytocis. Compaction of DNA also reduces its access to nucleases, which means that the transgene has a better chance of reaching the cell intact.

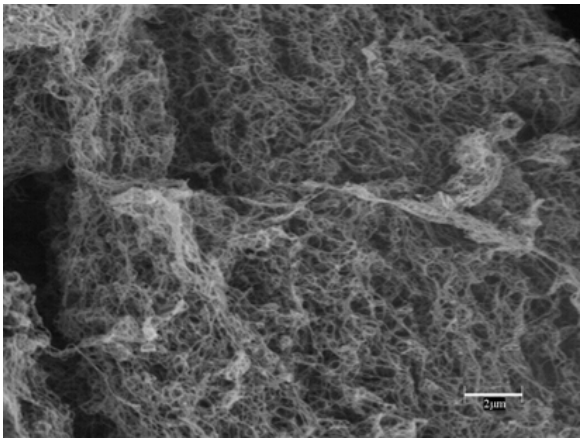

Figure 3. Scanning Electron Micrographs (SEM) of carbon nanotubes.

In addition, many of the carriers impart an excess positive charge on the surface of the particles. This promotes interaction between the particles and cell surface and thereby aids in cellular uptake. Since DNA can be degraded by cytosolic nucleases also, nanoparticulate non-biological vectors should also be able to escape from endosomes. Although viral vectors have the advantages over non-viral vectors in terms of gene transfer efficiency, the non-viral vectors have a merit in their low immunogenecity, the absence of endogenous virus recombination, low production cost, and reproducibility. Several different approaches have been used to improve this step, ranging from using endosomolytic agents such as chloroquine to using pH-sensitive lipids and polymers (50, 51).

4.2.2. Liposomes

Liposomes, 100-300 nm vesicles of lipids, were first proposed as a unique drug delivery system in the late 1960's by A.D. Bangham. DOTMA (N- (1- (2, 3-dioleyloxy) propyl)-N,N-trimethylammonium chloride) was the first synthetic cationic lipid to be used as a gene delivery vector in 1987 by Felgner *et al.* (52). Recently autologous mesenchymal stem cell based cartilage regeneration has been shown in rabbit bone marrow-derived mesenchymal stem cells (MSCs) which were stably transfected with the TGF-β1 gene in monolayer culture using a liposome Lipofectamine 2000. After transfection, the expression of cartilage-specific extracellular matrix was upregulated, whereas matrix metalloproteinases 1 and 3 (MMP 1 and 3) protein expressions and enzymatic activities were downregulated. This resulted in accelerated repair of the cartilage (53).

4.2.3. Polyplexes

Early studies were conducted using naturally occurring polymers such as chitosan and proteins such as histones and cationized human serum albumin. Other polymers used include polypeptides such as poly-L-lysine and poly-Lornithine, and polyamines such as polyethylenimine (PEI) and starburst polyamidoamine (PAMAM) dendrimers. Both linear and branched forms of polymers have been used. Several synthetic copolymers containing hydrophilic segments (such as polyethylene glycol, PEG or dextran) have also been evaluated. A study shows after genetically engineering MSC by the spermine-dextran complex with plasmid DNA of adrenomedullin (AM), a large amount of AM (an anti-apoptotic and angiogenic peptide) is secreted. Transplantation of AM gene-engineered MSCs improved cardiac function after myocardial infarction significantly more than MSCs alone. Thus, this genetic engineering technology using the non-viral spermine-dextran is one of the promising strategies to improve MSC therapy for ischemic heart disease (54).

4.2.4. Carbon nanofibers (CNF) and carbon nanotubes (CNT)

Carbon-based nanotechnology has been rapidly emerging as a platform technology for a variety of medical applications. Carbon nanofibers (CNF) are cylindric nanostructures with graphene layers. CNF with graphene layers that are wrapped into perfect cylinders are called carbon nanotubes, represent along with fullerenes, the third allotropic crystalline form of carbon. CNT are cylindrical carbon tubes with diameters at nanometer range with very large aspect ratios (see Fig.3). There has been a growing interest in utilizing CNT for biomedical applications owing to their high aspect ratio and wide range of structural, electrical, and thermal properties. It was not until 2004 that CNTs were first used in tissue engineering (55).

CNT are of two types – Single walled (SWNT) or multi walled (MWNT). The diameter of SWNT varies from 0.4 to 2 nm whereas for MWNT it is 1.4 to 100nm, with each concentric ring separated by about 0.34 nm. CNT exhibit unique mechanical, chemical, physical, and biological properties and are amenable to chemical modifications. CNT have been successfully used for stem cell scaffolding to enhance the therapeutic efficacy of stem cell therapy (56). Several studies have suggested that direct injection of stem cells (no scaffolding) is of low efficacy due to the wash out of stem cells at the site of injection inside the target tissue. However, if stem cells form networks along with scaffold materials such as CNT, their efficacy will significantly increase upon injection *in vivo*. Another study revealed the promise of using CNT and carbon nanofibers for treating a variety of neural disorders and diseases (57). More specifically, stem cells along with carbon nanofibers were injected into stroke damaged neural tissue in rat brains. Results showed that stem cells differentiated into neurons and motor function was returned to stroke-induced rats with little scar tissue forming surrounding nanofibers after 3 weeks of implantation as shown in Figure 4; such results were not observed using stem cells alone (58).

CNT are also being investigated in the field of cardiac tissue engineering and regeneration. In an effort, Mooney *et al.* (59) studied the effect of CNT on human mesenchymal stem cells (MSC) responses including biocompatibility, proliferation, and multipotency. A range of different types of CNTs, including single-walled nanotubes (SWCNT), multi-walled nanotubes (MWCNT) and functionalized CNT were studied.

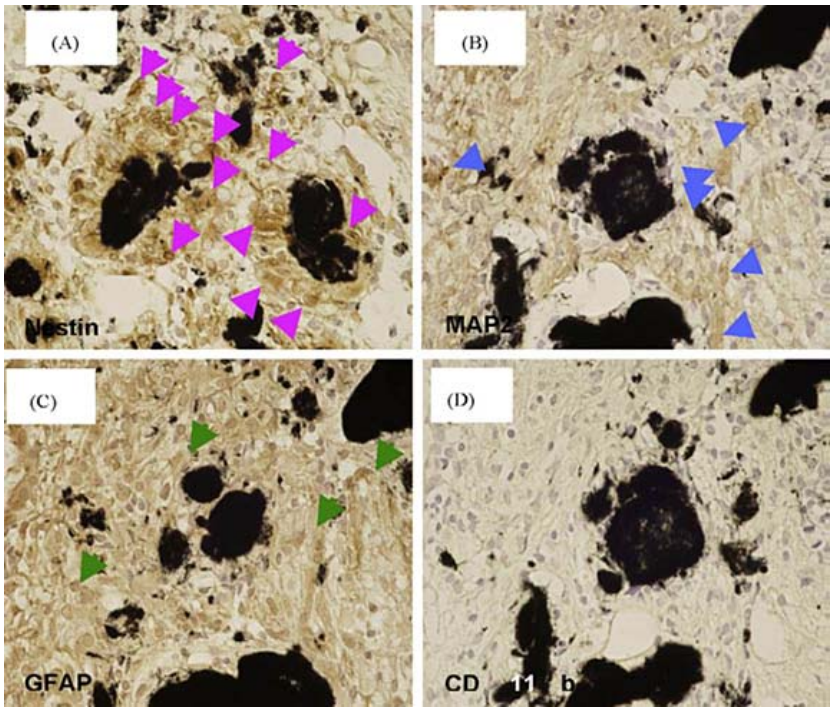

Figure 4. Histology of carbon nanofibers (black spots) impregnated with stem cells into stroke damaged rat neural tissue after 3 weeks. (A) and (B), numerous active neuroprogenitor cells and fully differentiated neurons (brown stained cells) were found around CNFs. (C) and (D), few glial cells interacting with CNFs led to little or no scar tissue formation. Adapted with permission from (58).

It was found that COOH-functionalized SWCNT had no significant effect on cell viability or proliferation at low concentrations. Furthermore, COOH functionalized SWCNT, which were fluorescently labeled, migrated into the nuclear location within the cell after 24h without adversely changing the cellular ultrastructure as shown in Fig.5. In addition, the CNT were found to have no effect on adipogenesis, chondrogenesis or osteogenesis. Studies like this highlight the importance of stem cells nanotechnology and its potential to advance the field in many venues including manipulation of MSC differentiation, development of nanocarriers for delivery of bioagents to stem cells, and development of new biomaterials for regenerative medicine applications. Many studies have shown the good potential of CNT for delivery of genes and peptides into cells (60, 61).

4.2.5. Nanoparticles

A recent research by Kharlamov *et al.* (62) presented at the 2010 Scientific Session of American Heart Association has shown that a technique that combines nanoparticles with adult stem cells can destroy atherosclerotic plaque and rejuvenate the arteries. In the study, silica-gold nanoparticles were infused into the heart of pigs along with adult stem cells. After laser light heated the nanoparticles, they burned away arterial plaque. The study also showed that nanoparticles were more effective at eliminating plaque when combined with adult stem cells compared to nanoparticles alone.

5. NANOTECHNOLOGY FOR CREATING STEM CELL NICHES

Stem-cell populations are established in 'niches', specific anatomic locations that regulate how they participate in tissue generation, maintenance and repair. The niche saves stem cells from depletion, while protecting the host from over-exuberant stem-cell proliferation. It constitutes a basic unit of tissue physiology, integrating signals that mediate the balanced response of stem cells to the needs of organisms (63). Creating 3D tissue and organs from stem cells is a principal endeavor of tissue engineering. In order to control and direct stem cells toward this goal, a defined biomimetic environment is essential. This is achieved by a combination of signaling molecules and scaffolds. Among many properties of scaffold, one of utmost importance is the size and structural characteristics of the matrix. This is because the native ECM is a dynamic and hierarchically organized nanocomposite. While the components of ECM provide cues for cell adhesion etc., appropriate size is responsible for modeling of tissue and determines their orientation. In a typical connective tissue, protein fibres such as collagen, laminin and elastin have dimensions ranging from 10 to several hundred nanometers and entangle with each other to form a non-woven mesh providing the necessary strength to growing cells. Interestingly, only 1% of the ECM is solid content thus leaving nature to modulate mechanical properties of matrix by alterations of its nano-scale organization. Structural ECM features (pore size, pore density, connectivity etc.) are also responsible for determining cellular migration.

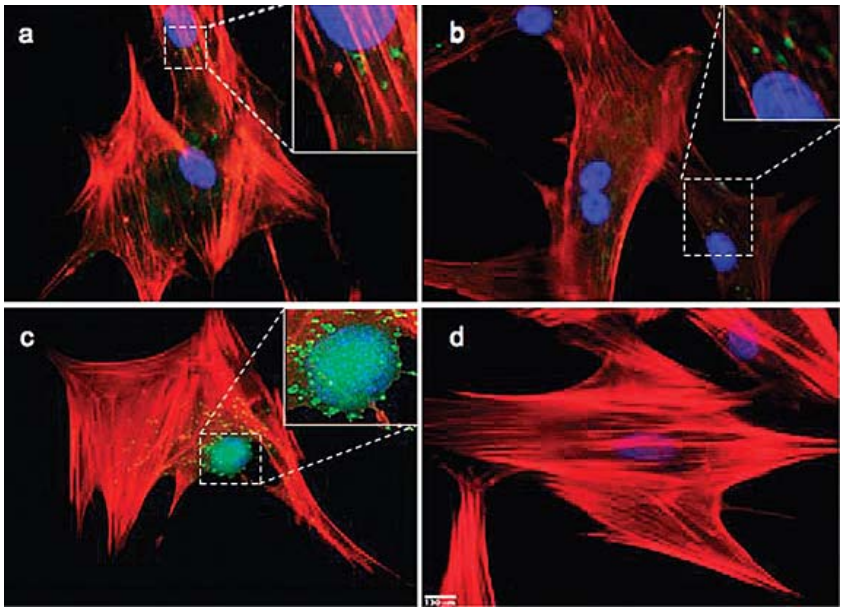

Figure 5. Uptake of COOH-functionalized SWCNT by stem cells. Fluorescent images of biotinylated CNT within the cell after (a) 24 h, (b) 48 h, and (c) 6 days and (d) hMSC alone. Adapted with permission from (59).

Thus, it is evident that simulating nano-scale organization and topography in laboratory conditions will be key to the success of stem cells in tissue engineering (64).

Various technologies are experimented with to mimic the exact ECM. Electro-spinning process and molecular self-assembly are two of the popular approaches. Electron Beam Lithography (EBL) has been used to fabricate ultraprecise nanotopographies of ordered arrays down to 10nm in size for the examination of cell–nanoenvironment interactions (65). A group of investigators has shown nanometer-scale metal patterns can be fabricated on quartz glass substrates (with suitable chemical modifications to study protein adsorption) by electron-beam lithography and metal sputtering methods. Briefly, nanopatterns were drawn by electron-beam lithography

(ELS-7500, Elionix) on a quartz glass substrate with an electron-beam resist coating. After development, titanium and gold were sputtered on the substrate, and the remaining resist was removed. Metal stripes that were 150 nm in height and as small as 250 nm in width were fabricated. The patterned surface has topographical and chemical patterning, maintained its ability to control protein and cell adsorption for more than 10 days, and realizes a variety of conditions for cell-surface adhesion. This study shows that when the pattern width is smaller than 750 nm, many cell monolayers detach from the surface.

In addition, when the width is 250 nm, cells don not detach thus emphasizing the need of nanofabrication techniques to create stem cell niche (66).

Dalby and co-workers have used EBL to the substratum, polymethylmethacrylate (PMMA), was embossed with 120-nm-diameter, 100-nm-deep nanopits over 1 cm^2 from an original pattern defined using EBL. Five different arrays were used, all with either absolute or average centre–centre spacing of 300 nm and response was monitored with MSC and progenitor cells, and show the differences observed with nanotopographies for bone formation (67). Other methods of soft lithography can also be adapted for nano-structuring biopolymers and evaluating their response. Most of these use p olydimethylsiloxane (PDMS) and its various modifications. A review describes the leading techniques for generating nanopatterns with biological function including parallel techniques such as extreme ultraviolet interference lithography (EUV-IL), soft-lithographic techniques (e.g., replica molding (RM) and microcontact printing (muCP)), nanoimprint lithography (NIL), nanosphere lithography (NSL) (e.g., colloid lithography or colloidal block-copolymer micelle lithography) and the nanostencil technique, in addition to direct-writing techniques including e-beam lithography (EBL), focused ion-beam lithography (FIBL) and dip-pen nanolithography (DPN) as applied in nanobiotechnology. Details on how the patterns are generated, how biological function is imparted to the nanopatterns, and examples of how these surfaces can and are being used for biological applications can be explored. It is a matter of time that all these techniques will be standardized for application to stem cells (68).

6. CONCLUDING REMARKS

Stem cells are potential sources of therapeutics and useful tools for understanding processes of human physiology and pharmacological interventions. However, the state of art in stem cell research is yet nascent and

requires elaborate studies before reaching the end-user. In quest to attain this goal, nanotechnology and allied techniques will be an indispensable approach for investigators.

7. ACKNOWLEDGMENTS

Kaushik Dilip Deb and Mehrdad Rafat equally contributed to this article.

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