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Surface Engineering to Control Embryonic Stem Cell Fate

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1. Introduction

The niche established by supportive cells and the extracellular polymeric matrix (ECM) probably regulates stem cell fate through multiple, complimentary mechanisms, including the spatiotemporally defined presentation of immobilized signaling molecules, the modulation of matrix stiffness, the physicochemical characteristics of the environment, and the creation of cytokine gradients. In contrast to tissue-specific stem cells, embryonic stem (ES) cells are present only transiently in the developing embryo, and therefore, do not have a stable niche in vivo. ES cells also differ from tissue-specific stem cells in their ability to be readily expanded in culture over long time periods. However, the culture systems that have been used successfully for ES cell expansion suggest that ES cell self-renewal versus differentiation is regulated in a similar manner to tissue-specific stem cells, via interactions with other cells, ECM components, soluble factors, and the physicochemical environment (McDevitt & Palecek, 2008). ES cells commute between metastable states from the inner cell mass (ICM) to the epiblast stage, and these reversible states are associated with distinct differentiation potentials (Toyooka et al., 2008; Hayashi et al., 2008; Pelton et al., 2002). Thus, ES cells represent a highly dynamic, self-renewing population that responds to environmental cues to maintain its pluripotency or to differentiate. In ES cell cultures, these cues include growth factors in the culture medium surrounding the ES cell colonies or secreted by the colonies themselves, and signals arising from the ES cells' adhesion to the substrate and the stiffness of the substrate (Discher et al., 2009).

ES cells are anticipated to serve as an unlimited cell source for cell transplantation therapy. However, the most common techniques for controlling ES cell fate using soluble biochemical and biological factors (cytokines and growth/differentiation factors) in the growth medium are often inefficient, and the resulting cell population (either undifferentiated or differentiated) is not homogenous. The idea that ES cell populations are homogenous was first challenged by Cui et al., who observed differential spatial distributions of adhesion molecules within ES cell colonies (Cui et al., 2004), and more recently by the derivation of epiblast stem cells from ES cell (Brons et al., 2007) and the identification of ES cell subpopulations in mouse ES cell cultures (Toyooka et al., 2008).

To improve the efficiency for controlling the ES cell fate, researchers have recently focused on the stimulation of receptors on the ES cell membrane through interactions with solid surfaces. In particular, the interactions of biologically active components with cells can be strengthened by fixing the signals on a surface in close contact with their targets on the cell membrane, because when the signaling components are dispersed in a bulk liquid (medium), they are less likely to encounter their targets.

This chapter will present various surface design strategies for regulating ES cell morphology and function that use micro/nanoscale technologies and a wide range of natural and synthetic materials. First, we will introduce the principles for modifying the culture surface with reference to recent studies that have used various surface design strategies (reviewed in Dellatore et al., 2008; Keung et al., 2010; Saha et al., 2007) and their corresponding effects on ES cell behavior. The latter part of the chapter will describe dendrimer-immobilized surfaces designed in the authors' studies and their effects on the in vitro culture of mouse ES cells.

2. Surface-based control of the morphology and function of cultured ES cells

In this section, we provide an overview for designing the culture surface, as categorized into four general approaches for controlling ES cell fate (Table 1).

Modification	Examples	Observations	Reference
Chemical modification	Plasma etched polystyrene	Maintenance of hESC pluripotency	Mahlstedt et al., 2010
	Plasma-deposited gradients of octadiene to acrylic acid	Effect on mESC adhesion and differentiation	Wells et al., 2009
	PDMS or SAM surfaces presenting terminal hydrophobic moieties	Enhancement of the differentiation yields of hESCs	Valamehr et al., 2008
	Combinatorial library of biomaterials formed from acrylate and methacrylate monomers	Uniform hESC differentiation into epithelial cells	Anderson et al., 2004
Biofunctionalization	ECM, such as matrigel, laminin, fibronectin	Expansion and maintenance of hESCs and mESCs	Stewart et al., 2008; Meng et al., 2010; Flaim et al., 2008
	Laminin, fibronectin, and gelatin	Promotion of mESC differentiation toward neural lineages	Goetz et al., 2006
	Decellularized bone-specific ECM	Promotion of mESC differentiation toward the osteogenic lineage	Evans et al., 2010

Table 1. Various strategies for surface engineering to control ES cell fate

Modification	Examples	Observations	Reference
Biofunctionalization (Continued)	ECM molecules on a Layer-by-layer self-assembled surface of HA and chitosan	Efficient attachment of hESCs	Doran et al., 2010
	E-cadherin-coated surface	Increased proliferative ability and transfection efficiency for mESCs	Nagaoka et al., 2006
	Laminin peptides presented in SAMs on gold	Support of hESC expansion by different peptides from the laminin γ and β chain	Derda et al., 2007
	RGD-modified materials	Promotion of hESC differentiation toward the chondrogenic lineage	Hwang et al., 2006
	RGD and CRGDC-modified materials	Support of hESC culture	Kolhar et al., 2010
	Random peptide libraries using phage display	Expansion and maintenance of hESCs on SAMs presenting specific peptide sequences	Derda et al., 2010
	Immobilized LIF	Expansion and maintenance of ESCs	Nagaoka et al., 2008; Makino et al., 2004; Alberti et al., 2008
	Covalent binding of FGF-2 to polyamide nanofibrillar surfaces	Support of hESC expansion and colony formation	Nur-E-Kamal et al., 2008
	Immobilized VEGF	Promotion of mESC differentiation toward endothelial cells	Chiang et al., 2010
Geometric modification	Topographically microstructured surface libraries	Effect on proliferation and differentiation of mESCs	Markert et al., 2009
	Electrospun polyamide nanofibers	Expansion and maintenance of mESCs	Nur-E-Kamal et al., 2006
	Nanoscale ridge/groove pattern arrays	Promotion of hESC differentiation toward the neuronal lineage	Lee et al., 2010
	Electrospun fibrous scaffolds	Promotion of mESC differentiation toward the neuronal lineage	Xie et al., 2009

Table. 1. (Continued)

Modification	Examples	Observations	Reference
Geometric modification (Continued)	Nanofibrous architecture	Promotion of hESC differentiation toward the osteogenic lineage	Smith et al., 2010
Mechanical modification	Nanofilms made of PLL and HA	Promotion of mESC differentiation toward the epiblast lineage by surface stiffness	Blin et al., 2010
	PDMS substrates	Promotion of mESC differentiation toward the osteoblast lineage by surface stiffness	Evans et al., 2009

Acronyms:
mESC: mouse embryonic stem cell; hESC: human embryonic stem cell; PDMS: Polydimethylsiloxane; SAM: Self-assembled monolayer; ECM: Extracellular polymeric matrix; HA: Hyaluronic acid; RGD: Integrin-binding Arg-Gly-Asp; CRGDC: Cyclic RGD; LIF: leukemia inhibitory factor; FGF-2: Fibroblast growth factor; VEGF: Vascular endothelial growth factor; PLL: poly (L-lysine).

Table. 1. (Continued)

2.1 Control of cells by chemical modification of the substrate

The chemical properties of substrates (e.g., hydrophobicity) play an important role in the kinetics of protein adsorption and folding, which in turn influence cellular activities. Mahlstedt et al. demonstrated that the physicochemical modification of polystyrene by plasma etching can improve the culture surface's ability to maintain human ES cell pluripotency (Mahlstedt et al., 2010). Elsewhere, plasma-deposited gradients of octadiene to acrylic acid were fabricated to investigate the effect of carboxylic group (COOH) concentration on mouse ES cell adhesivity and differentiation status (Wells et al., 2009). In addition, by altering the hydrophobicity of a surface, the formation and differentiation potential of ES cells within embryoid bodies (EBs) can be tuned to promote a desirable EB size and composition (Valamehr et al., 2008).

Because it is often difficult to predict how a stem cell will respond to environmental cues, methods have been developed for the rapid screening of interactions between biomaterials and stem cells. A combinatorial library of biomaterials formed from different acrylate and methacrylate monomers has proved to be useful for identifying environments suitable for the uniform differentiation of ES cells into epithelial cells (Anderson et al., 2004).

2.2 Control of cells by biofunctionalization

Artificial materials can be endowed with precise biological functionalities by immobilizing bioactive molecules such as cytokines, growth factors, ECM proteins, and adhesive peptides on their surface. These biomolecules can be simply adsorbed onto the material's surface or covalently linked via chemical groups previously created on the surface. The biological response following the surface biomodification of a material depends on structural parameters, such as the density of the ligands, their spatial distribution, their colocalization with synergistic ligands, etc.

2.2.1 Cell-adhesive peptides and proteins

Specific ECM-cell and cell-cell interactions are important for providing spatial anchors as well as signals that regulate stem cell maintenance, survival, and differentiation. Cell adhesion is also required for a cell to sense other contextual information, such as the mechanical properties of the microenvironment. Here we review the ways that engineered systems have been used to identify functional adhesive peptide sequences or proteins and to investigate their interactions with ES cells.

ECMs can be used either for feeder-free culturing or for stimulating ES cell differentiation toward a desired cell lineage by incorporating tissue-specific ECM signals. Stewart et al. reviewed the feeder-free conditions that have been successfully applied to culture human ES cells using various types of ECM, such as matrigel, laminin, and fibronectin (Stewart et al., 2008). For example, matrigel, a complex mixture of hundreds of ECM and other proteins, has emerged as a common substrate for human ES cell and human induced-pluripotent stem (iPS) cell culture. Meng and colleagues (Meng et al., 2010) investigated the adhesive interactions in matrigel involved in the maintenance of human ES cell pluripotency. They found that whereas three peptides were able, individually, to support human ES cell growth and pluripotency for short periods of time, their combination enhanced the quality of the culture and the duration of the cells' pluripotency. This finding illustrates how engineered systems can be used to parse out the synergistic contribution of individual motifs within full-length natural proteins, which may inspire future mechanistic studies.

Flaim and co-workers (Flaim et al., 2008) analyzed combinatorial mixtures of ECM molecules to understand their cooperative control of murine ES cell differentiation, and rapidly identified key mixtures with synergistic properties. Other groups have directed stem cell differentiation toward neural lineages by using laminin, fibronectin, and gelatin (Goetz et al., 2006). In another report, decellularized bone-specific ECM promoted the osteogenic differentiation of ES cells (Evans et al., 2010). Recently, Doran et al. used a simple, effective, and efficient method to design a defined high-protein-content surface for stem cell culture (Doran et al., 2010). They demonstrated the highly efficient attachment of human ES cells to various extracted and recombinant ECM molecules presented on a layer-by-layer self-assembled surface of hyaluronic acid and chitosan.

In another study, Nagaoka et al. demonstrated that mouse ES cells cultured on an E-cadherin-coated surface maintained unique morphological characteristics, retained the full complement of ES cell features, and showed a higher proliferative ability and transfection efficiency than those grown under conventional conditions. Furthermore, when grown on the E-cadherin-coated surface, the ES cells also required less leukemia inhibitory factor (LIF) than those grown under conventional conditions, probably due to the homogenous exposure to LIF achieved in this culture system (Nagaoka et al., 2006).

Cell-adhesive ligands can, when incorporated into biomaterials, be used to mediate specific receptor–ligand interactions, and thereby to activate selected receptor-mediated signaling pathways to control cell behavior and differentiation. Several cell-adhesive peptides, such as the integrin-binding Arg-Gly-Asp (RGD) motif, have been incorporated into materials to enhance the cell–matrix interaction. For instance, RGD promotes the chondrogenic differentiation of human ES cells (Hwang et al., 2006). In another study, Kolhar et al. demonstrated that both RGD and cyclic RGD (CRGDC) can support the culture of human ES cells, with CRGDC increasing their adhesion 4-fold over the linear RGD peptide (Kolhar et al., 2010). The identification of peptide sequences such as RGD has been pivotal in

advancing biomaterial research, because of the ease of synthesizing, manipulating, and tuning the properties of such materials (Hersel et al., 2003). Nevertheless, only a few adhesive peptide sequences have been found in natural proteins. It is likely that the identification of cell growth substrates would be accelerated by the discovery of new peptide ligands for cell-surface receptors.

In addition, several peptide mimics of the laminin cell-binding domain have been evaluated in stem cell cultures. Derda et al. evaluated a wide variety of laminin peptides presented in self-assembled monolayers (SAMs) on gold for their ability to support human ES cell adhesion and proliferation (Derda et al., 2007). Four different peptides from the laminin γ chain and one peptide from the β chain supported ES cell expansion and the expression of the primitive markers Oct4, alkaline phosphatase, and SSEA4, to a similar extent as matrigel in six-day cultures (Derda et al., 2007). In another recent study, Derda et al. screened random peptide libraries using phage display to identify novel ligands to support the proliferation of pluripotent cells. When human ES cells were cultured on SAMs presenting the sequence TVKHRPDALHPQ or LTTAPKLPKVTR in a chemically defined medium, they expressed pluripotency markers at levels similar to those of cells cultured on matrigel (Derda et al., 2010). These results indicate that this screening strategy is a productive avenue for generating new materials that control the growth and differentiation of cells.

The combined use of rational and library-based screening methods should provide an increasing number of ligands for the functionalization of synthetic systems, and may aid the mechanistic investigation of specific receptors and signaling events that regulate the responses of stem cells to their microenvironment.

2.2.2 Cytokines and growth factors

The ECM not only offers sites for cell adhesion, but it can also serve as a platform for the presentation of other biochemical factors that orchestrate cell-cell interactions. Whereas stem cell researchers have often investigated growth factors and cytokines as soluble factors, many of these proteins have matrix-binding domains that may enable them to be presented within the niche as "solid phase" ligands.

For example, several studies have immobilized LIF by various strategies to maintain ES cells in an undifferentiated state. An immobilizable fusion protein consisting of LIF and the IgG-Fc region, named LIF-FC, can maintain the ES cells in the undifferentiated state (Nagaoka et al., 2008). Similarly, a photo-immobilized LIF stimulates the activation of STAT3 for a longer time than does soluble LIF, and as a result, maintains ES cells in an undifferentiated state (Makino et al., 2004). In another study, both LIF and stem cell factor (SCF) were immobilized, and the threshold effects of these factors on stem cell maintenance were analyzed (Alberti et al., 2008). These studies demonstrated that using immobilized LIF reduces the need to add soluble LIF frequently to the medium.

Finally, the covalent binding of growth factors has proved to be helpful in controlling human ES cell growth and differentiation. Fibroblast growth factor (FGF)-2 immobilized on polyamide nanofibrillar surfaces inhibits the rapid degradation of FGF-2 in solution and supports the expansion and colony formation of human ES cells (Nur-E-Kamal et al., 2008). Another study demonstrated that the cultivation of mouse ES cells on surfaces with immobilized vascular endothelial growth factor-A (VEGF) yields primarily endothelial cells, whereas their cultivation on such surfaces without VEGF yields primarily vascular smooth muscle-like cells (Chiang et al., 2010).

2.3 Control of cells by geometric modification

Topographical structures such as grooves, ridges, and pits are present in many natural structures at the nanoscale level, as in the fibrous structure of collagen and other ECM proteins, and at the microscale level, as in the pores in bone marrow and the undulating basement membranes in the epidermis. The presence of topographical information in natural systems has motivated the use of technologies such as soft lithography, microfluidics, electrospinning, and the deposition of nanostructures (Khademhosseini et al., 2006; Pirone & Chen, 2004; Yang et al., 2005) to engineer substrate materials' topography to affect stem cell responses at both the nano and micro levels.

How cells sense topographical cues from the environment has been debated, but the cellular response to surface topographies is known to involve cytoskeletal changes and the modulation of focal adhesion formation (Lim & Donahue, 2007; Biggs et al., 2008). A recent study indicated that integrins may be involved in these cellular responses (Wood et al., 2008), suggesting that established adhesion signaling pathways are involved.

Little is known about the effect of artificial micro- and nanoscale topographical surfaces on the ES cell differentiation state. Recently, Markert et al. investigated the influence of topographical microstructures on the proliferation and differentiation of mouse ES cells. Their findings indicated that one class of microstructures sustains the feeder-free proliferation of undifferentiated ES cells and another class enforces differentiation, as indicated by the spreading of the cells (Markert et al., 2009). Murine ES cells cultured on electrospun polyamide nanofibers that mimic the basement membrane texture showed twice the cell expansion of those cultured on coverslips, while retaining their Nanog expression and differentiation potential (Nur-E-Kamal et al., 2006). Lee et al demonstrated that nanoscale ridge/groove pattern arrays alone can effectively and rapidly induce the differentiation of human ES cells into a neuronal lineage, without the use of any differentiation-inducing agents. They proposed that elongation of the cytoskeleton during the morphological changes in cells guided by ridge/groove patterns results in a transfer of tensional force to the nucleus, which influences gene expression and signal transduction (Lee et al., 2010). Similarly, another study demonstrated that mouse ES cells can be induced to differentiate into specific neural lineages, that is, neurons, oligodendrocytes, and astrocytes, when seeded onto electrospun fibrous scaffolds (Xie et al., 2009). In another study, the nanofibrous architecture of the substrate enhanced the osteogenic differentiation of human ES cells compared to a more traditional scaffolding architecture (Smith et al., 2010).

Thus, surface engineering approaches that alter the topographical structure of the substrate surface can be used to modulate ES cell behavior and fate.

2.4 Control of cells by modification of material mechanics

Of the many mechanical properties of biological systems, stiffness or rigidity is perhaps the most apparent and widely studied. Mechanical stiffness reflects a material's ability to store and frictionally dissipate applied mechanical energy, as reflected by storage (elastic) modulus and loss (viscous) modulus, respectively. The elastic modulus is the measure of the stress required to achieve a specific strain in a material without permanent deformation, and has emerged as an important regulator of stem cell function. Upon mechanical stimulation, cells convert mechanical signals into biochemical responses through a mechanism called, "mechano-transduction" (Orr et al., 2006). Cells interact with their surroundings via ECM

receptors such as integrins and laminin receptors. Specifically, the ECM dynamics and matrix stiffness are translated into cytoskeletal tension mediated by integrin–ECM interactions (Katsumi et al., 2004). Integrin signaling is principally mediated by focal adhesion kinases, and the cell's responses to these signals can modulate a number of intracellular pathways that may cooperatively affect the activation SMADs, Rho GTPases, ERK, and other downstream signaling pathways that lead to transcriptional and epigenetic changes (Miyamoto et al., 1995). For example, integrin-mediated adhesion signaling cooperates with soluble-factor signaling to regulate Rho GTPases and generate actin cytoskeletal tension (Clark et al., 1998).

Recently, Blin et al used nanofilms made of poly(L-lysine) and hyaluronan (HA), named PLL/HA, which were cross-linked to various extents, to modulate the nanoenvironment of ES cells. The adhesion of ES cells to the nanofilms increased from the native film to the highly cross-linked films. The adhesion process was associated with cell proliferation. The dynamic balance of the ES cells between the ICM and the epiblast states was also dependent on the cross-linking of the nanofilms. The more cross-linked and thus stiffer the film was, the more cells were driven toward the epiblast fate. This finding suggests that the stiffness of the nanofilm can play a key role in modulating the ES cell niche to govern the ES cell self-renewal and fate (Blin et al., 2010).

Similarly, in another study, the behavior of ES cells grown on a flexible polydimethylsiloxane substrate of varying stiffness was examined. While cell attachment was unaffected by the stiffness of the growth substrate, both cell spreading and cell growth increased with increasing substrate stiffness. Moreover, several genes expressed in the primitive streak during gastrulation and implicated in early mesendodermal differentiation were upregulated in cell cultures on the stiffer substrates than on the softer ones. Finally, the osteogenic differentiation of ES cells was enhanced on stiff substrates compared to soft ones, demonstrating that the mechanical environment can play a role in both early and terminal ES cell differentiation (Evans et al., 2009).

3. Strategies for culture surface design using glucose-displaying dendrimer substrates

3.1 Surface design and characterization

A schematic illustration showing preparation of culture surfaces based on dendrimer substrates is shown in Fig. 1.

Starburst polyamidoamine (PAMAM) dendrimers are highly branched spherical polymers with well-defined structures and primary amino groups at their terminals. It is quite easy to modify the chemical properties of dendrimers by adjusting their terminal groups (Kawase et al., 2000; Tomalia et al., 2003). When an additional layer or generation is polymerized on the dendrimer molecules, the number of terminal amino groups is doubled. The defined dendrimer structure and large number of terminal amino groups allow great flexibility in the design variables, including the ligand species presented on the terminal groups, dendrimer size, and ligand density, making these polymers suitable for use as biocompatible nanometer-sized capsules in gene- or drug-delivery systems, as well as in scaffolds for cell culturing (Tomalia et al., 2003).

Dendrimers deposited on a solid surface have unique properties that yield physical and chemical variations in the surface; these properties are also affected by the ligand species and amounts displayed on the dendrimers, and the locations of the displayed ligands.

Surfaces with different topographies can be obtained by changing the dendrimer density and generation number, as illustrated in Fig. 1. In addition, dendrimers can offer extended design parameters, such as an altered ligand ratio of D- to L-glucose isomers (termed one-ligand display for cell anchoring) or the co-display of an adhesive ligand (D-glucose) and a functional ligand (e.g., growth factor) on the surface (termed multi-ligand display for cell anchoring and stimulation) (Fig. 1).

D-Glucose molecules on the culture surface and glucose transporters (GLUTs) on the cytoplasmic membrane are assumed to function as binding and receptor sites, respectively. GLUTs show sharp specificity in their binding affinity for glucose isomers: they exhibit high affinities for D-glucose but extremely low affinities for L-glucose. D-Glucose itself does not induce cell signaling. However, it is likely that such high-affinity GLUTs can act as a cell-anchoring mechanism by binding D-glucose molecules displayed on the surface.

Evidence suggests that the nanoscale geometry of dendrimer substrates plays crucial roles in determining cellular responses to the substrate. The generation number of dendrimers and their density yield varying, cell-specific responses. Kim et al characterized various dendrimer-immobilized surfaces with different architectures in terms of their surface roughness using an atomic force microscope, and found their mean roughness to range from 1.8-11.0 nm. The combination of displayed D-glucose and roughness promoted cytoskeletal formation, accompanied by the elongation of cells on the culture surface. The authors concluded that a dendrimer substrate with a D-glucose display offers a solid environment that permits the partial anchoring of the cells via the temporarily grasping of the GLUTs by D-glucose (Kim et al., 2007a).

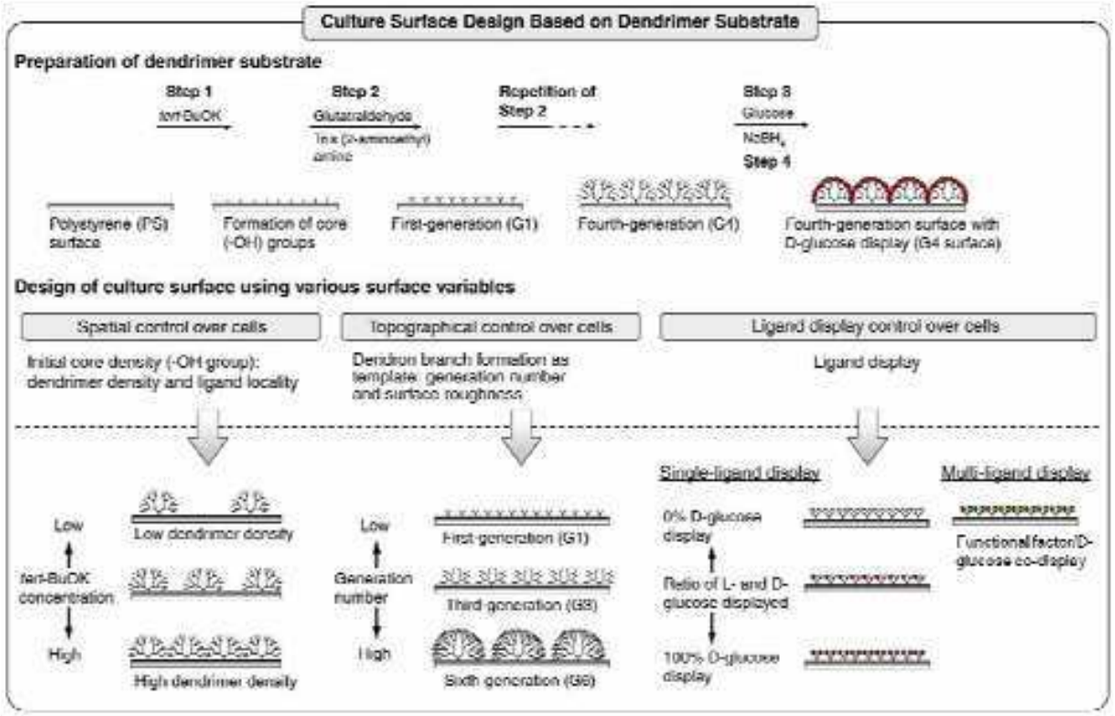

Fig. 1. Schematic illustrations showing the preparation of culture surfaces based on dendrimer substrates (reproduced with permission from Kim et al., 2010a)

In another study, an extended substrate design with improved cell anchoring and migration using the concurrent display of D-glucose and EGF was reported (Kim et al., 2007b). The displayed D-glucose molecules permit the cells to be in close contact with the surface via the grasping of GLUTs on the cytoplasmic membrane, thereby leading to increased focal contacts that can induce the up-regulation of EGF receptor signaling. This study used an advanced design to target cells by plating them on dendrimer-immobilized substrates that strongly stimulated cell behaviors.

These studies demonstrated the potential for dendrimer-immobilized surfaces to regulate cell morphology and subsequently cell functions, via morphologic priming. Recent strategies and concepts for culture surface designs based on cell anchoring mechanisms, and using glucose-displaying dendrimer substrates to regulate cell morphology and function, are reviewed elsewhere (Kim et al., 2010a). In the next section, we will describe the morphological and functional responses of mouse ES cells cultured on a D-glucose-displaying dendrimer (GLU/D) surface.

3.2 Enrichment of undifferentiated mouse ES cells on dendrimer-immobilized surface

ES cells are pluripotent cells that are characterized by their ability to propagate indefinitely in culture as undifferentiated cells with a normal karyotype, and to differentiate into derivatives of the three primary germ layers. Although ES cells are expected to serve as an unlimited cell source for cell-transplantation therapy, great care is required to maintain undifferentiated ES cell cultures, since the cells can spontaneously differentiate via seemingly random pathways under normal ES cell culture conditions, especially in the course of expanding the colony density and size (Watt & Hogan, 2000). Therefore, cultured ES cells may develop into colonies of heterogeneous cell types that include cells with less pluripotency. Our group has been investigating the possibility of using the dendrimer surface as a tool for obtaining cell preparations enriched in undifferentiated ES cells (Mashayekhan et al., 2008).

Here we present our results showing the enrichment of undifferentiated ES cells by serial passaging on a fourth-generation GLU/D surface. The morphologies of the ES single cells as well as the ES cell colonies on different culture surfaces were compared as indicated in Fig. 2. The single-cell observation on day 1 showed that most of the cells on the GLU/D surface were round, while those on the gelatinized surface exclusively showed a stretched morphology (Fig. 2 A, B). Moreover, the cells on the GLU/D surface formed loosely attached spherical colonies, while those on the conventional surface formed flatter colonies that were firmly attached to the surface (Fig. 2 C, D).

Time-lapse observations showed that on the gelatinized surface, the cells started to divide while spreading, and they experienced contact inhibition upon becoming confluent on the surface, resulting in the formation of dome-shaped colonies. In contrast, the cells on the GLU/D surface made spherical colonies as they divided, probably because of the increased frequency of cell-cell contacts. As shown in Fig. 2E, the outermost layer of the spherical colonies near the GLU/D surface consisted of much fewer cells than in the colonies (either flat or compact) on the gelatinized surface, which can explain the difference in the colonies' attachment strength to the two surfaces.

Cell morphology is one of the most important parameters in the regulation of stem cell growth and differentiation, and is determined through signaling that reorganizes the actin cytoskeleton. The cytoskeleton is implicated in mechanotransduction, since it links the

stimulation from an extracellular environment (e.g., solid surface) with an intracellular signaling mechanism that regulates cell functions. Cellular mechanotransduction requires the rearrangement of membrane constituents, focal contact formation, and an association with a dynamic actin cytoskeleton and Rho family GTPase-mediated signal pathways, which have emerged as key regulators of cadherin-mediated cell-cell adhesion (Fukata & Kaibuchi, 2001).

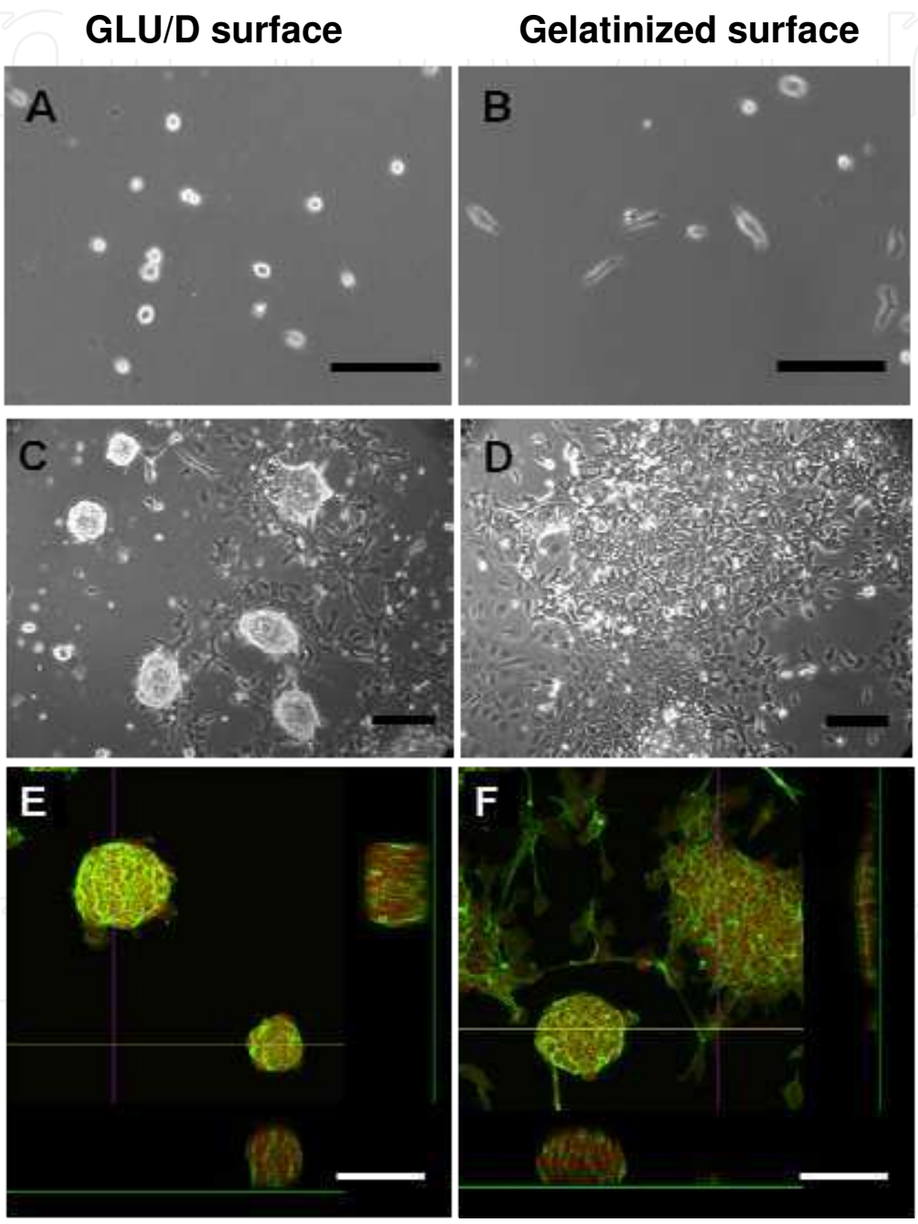

Fig. 2. Morphology of ES cells on different surfaces. ES cell colonies in A and B are shown 1 day after seeding, and those in C, D, E, and F are shown 4 days after seeding. The images at the bottom and right sides in E and F show the tomograms sectioned at the x-z (yellow line) and y-z (pink line) planes, respectively. The scale bars represent 100 μm

Kim et al. suggested that dendrimer-immobilized surfaces with a D-glucose display can induce a moderate activation of Rho family GTPases during the induced migration of rabbit chondrocytes. The activated Rho family GTPases can consequently promote cell-cell interactions via N-cadherin-mediated adhesion during cell aggregation to facilitate the development of chondrogenic phenotypes (Kim et al., 2009). Moreover, Kim et al. observed the spatiotemporal activation of N-cadherin expression when they altered the Rho family GTPase activity in human mesenchymal stem (hMS) cells by plating them on a GLU/D surface; this change promoted the formation of cell aggregates, which in turn directed hMS cell differentiation toward a cardiomyocyte phenotype (Kim et al., 2010b).

Recent studies showed that the morphology of single cells and of loosely attached spherical colonies of ES cells on a fourth-generation GLU/D surface were similar to those observed in hMS cells cultured on a fifth-generation GLU/D surface. Moreover, examination of the cytoskeletal and focal adhesion formation revealed that the development of stress fibers and vinculin plaques was suppressed for both ES and hMS cells cultured on GLU/D surfaces (Mashayekhan et al, 2008; Kim et al., 2010b). Although the detailed mechanism for the formation of ES cell aggregates on GLU/D is still unclear, we suggest that the mounded shape of the cell clusters that forms on dendrimer-immobilized surfaces promotes the expression of E-cadherin, a crucial cell-cell adhesion element in ES cells (Larue et al., 1996), which leads to the formation of spherical colonies.

Since the majority of colonies that formed on the GLU/D surface showed a morphology typical of undifferentiated cells (round and compact colonies with poorly delineated cell-cell borders), and were loosely attached to the surface, we tested whether preparations enriched in undifferentiated ES cells could be obtained by performing several passages of the cells on the GLU/D surface.

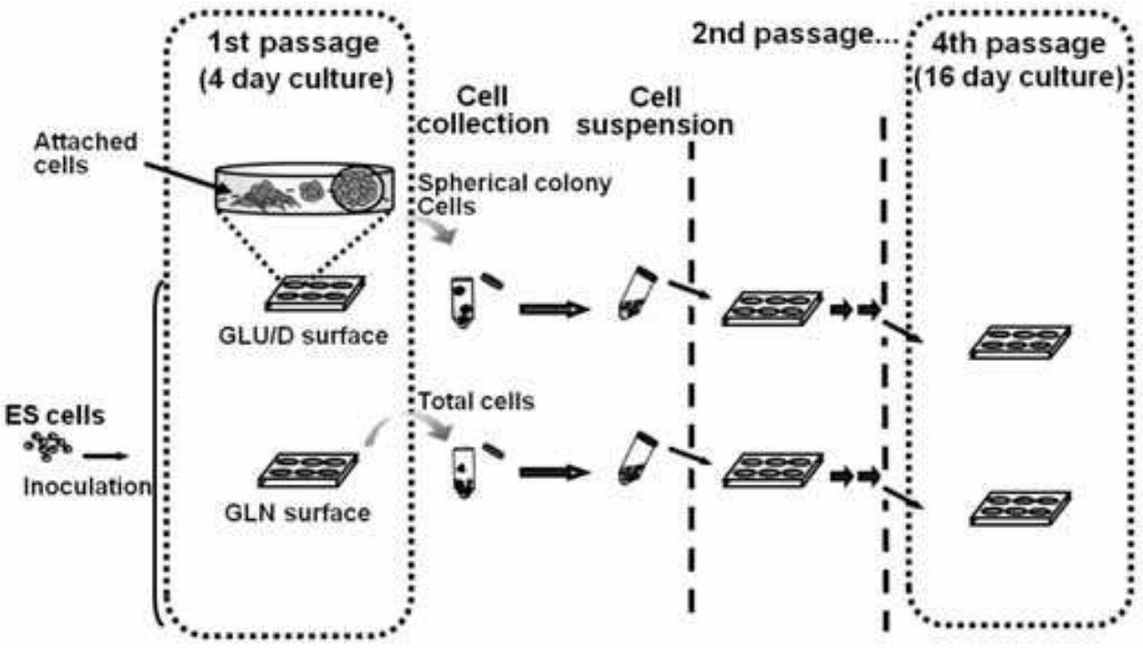

Fig. 3. Passaging protocol for the enrichment of ES cells in the undifferentiated state

The passaging protocol is illustrated in Fig. 3. The spherical colonies that were loosely attached to the GLU/D surface were harvested by tapping on day 4, dissociated into single cells by trypsin/EDTA treatment, and replated. These procedures were repeated every 4 days. For comparison, ES cells were also cultured on a gelatinized surface; in this case, the entire cell population was collected on day 4, and subjected to the enzymatic treatment for replating. The differentiation states of the cells cultured on the different surfaces were then compared by alkaline phosphatase (ALP) staining and gene expression analysis. For the ALP analysis, the spherical colony cells grown on the GLU/D surface and the cells grown on the gelatinized surface at passages 1 (4-day culture) and 4 (16-day total culture) were harvested, trypsinized, and replated onto gelatinized plates.

During the long-term passaging, the frequency of colonies with a spherical shape and the ALP activity of the spherical colony cells grown on the GLU/D surface increased gradually with the number of passages. Moreover, at passage 4, the percentage of ALP-positive colonies was significantly greater on the GLU/D surface than that on the gelatinized surface (Mashayekhan et al., 2008).

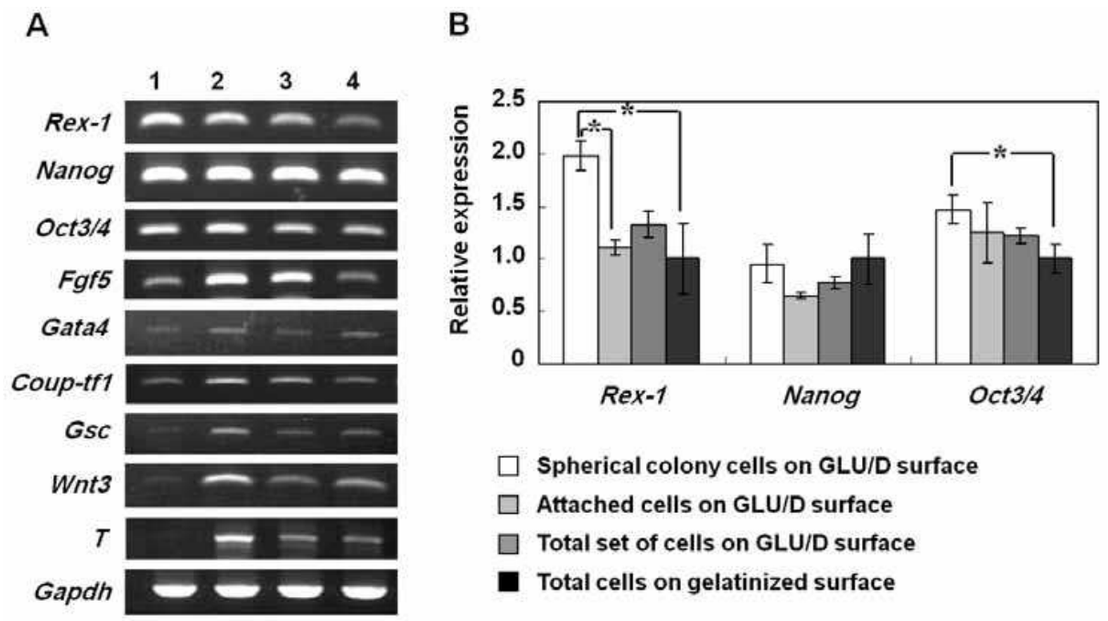

Fig. 4. RT–PCR analysis of ES cells cultured on different surfaces after four passages. (A) Conventional RT–PCR analysis for three markers of undifferentiated stem cells (Rex-1, Nanog, and Oct3/4) and six markers of early differentiation (Fgf5, Gata4, Coup-tf1, Gsc, Wnt3, and T). Lanes 1, 2, 3, and 4 correspond to the spherical colony cells, attached cells, and total cells cultured on the GLU/D surface, and the total cells collected from the gelatinized surface, respectively. (B) Quantitative RT–PCR analyses for the three stem cell markers (Rex-1, Nanog, and Oct3/4) in ES cells cultured on the GLU/D or gelatinized surface. The data were obtained from three independent experiments. The vertical bars show the standard deviation (*$p < 0.05$)

As shown in Fig. 4, we performed RT–PCR on cells from the GLU/D cultures, separating them into three groups: spherical colony cells, cells that remained attached to the surface

after tapping, and cells belonging to both types of colonies. We used cells grown on a gelatinized surface for comparisons. First we found that the markers for undifferentiated cells, Rex-1 and Oct3/4, were more highly expressed in the spherical colony cells grown on the GLU/D surface than in the other sets of cells or those grown on the gelatinized surface. Quantitative RT–PCR analysis confirmed that the cells from the spherical colonies on the GLU/D surface had higher expression levels of Rex-1 and Oct3/4 than the other cells. We also tested the different cell groups for the expression of early differentiation markers, by conventional RT–PCR. We found that early endodermal (Gata4), mesendodermal (Gsc), and mesodermal (T and Wnt3) differentiation markers were expressed at lower levels in the spherical colony cells from the GLU/D surface than in the attached cells or those grown on the gelatinized surface. Among all the cells of the different states tested, the expression levels of all the early differentiation markers were highest in the cells that were attached to the GLU/D surface, which appeared as flattened colonies. In contrast, the markers of undifferentiated cells, Rex-1 and Nanog, were expressed at significantly lower levels in the attached cells than in the spherical colony cells cultured on the GLU/D surface (Fig. 4). Thus, ES cells exhibiting various degrees of differentiation existed on the GLU/D surface, in a localized or enriched manner.

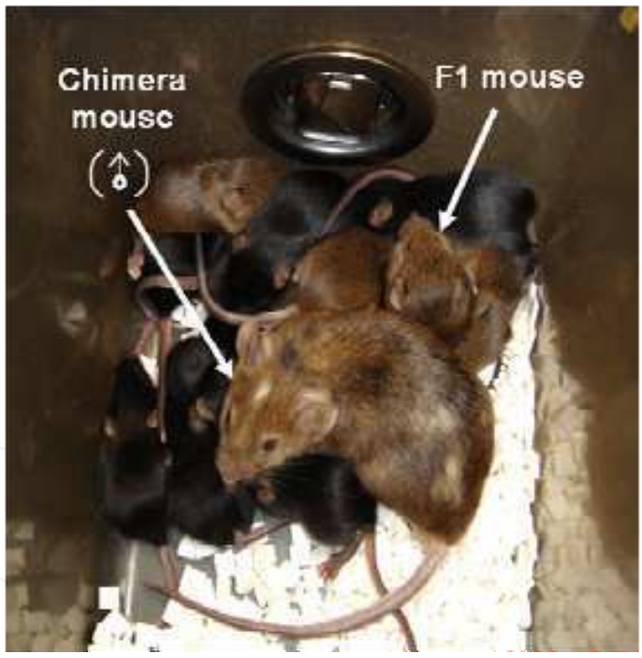

Fig. 5. Chimeric mice generated by blastocyte injection of ES cells cultured on the GLU/D surface. Dissociated ES cells from the spherical colonies on the GLU/D surface were injected into the blastocysts of C57BL/6J mice. The blastocysts were then transferred into the uteri of pseudopregnant MCH/ICR female mice. The resultant chimeric males with a white/agouti coat color ratio greater than 50% were bred with C57B/6J females to test for germ-line transmission

Overall, our RT–PCR analysis revealed that the markers for the undifferentiated state and for early differentiation were expressed at higher and lower levels, respectively, in the

spherical colony cells passaged on the GLU/D surface, than in the cells grown on gelatinized surface. These results support the view that the GLU/D surface is more effective than the gelatinized surface for maintaining ES cells in an undifferentiated state. In the flattened colonies on the GLU/D surface, all the markers of early differentiation were detected at much higher levels than in the cells from the spherical colonies. Thus, by using the proposed protocol of serial passaging on the GLU/D surface, which excluded the cells with a relatively stretched shape and flattened colonies and selectively transferred the loosely attached spherical colony cells to the next passage, the ES cells could be maintained in the undifferentiated state.

Finally, to confirm that the pluripotency of the ES cells grown on the GLU/D surface was maintained, we generated chimeric mice and checked the germ-line transmissibility of these cells. The ES cells were passaged four times on the GLU/D surface, and the spherical colonies were then dissociated into single cells prior to blastocyst injection. Among 43 progeny mice, 16 had the agouti coat color, indicating successful germ-line transmission, as typically shown in Fig. 5.

Differentiated cells are known to appear spontaneously on a gelatinized surface even in a complete ES medium containing serum, and the expression of mesodermal and extra-embryonic marker genes is slightly up-regulated under these conditions, due to the activation of integrin signaling, which is known to inhibit mouse ES cell self-renewal by increasing the LIF-induced activation of ERK1/2 (Hayashi et al., 2007). Considering the difficulty in culturing undifferentiated mouse ES cells without feeder cells in serum-containing medium, the GLU/D surface used in this study may be a useful biomaterial for culturing mouse ES cells. In the case of human ES cells, it is especially desirable to exclude foreign culture components like feeder cells and nonhuman-derived serum, to minimize the risk of pathogens such as retroviruses in therapeutic applications (Beattie et al., 2005; Amit & Itskovitz-Eldor, 2006; Chin et al., 2007). In this context, the application of the dendrimer-immobilized surface is a promising novel strategy for overcoming the difficulties in propagating human ES cells.

4. Conclusions and outlook for the future

The current chapter described general strategies for designing culture surfaces to control the morphology and function of ES cells. In addition, we introduced our approach to designing a culture surface using dendrimer substrates displaying D-glucose as a ligand to enrich the undifferentiated state of ES cells. The results suggest that the GLU/D surface is a potential tool for changing both the topography and the biochemistry of the surface, which play key roles in modulating the niche of ES cells and in turn govern their morphology and fate.

Although ES cells are potentially powerful tools in therapeutic applications for tissue regeneration, we still have little understanding of the microenvironment-specified molecular mechanisms and signaling pathways that lead to their efficient differentiation and to tissue formation. Identifying specific cues in the microenvironment and understanding how neighboring cells and the ECM control developmental fates will be required to promote the differentiation of ES cells into targeted cell lineages. As bioengineers learn more about how the microenvironment directs stem cell fate decisions, these factors can be incorporated into the culture conditions to better control ES cell growth and differentiation.

In general, the knowledge garnered using engineered systems will advance stem cell biology and provide prototypes for tissue engineering and strategies for therapeutics.

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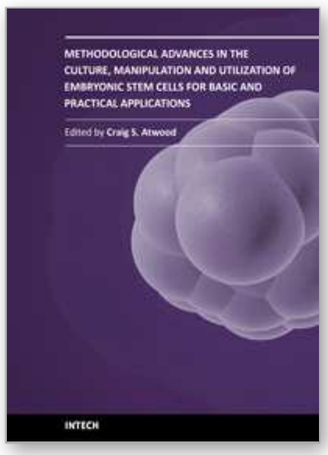

Methodological Advances in the Culture, Manipulation and Utilization of Embryonic Stem Cells for Basic and Practical Applications

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Pluripotent stem cells have the potential to revolutionise medicine, providing treatment options for a wide range of diseases and conditions that currently lack therapies or cures. This book describes methodological advances in the culture and manipulation of embryonic stem cells that will serve to bring this promise to practice.

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