Biotechnology and Genetic Engineering Reviews - Vol. 24, 243-262 (2007)

Engineering Human Embryonic Stem Cell Differentiation

NATANEL KORIN AND SHULAMIT LEVENBERG*

Department of Bio-medical Engineering, Technion, Haifa, Israel, 32000

Introduction

Stem cell research is one of the promising areas of biotechnology, which offers the prospect of developing new methods to repair or replace tissues or cells damaged by injuries or diseases. The therapeutic potential of stem cells includes chronic heart disease, end-stage kidney disease, liver failure, diabetes, cancer and many other are diseases. Stem cells isolated from adults or developing embryos may serve as a source of cells for tissue engineering therapies. Human embryonic stem cells (hESC), first derived in 1998 (Thompson *et al*, 1998), have generated great interest in tissue engineering and biomedicine due to their ability to give rise to differentiated cells of all adult tissues. However, despite their therapeutic potential, both adult and embryonic stem cells present a number of challenges associated with their clinical application. Possibly the greatest challenge in using hESC in clinical applications is the lack of knowledge in directing their differentiation ability. All studies that have shown the generation of specific cell types have not shown a uniform differentiation into a particular cell type. This may be attributed to the absence of proper temporal and spatial signals from the surrounding microenvironment.

Novel engineering approaches will play a key role in studying ESC differentiation and developing ESC tissue engineering therapies. This bio-engineering challenge is a multi-disciplinary task which combines biology, chemical, material, mechanical and electrical engineering. The development of improved technologies in various engineering fields such as: scaffolds engineering, high throughput devices, microfabrication & micromanipulations, microfluidics, genetic engineering and others

*To whom correspondence may be addressed (shulamit@bm.technion.ac.il)

Abbreviations: EG: Embryonic Germ, ES: Embryonic Stem, ESC: Embryonic Stem Cells, hESC: human Embryonic Stem Cells, ICM: Inner Cell Mass, mESC: mouse Embryonic Stem Cells, EBs: Embryoid Bodies, MEF: Mouse Embryonic Fibroblasts.

may lead to breakthroughs in understanding hESC differentiation mechanism and to the fulfillment hESC clinical therapeutic potential.

STEM CELLS

Tissue engineering and cellular therapies have the potential to significantly affect medicine. These approaches require a readily available source of cells outside a living body. A stem cell can be defined by two properties: the capacity for long-term self-renewal and multipotency - the ability to differentiate into one or more specialized cell types (Blau *et al*, 2001). Hence, theoretically, stem cells could serve as an ideal supply of cells for tissue engineering therapies.

Stem cells can be derived from multiple stages of development as well as numerous adult tissues. The embryonic stem cell (ESC) is defined by its origin from one of the earliest stages of the development of the embryo, called the blastocyst. The embryonic stem cell can self-replicate and is pluripotent - it can give rise to cells derived from all three germ layers. Embryonic-like stem cells, called embryonic germ (EG) cells, can also be derived from primordial germ cells (the cells of the developing fetus from which eggs and sperm are formed) of the mouse and human fetus. The adult stem cell is an undifferentiated cell that is found in a differentiated tissue; it can renew itself and become specialized to yield all of the specialized cell types of the tissue from which it originated. Adult stem cells were found in various positions throughout the body, such as bone marrow, brain, liver and skin (Zandstra & Nagy, 2001). Adult stem cells are capable of self-renewal for the lifetime of the organism. The adult stem cells were originally thought to be committed to regenerating only a very limited set of cell lineages; however, it is becoming clear that they can exhibit more plasticity.

Adult stem cells therapies may bypasses the need for immunosuppression, however there are major obstacles in their practical use. For example, most adult stem cells are difficult to collect and isolate. In addition, the isolated cells have poor growth *in vitro* and a limited lineage potential. In these cases, ESC are expected to provide a more suitable cell source.

HUMAN EMBRYONIC STEM CELLS (HESC)

ESC are cells derived from the inner cell mass (ICM) of the mammalian blastocyst, initially derived from the mouse blastocyst. Thomson and colleagues (1998) reported the first derivation of hESC lines. HESC have been shown to be capable of being induced to develop into a variety of cell types, including neural cells (Schuldiner *et al*, 2001), endothelial cells (Levenberg *et al*, 2002), hepatocytes (Lavon *et al*, 2004), heart muscle cells (Kehat *et al*, 2001) osteogenic cells (Sottile *et al*, 2003), blood cells (Kaufman *et al*, 2001), epidermis (Green *et al*, 2003) and insulin producing cells (Segev *et al.* 2004).

HESC characteristics

ESC are derived from the inner cell mass/epiblast of the blastocyst. ESC are capable of undergoing an unlimited number of symmetrical divisions without differentiating

(long-term self-renewal), and can give rise to differentiated cell types that are derived from all three primary germ layers of the embryo (endoderm, mesoderm, and ectoderm). Additionally, single ESC can give rise to a colony of genetically identical cells. ESC express the transcription factor Oct-4, which then activates or inhibits a host of target genes and maintains ESC in a proliferative, nondifferentiating state (Pesce and Scholer, 2001).

HESC are currently evaluated by a set of markers and their capacity to differentiate. The criteria for this estimation include the expression of surface markers and transcription factors associated with an undifferentiated state. In addition, extended proliferative capacity, pluripotency and a euploid karyotype are important characteristics of the cells. Examination of hESC over extended periods *in vitro* should also be used to verify that hESC characteristics do not change over time, and that the lines are stable in their expression of markers, expression of telomerase, ability to differentiate and maintenance of a normal karyotype.

A number of surface markers are currently used to characterize hESC. It has been well established that hESC express stage-specific embryonic antigen (SSEA)-3, SSEA-4, as well as TRA-1-60 and TRA 1-81, and downregulate these markers upon differentiation. Unlike mouse embryonic stem cells (mESC), undifferentiated hESC do not express SSEA-1. Undifferentiated hESC also stain positively for alkaline phosphatase, and demonstrate telomerase activity. The human gene OCT-4 is highly expressed in undifferentiated hESC and is necessary to maintain the pluripotent state of hESC. As hESC differentiate, OCT-4 gene expression decreases to low levels.

HESC culture

HESC show several important differences from mESC in culture. Although both hESC and mESC lines require special culture conditions in order to remain undifferentiated, mESC lines may be propagated in the absence of a feeder layer when leukemia inhibitory factor (LIF) is added to the culture medium. hESC are insensitive to LIF and usually require culture on feeder cells. In addition, hESC grow more slowly and tend to form flat colonies. HESC and mESC also differ in some antigenic phenotype (e.g., SSEA-1- expressed in mouse, SSEA-3 and 4- expressed in humans).

The methods and techniques used to maintain hESC in an undifferentiated proliferative state are still under study (Amit *et al*, 2003, 2004; Bonder *et al*, 2004; Draper *et al*; 2004, Levenberg *et al*, 2006a,b; Stojkovic *et al*, 2004). Several variables are thought to contribute to the growth and differentiation *in vitro*, and these variables are discussed below.

Feeder cell layers

In order to maintain their pluripotency HESC are routinely grown on a layer of feeder cells, as shown in *Figure 1*. Traditionally mitotically inactivated mouse embryonic fibroblasts (MEFs) are used to support hESC growth and maintenance. Feeder cells are normally mitotically inactivated using irradiation or through incubation with mitomycin C. For therapeutic purposes, it is unlikely that hESC grown on murine

feeder layers will be acceptable due to the risk of transferring animal pathogens. Therefore, hESC are now beginning to be derived on human feeder cells (Amit *et al*, 2003, Choo *et al*, 2004) or without any feeder layers (Amit *et al*, 2003; Richards *et al*, 2002; Xu *et al*, 2001; Ludwig *et al*, 2006). However, currently the use of mouse or human feeder cells is the most widely used method of providing the soluble factors necessary to support the growth and maintenance of undifferentiated hESC.

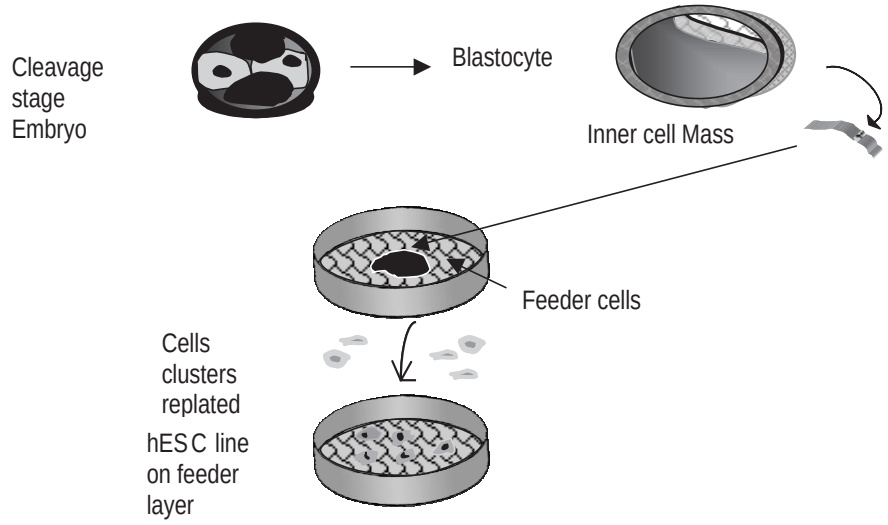

Figure 1. HESC culture on a feeder layer.

Medium

HESC require a medium with specific properties to maintain growth in an undifferentiated state. Fetal bovine serum (FBS), which is often used to supplement cell culture medium, can contain many compounds both valuable and harmful to hESC growth, and the concentration of these compounds may differ among batches. A defined serum substitute in hESC medium should be used in order to minimize the variability of hESC culture conditions and the occurrence of spontaneous differentiation of hESC. It should be noted that although KnockOut Serum Replacement (SR) contains fewer components than FBS, it is still not fully defined and has animal components. Recently, Ludwig *et al* (2006) have successfully cultured an hESC line under defined conditions.

Extracellular matrix (ECM)/substrates

Culturing hESC also requires a suitable substratum. Removal of an appropriate substrate, may result in the differentiation of hESC. HESC have been maintained in the undifferentiated state on feeders cells (MEFs, or various human cells such as human foreskin, Amit *et al*, 2003), or in conditioned medium containing bFGF on Matrigel, laminin (Xu *et al*, 2002) or fibronectin ,on human fibronectin in a feeder free configuration (Amit *et al*, 2003) and on a combination of collagen IV, fibronectin, laminin and vitronectin (Ludwig *et al*, 2006).

HESC density in vitro and passaging

HESC have a very low survival rate when they are dissociated to a single-cell suspension. Therefore when hESC colonies are passaged, they are dissociated into clusters of approximately 100 cells. Passaging of hESC is obtained using either mechanical or enzymatic methods. Regardless of the passaging technique, it is important that the hESC remain in clusters. However, cluster that are too big or too crowded tend to differentiate. This situation makes it difficult to generate cultures with consistent cell density from passage to passage.

Ideal culture conditions

Ideal culture conditions include a defined matrix, defined media and passaging which allows cell seeding at a constant cell density. The culture conditions should maintain phenotypically and karyotypically the HESC characteristics and insure their reproduction and differentiation abilities.

HESC DIFFERENTIATION

HESC can be differentiated in culture through a number of different techniques. These techniques involve the use of proper chemical signals and molecular cues that induce stem cell differentiation (Rippon and Bishop 2004). Typically stem cells are differentiated in two dimensional cultures or within a suspension culture of cell aggregates or spheroids (Figure 2). These cell aggregates are called embryoid bodies (EBs) since they mimic some features of normal embryonic development. The EBs contain derivatives of all three embryonic germ layers (Itskovitz-Eldor *et al*. 2000). The length of EB culture duration is dependent upon the desired cell type. For example, mesodermal and ectodermal precursors form within a few days, while some endodermal cell types require a longer culture time. Following the formation of EBs, they are usually re-cultured in adherent culture conditions in which specialized cells develop.

Though the majority of hESC differentiation protocols *in vitro* have so far focused on differentiation of cells in 2D culture condition, the use of 3D structures is essential for most tissue engineering applications.

All differentiation methods, in 2D and 3D, require understanding/controlling highly complex cell biology processes. 3D culture requires additional challenges for inducing cell attachment, survival, differentiation and organization. Engineering hESC constructs and controlling their differentiation is a key step in the realization of hESC therapeutic potential.

Engineering methods and approaches

SCAFFOLDS

The use of scaffolds provides three-dimensional (3D) environments and sets the cells in the vicinity of each other; thus enabling self-assembly and formation of tissue

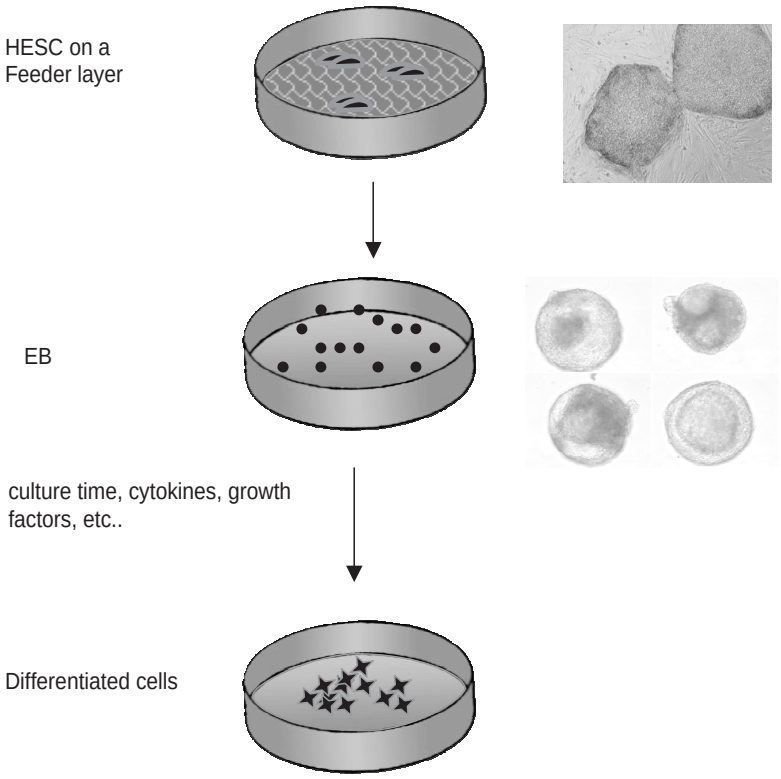

Figure 2. ES cell differentiation protocols have generally used two dimensional cultures and/or embryoid bodies.

microenvironment components. The ability to culture cells in 3D construct is essential for understanding cell interactions *in vivo* and for understanding the complex mechanisms that control cell differentiation to form tissue structures.

Scaffolds can be formed by synthetic, natural materials or both. Oxygen and nutrients transport, mechanical properties and degradation rate should be considered and designed according to cell culture requirements. For example, in porous scaffolds the size of the pores controls the initial space between the cells and the space left for further proliferation (Levenberg *et al*, 2002, 2003). Scaffolds can also provide a 3D environment in the form of gels or hydrogels, where cells are embedded within the gel and can migrate and organize within the gel as it degrades (Elisseff *et al*, 2006).

hESC scaffolds

Scaffolds may be able to play an important role in understanding ESC differentiation and provide 3D tissue structures that are essential for most tissue engineering applications.

However, there have been very few scaffold based tissue engineering studies that use hESC.

There are two approaches for hESC use in tissue engineering constructs (*Figure 3*). The first is to differentiate stem cells *in vitro* into the desired cell types and then assemble the cells into scaffolds. The other approach is to seed undifferentiated stem cells directly onto scaffolds and then differentiate them.

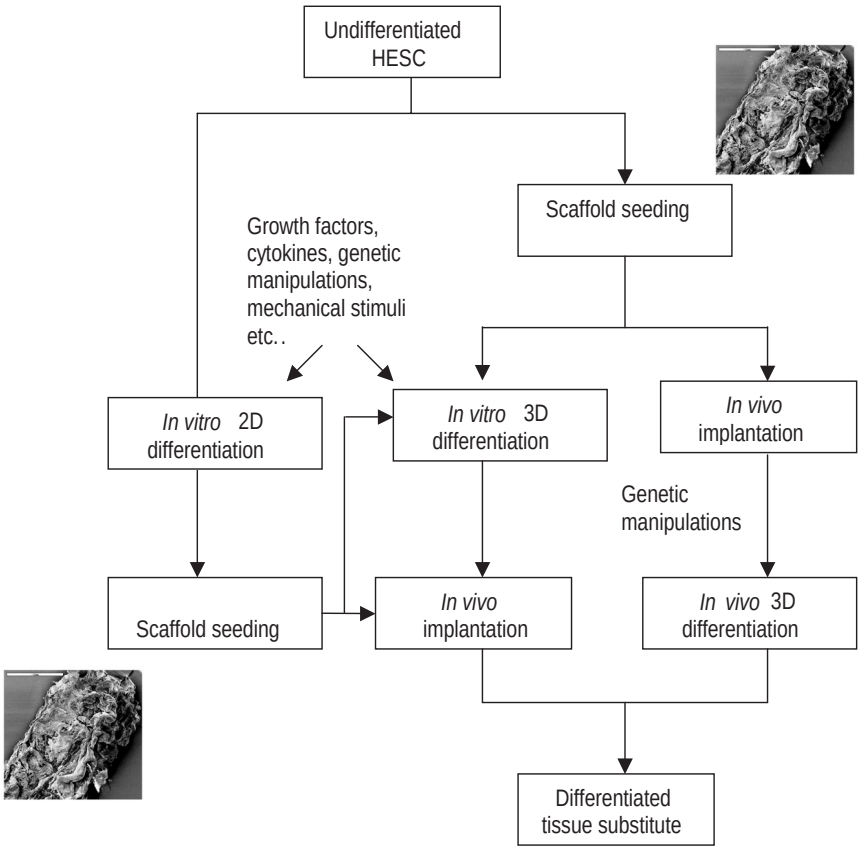

Figure 3. HESC based tissue engineering constructs can be formed by various approaches. HESC can be differentiate *in vitro* into the desired cell types and then assemble the cells into scaffolds or they can be seeded directly onto scaffolds and then differentiating in them (*in vitro* or *in vivo*).

The first approach, in which differentiate stem cells are seeded in a scaffold, has been used by us to study the behavior of ESC derived endothelial cells in tissue engineering constructs (Levenberg *et al*, 2002). HESC derived endothelial progenitors that were seeded onto highly porous PLLA/PLGA biodegradable polymer scaffolds formed blood vessels that appeared to merge with the host vasculature when implanted into immunodeficient mice.

The second approach, to directly differentiate hESC on scaffolds in culture, has the possible advantages that the assembly of the cells as they differentiate may imitate physiological differentiation. We demonstrated the differentiation of hESC on a

polymeric scaffold (Levenberg *et al*, 2003). Eight to nine days EBs were dissociated to release individual cells and seeded onto porous biomaterial scaffolds such as poly(lactic-co-glycolic acid) poly(L-lactic acid) polymer scaffolds. The ES–biomaterial constructs were cultured in the presence of different growth factors to direct differentiation. Tissues resembling cartilage, neural, endodermal (liver and pancreas) and vascular structures were created. Organization of the structures could be enhanced by conditioning the scaffold with specific growth factors and by implantation into immunodeficient mice, in which the constructs were observed to be viable for at least 2 weeks. Gerecht-Nir *et al* (2004) have reported that three-dimensional porous alginate scaffolds provide a favorable environment for generation of well-vascularized embryoid bodies from hESC. The culture in alginate scaffolds induced vasculogenesis to a greater extent than in static or rotating cultures. Imamura *et al* (2004) and Baharvand *et al* (2006), demonstrated the differentiation of hESC into hepatocyte-like cells within 3D collagen scaffolds containing exogenous growth factors. The cells displayed morphological features, gene expression patterns and metabolic activities characteristic of hepatocytes. We have used 3D scaffolds to investigate the neuronal differentiation of hESC (Levenberg *et al*, 2005). Neuronal differentiation of hESC on three-dimensional polymer scaffolds was enhanced in the presence of neurotrophins nerve growth factor and neurotrophin 3 combined with retinoic acid. Elisseeff *et al* (2006) examined culturing hESC in hydrogel biomaterials for engineering musculoskeletal tissues. In that study Elisseeff and coworkers found that for chondrogenic differentiation, an adhesion peptide sequence was required, which was not necessary for adult stem cells. Unexpectedly, the various examined hESC lines showed different biomaterial requirements.

The findings of these studies demonstrate the complexity of hESC culture in 3D scaffolds. Much work should be conducted in order to achieve understanding and control of hESC differentiation in 3D structures. Future work may include engineered scaffolds with improved properties and functions. Scaffolds that control the temporal release of various molecules and/or contain immobilize ligands can be further used. Additionally, more complex biomaterials design, using microfabrication, microfluidics, or other structure-organizing approaches may be used to create defined and complex scaffolds. The use of perfusion systems in which the medium is flown through the scaffold may also be examined to improve hESC culture in scaffolds.

An additional approach, which is highly problematic, is to use the adult body's microenvironment to induce the differentiation of hESC. *In vivo* differentiation of hESC is not a realistic option due to the tumourgenic capabilities of hESC and the variety of cell types obtained in non-directed differentiation. In the future it may be possible to use genetically modified ESC that undergo apoptosis when differentiated into undesirable cell types and other similar genetic approaches to improve desired cell differentiation *in vivo* (Hook *et al*, 2005; Eiges, 2006). However, currently this approach remains impractical.

HIGH THROUGHPUT DEVICE

High-throughput screening has contributed significantly to modern biology and drug discovery.

HESC differentiation is controlled by the microenvironment which conveys multiple complex signals of various sorts to the cell. The cells sense and respond to applied combinations of signals. Since the number of combinations increases significantly with the number of individual factors, high-throughput approaches are required to examine the signaling space. Thus, high throughput devices may shade light on important parameters in hESC differentiation. Arrays of small soluble molecules (Wu *et al*, 2004), cDNA (Ziauddin and Sabatini, 2001; Wu *et al*, 2002), biomaterials (Anderson *et al*, 2004), and extracellular matrix proteins (Flaim *et al*, 2005) have proven to be powerful approaches to exploring cell responses to varying signals.

Small molecules

High throughput screening of synthetic small molecules has provided useful chemical tools to modulate and to study complex cellular processes (Ding *et al*, 2004). For example, it has enabled the identification of heterocyclic small molecules that can direct ESC toward particular lineages, such as cardiomyocytes (Wu *et al*, 2004), and even of molecules that can induce the dedifferentiation of adult cells into pluripotent progenitor cells (Chen *et al*, 2004).

Genomic studies

Cell microarrays are used also for functional genomic studies. Each cell microarray contains thousands of cell clusters that are each transfected with a defined DNA, which leads to overproduction (cDNAs for gain-of-function) or inhibition (siRNA for loss-of-function) of a specific gene product. Experiments using this technique have been used to characterize undifferentiated hESC and provide a unique set of markers to profile and better understand the biology of hESC (Bhattacharya *et al*, 2004). Additionally, various studies have focused on differentiation gene products (Calhoun *et al*, 2004; Devash *et al*, 2004). In the future, the differentiation process should be further studied using these techniques.

Feeder cells

The potential of the microarray methodology for high-throughput screening has been also used to examine how feeder cells may affect differentiation of hESC (Yamazoe & Iwata, 2005).

Biomaterials

The effect of biomaterials on the behavior of hESC is important for developing hESC tissue engineering methods (Hubble, 2004). In this issue, Anderson *et al* (2004) described screening combinatorial biomaterials in an array format in the purpose of examining materials that allow controlled differentiation of hESC. In this study we examined a library of nearly 600 materials and found that specific materials induced ES-cell differentiation toward epithelial cells much more effectively than others.

ECM

Flaim *et al* (2005) presented an extracellular matrix (ECM) microarray platform for the culture of patterned cells atop combinatorial matrix mixtures. Flaim *et al*, applied this platform to study the effects of 32 different combinations of five extracellular matrix molecules (collagen I, collagen III, collagen IV, laminin and fibronectin) on cellular differentiation. The system was used to examine the maintenance of primary rat hepatocyte phenotype and the differentiation of mESC toward an early hepatic fate.

Microfabricated microwells

A platform which enables parallel, automated, long-term live-cell microscopy of single cells in culture and tracking of individual cell fates over time has been demonstrated by Chin *et al* (2005). The system is based on a microfabricated array of 10,000 microwells on a glass coverslip to analyze the proliferation dynamics of adult rat neural stem cell.

Immobilized ligands

Recently, Soen *et al* (2006) developed a microarray system to systemically investigate the effects of numerous morphogens, growth factors, cell adhesion molecules, and extracellular matrix components on the fate of primary bi-potent human neural precursor cells that can differentiate into neurons or glial cells .Their approach focused on the effects of the immobilized ligands on cell function. Cellular behaviors (i.e. differentiation and proliferation) were then monitored by immunostaining for bromodeoxyuridine and cell fate markers, followed by high-throughput quantitative imaging analysis at the single-cell level. Multi-parameter analysis of responses to conflicting signals revealed interactions more complex than previously envisaged, including reciprocal dominance relations.

Taken together, a system of high-density, microarrayed, immobilized and soluble microenvironments provides a robust method to investigate the effects of multiple signals on cell behavior (*Figure 4*). The use of high throughput devices to explore the subject of hESC differentiation has not yet fulfilled its potential. Novel approaches being developed will provide new capabilities for the study of basic hESC biology and the advancement of regenerative medicine.

MEMS AND MICROFLUIDICS

One of the major challenges associated with the use of stem cells is the identification and understanding of microenvironmental cues that regulate their fate. Microscale approaches can also be used to control culture conditions and perform high-throughput experimentation, hence providing a suitable tool to study cell–microenvironment interactions *in vitro* (Voldman *et al*, 1999; El-Ali *et al*, 2006).

Microfabrication techniques offer the ability to control the molecular structure of surfaces and to pattern complex molecules, to fabricate microchannels, and to pattern

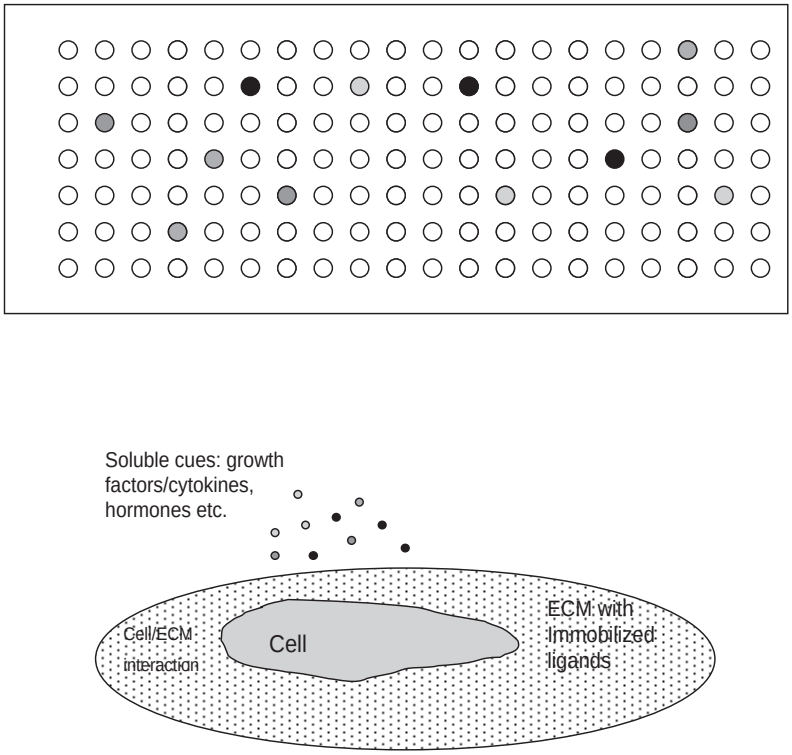

Figure 4. Novel high throughput screening of different immobilized and soluble microenvironments can be used to systemically investigate hESC differentiation.

and manipulate cells. The isolation of cells on a surface makes it possible to study events occurring in each individual cell. The combination of patterned cells and reagents delivered to the cells in laminar flows in microchannels makes it possible to study the influence of these reagents on cells with high accuracy. Various microenvironmental manipulations can be preformed using microfabrication and microfluidics techniques to create a controllable microenvironment. Hence, the culture of stem cell in a controllable microenvironment may be highly beneficial for stem cell study and may in light the mechanisms which govern stem cell behavior, such as stem cell differentiation.

Micro-bioreactors and microfluidics

The culture of cells in a microchannel system can be highly beneficial for cell biology studies and tissue engineering applications. Leading research teams in the field of tissue engineering utilize cell culture in microchannels for various applications. For example, culture of endothelial cell in microchannels is used for studying, optimizing and developing vascular systems with engineered geometries - a key challenge in the regeneration of vital organs (Bettinger *et al*, 2005; Shin *et al*, 2004; Borenstein *et al*,

2002). Another example is the development of microfluidic devices for long-term culture of hepatocytes, which is very important for realizing drug metabolism studies, bioreactors and artificial organs (Leclerc *et al*, 2003 a,b).

Microfluidics is also a promising technology for stem cell culture because of the length scales involved (Bhatia & Chen, 1999). The scale of the channels allows important factors (e.g., growth factors) to accumulate locally forming a stable microenvironment for the cells. It has been shown that the culture of mammalian embryos in microchannels results in more natural developmental kinetics and improved developmental efficiency (Beebe at al. 2002a, Glasgow *et al*, 2001). Similar benefits may be possible by culturing stem cells in microchannels. Abhyankar *et al*, (2003) showed the feasibility of short term culturing of hESC in microchannels using two different layers: (MEF) and Matrigel. Khademhosseini (2004c) patterned mESC in a microchannel using a new technique for patterning cells in shear protected microwells.

Recently there has been increasing interest in culturing cells in perfused microfluidic environments since continuous perfusion offers the ability to control cell-media interactions by controlling the chemical composition of the media that surrounds cells. Kim *et al*, (2006) designed a microfluidic arrays for logarithmically perfused embryonic stem cell culture, and cultured mES under constant perfusion in a microchannel for 4 days.

Another important advantage of microfluidics is that laminar flow fluids within microchannels can be used to control the spatial positioning of soluble factors relative to cells (*Figure 5*). Laminarly flowing fluids have been used to pattern cells and their microenvironments (Takayama *et al*, 1999, 2003). Linear or complex soluble gradients are generated by merging of inlet streams. Microfluidic gradients have been used to study complex biology system such as neural stem cell differentiation (Chung *et al*, 2005). However, currently, the benefits of culturing cells in microchannels have not been utilized to study or manipulate hESC culture.

Micro-paterrning

Cellular patterning methods organize the chemical properties of a surface to promote selective attachment of specified types of cells to defined regions on a substrate (Folch and Toner, 2000). Cellular patterning has been used to explore the effects of cellular geometry on proliferation, migration cell-cell interaction and differentiation. Patterned cocultures may be a useful tool for tissue engineered constructs and for studying cell–cell interactions *in vitro*. Coculture techniques have been used to study the interaction of hESC with other cell types (Khademhosseini *et al*, 2004).

High-throughput

As detailed in the previous section, the combination of high throughput devices with microfabrication may lead to the development of novel high throughput systems for isolating and monitoring single cells and monitoring hESC differentiation. The utilization of novel high throughput microdevices in stem cell biology studies may improve our understanding of hESC differentiation.

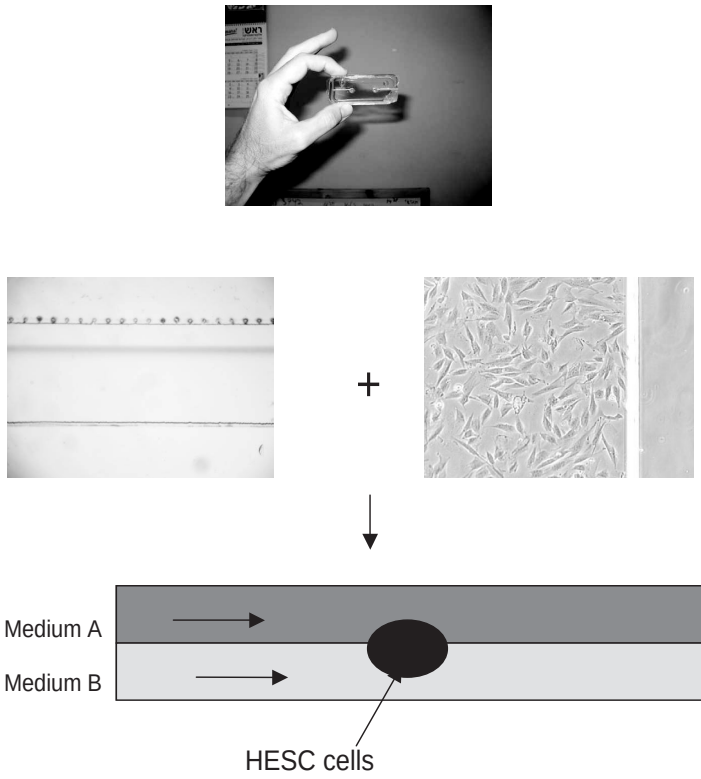

Figure 5. Microfluidics can be used to control the spatial positioning of soluble factors in Micro bioreactors. This can be used to control hESC microenvironment.

MECHANICAL STIMULI

Though the effect of mechanical stimuli on stem cells is a subject of great interest, only limited results has been presented in this field. The following section briefly reviews some of the latest works in this field.

McBeath *et al* (2004) have shown that human mesenchymal stem cells allowed to adhere, flatten, and spread under went osteogenesis, while unspread, round cells became adipocytes. Cell shape regulated the switch in lineage commitment by modulating endogenous RhoA activity.

Li *et al* (2004), showed that oscillatory fluid flow affects human marrow stromal cells by increasing the proliferation of the cells and the markers of osteogenic markers of differentiation. In addition, the human marrow stromal cells subjected to oscillatory fluid flow exhibited increased intracellular Ca^{+2}þ mobilization.

It has been reported that shear stress can induce mESC differentiation (Kimiko *et al*, 2005). Mouse Flk-1+-positive ESC were subjected to controlled levels of shear stress in a flow-loading apparatus and examined for changes in cell proliferation and differentiation. Flk-1-positive (Flk-1+) mESC density increased markedly when the

cells were subjected to shear stress. In addition, shear stress considerably increased the expression of the vascular endothelial cell-specific markers Flk-1, Flt-1, VE-cadherin, and PECAM-1, at protein level and mRNA level, but it had no effect on expression of the mural cell marker SM-α-actin, blood cell marker CD3, or the epithelial cell marker keratin. These findings indicate that shear stress selectively promotes the differentiation of Flk-1+ ESC into the endothelial cell lineage. The shear-stressed Flk-1+ ESC formed tube-like structures in collagen gel and developed an extensive tubular network significantly faster than the static controls.

The effect of mechanical stimuli on adult stem cell and mESC is currently under study. These ongoing studies show that mechanical stimuli can regulate stem cell fate and behavior. However, mechanisms that govern these effects continue to be unclear.

The effect of mechanical stimuli on hESC has been poorly explored and remains elusive. A new study by Saha *et al*, (2006) examined the rate of hESC differentiation in the presence and absence of biaxial cyclic strain. Above a threshold of 10% cyclic strain, applied to a deformable elastic substratum upon which the hESC colonies were cultured, hESC differentiation was reduced and self-renewal was promoted without selecting against survival of differentiated or undifferentiated cells.

As presented, mechanical forces have been reported to induce proliferation or differentiation in various cell types, but the role of mechanotransduction on embryonic stem cell fate decisions is unknown. The findings reviewed in this section imply that application of mechanical forces may be useful, in combination with biochemical signals, towards controlling differentiation of hESC for therapeutic applications.

GENETIC TECHNIQUES

Genetic manipulations techniques can be used to explore the fundamental genetic mechanisms governing hESC differentiation and to increase the yield of differentiation into desired lineages (Menendez *et al*, 2005; Moore *et al*, 2005; Hook *et al*, 2005; Eiges, 2006). Genetic techniques can be classified into positive or negative regulators. The positive regulators include controlled expression of transcription factors that contribute to differentiation into a tissue type. Negative regulators may be used to cause apoptosis of cells that differentiate to undesired pathways. For example, transcription factors that activate neomycin selection and suicide genes. Stable overexpression or inhibition of genes in mESC has contributed a great deal to the study of gene function in mammals. Genetic manipulations of hESC may shade light on gene function and cell differentiation process.

Summary and conclusions

HESC hold promise as an unlimited source of cells for tissue engineering therapies. Though hESC study is rapidly progressing, clinical applications are not practical since much research still needs to be done. A major challenge in hESC research is understanding and directing hESC differentiation. Novel interdisciplinary approaches, which combine biology, chemical, material, mechanical and electrical engineering aspects, may advance us to the day when hESC can be differentiated into a particular cell type in a controlled manner suitable to create/repair specific organs. The

fulfillment of hESC clinical therapeutic potential may revolutionize medicine and the treatment of human disease.

References

Abhyankar, V.V., Bittner, G.N., Causey, J.A., Kamp T.J. and Beebe D.J. (2003) Embryonic stem cell culture in microfluidics channels. In *Proceeding of 7th International Conference on Miniaturized Chemical and Biochemical Analyst System (u-TAS2003)*,Eds. Norhtrup, M.A., Jensen, K.F., Harrison, D.J., Kluwer Academic Publishers, Squaw Valley, California, USA, 17-20.

Anderson, D.G., Levenberg, S., Langer, R. (2004) Nanoliter-scale synthesis of arrayed biomaterials and application to human embryonic stem cells. *Nature Biotechnology* **22**, 863-866.

Amit, M., Margulets, V., Segev, H., Shariki, K., Laevsky, I., Coleman, R. and Itskovitz-Eldor, J. (2003) Human feeder layers for human embryonic stem cells. *Biology of Reproduction* **68**, 2150–2156.

Amit, M.,Shariki, C., Margulets, C. and Itskovitz-Eldor, J. (2004) Feeder Layer- and Serum-Free Culture of Human Embryonic Stem Cells. *Biology of Reproduction* **70**, 837–845.

Baharvand, H., Hashemi, S.M., Kazemi Ashtiani, S., Farrokhi, A. (2006) Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems *in vitro*. *International Journal of Development Biology* **50**, 645-652.

Beebe, D.J., Wheeler, M., Zeringue, H., Walters, E., Rat, S. (2002) Microfluidic technology for assisted reproduction. *Theriogenology* **57**, 125-l 35.

Beebe, D.J., Mensing, G.A., Walker, G.M. (2002) Physics and application of microfluidics in Biology. *Annual Review of Biomedical Engineering* **4**, 261–286.

Bershadsky, A.D., Balaban, N.Q., and Geiger, B. (2003) Adhesion Dependent Cell Mechanosensitivity. *Annual Review of Cell and Developmental Biology* **19**, 677–695

Bhatia, S.N. and Chen, C.S. (1999) Tissue Engineering at the Micro-Scale. *Biomedical Microdevices* **2**, 131-144.

Bhattacharya, B., Miura, T., Brandenberger, R., Mejido, J., Luo, Y., Yang , A.X., Joshi, B.H., Ginis, I., Thies, R.S., Amit, M., Lyons, I., Condie, B.G., Itskovitz-Eldor, J., Rao, M.S., Puri, R.K. (2004) Gene expression in human embryonic stem cell lines: unique molecular signature. *Blood* **103**, 2956-2964.

Blau, H.M., Brazelton, T.R., Weimann, J.M. (2001) The evolving concept of a stem cell: entity or function? C*ell* **105**, 829-841.

Bonder, M.S., Meneses, J.J., Rodriguez, R.T. and Firpo, M.T. (2004) Propagation and Maintenance of Undifferentiated Human Embryonic Stem Cells, *Stem Cell and Development* **13**, 243-253.

Borenstein, J.T., Terai, H., King, K.R., Weinberg, E.J., Kaazempur-Mofrad, M.J. and Vacanti, J.P. (2002) Microfabrication Technology for Vascularized Tissue Engineering, *Biomedical Microdevices* **4**, 167-175.

Calhoun, J.D., Rao, R.R., Warrenfeltz, S., Rekaya, R., Dalton, S., McDonald, J., Stice, S.L. (2004) Transcriptional profiling of initial differentiation events in human embryonic stem cells. *Biochemical and Biophysical Research Communications* **323**, 453–464.

CHEN, C.S., TAN, J. AND TIEN, J. (2004) Mechantransducation at cell-matrix and cell-cell contacts, *Annual Review of Biomedical Engineering* **6**, 275–302.

CHEN, C.S., MRKSICH, M., HUANG, S., WHITESIDES, G.M., AND INGBER D.E. (1997) Geometric control of cell life and death. *Science* **276**, 1425-1428.

CHEN, S., ZHANG, Q., WU, X., SCHULTZ, P.G. AND DING, S. (2004) Dedifferentiation of lineage-committed cells by a small molecule. *Journal of the American Chemical Society* **126**, 410-411.

CHICUREL, M.E., CHEN, C.S. AND INGBER,? D.E. (1998) Cellular control lies in the balance of forces, *Current Opinion in Cell Biology* **10**,232–239.

CHIN, V.I., TAUPIN, P., SANGA, S., SCHEEL, J., GAGE, F.H. AND BHATIA, S.N. (2004) Microfabricated platform for studying stem cell fates. *Biotechnology and Bioengineering* **88**, 399-415.

CHOO, B.H., PADMANABHAN, J., CHIN, C.P. AND OH, K.W. (2004) Expansion of Pluripotent Human Embryonic Stem Cells on Human Feeders. *Biotechnology and Bioengineering* **88**, 321-331.

CHUNG, B.G., FLANAGAN, L.A., RHEE, S.W., SCHWARTZ, P.H., LEE, A.P., MONUKI, E.S. AND JEON, N,L. (2005) Human neural stem cell growth and differentiation in a gradient-generating microfluidic device. *Lab on a Chip* **5**, 401-406.

DING, S. AND SCHULTZ, P.G. (2004) A role for chemistry in stem cell biology. *Nature Biotechnology* **22**, 833 – 840.

DRAPER, J.S., MOORE, H.D., RUBAN, L.N., GOKHALE, P.J. AND ANDREWS, P.W. (2004) Culture and Characterization of Human Embryonic Stem Cells, *Stem Cells and Development* **13**, 326-336.

DVASH, T., MAYSHAR, Y., DARR, H., MCELHANEY, M., BARKER, D., YANUKA, O., KOTKOW, K.J., RUBIN, L.L., BENVENISTY, N. AND EIGES, R. (2004) Temporal gene expression during differentiation of human embryonic stem cells and embryoid bodies. *Human Reproduction* **19**, 2875-2883.

EIGES, R. (2006) Genetic manipulation of human embryonic stem cells by transfection. *Methods in Molecular Biology* **331**, 221-239.

EL-ALI, J., SORGER, P.K. AND JENSEN, K.F. (2006) Cells on chips. Nature **442**,403-411.

ELISSEEFF, J., FERRAN, A., HWANG, S., VARGHESE, S. AND ZHANG, Z. (2006) The role of biomaterials in stem cell differentiation: applications in the musculoskeletal system. *Stem Cells Development* **15**,295-303.

FLAIM C.J., CHIEN, S. AND BHATIA, S.N. (2005) An extracellular matrix microarray for probing cellular differentiation. *Nature Methods* **2**, 119-125.

FOLCH, A AND TONER ,M. (2000) Microengineering of cellular interactions. *Annual Review of Biomedical Engineering* **2**, 227-256.

GLASGOW, I.K., ZERINGUE, H.C., BEEBE, D.J., CHOI, S.J., LYMAN, J.T., CHAN, N.G. AND WHEELER, M.B. (2001) Handling individual mammalian embryos using microfluidics. IEEE Transactions on Biomedical Engineering **48**, 570-578.

GERECHT-NIR, S., COHEN, S., ZISKIND, A. AND ITSKOVITZ-ELDOR, J. (2004) Three-Dimensional Porous Alginate Scaffolds Provide a Conducive Environment for Generation of Well-Vascularized Embryoid Bodies From Human Embryonic Stem Cells. *Biotechnology and Bioengineering* 88, 313-320.

GREEN, H., EASLEY, K. AND IUCHI, S. (2003) Marker succession during the development of keratinocytes from cultured human embryonic stem cells. *Proceedings of the National Academy of Sciences of the USA* **100**, 15625–15630.

HUBBEL, J.A. (2004) Biomaterials and high-throughput screening, *Nature Biotechnology* **22**, 828-829.

HOOK, L., O'BRIEN, C. AND ALLSOPP, T. (2005) ES cell technology: an introduction to genetic manipulation, differentiation and therapeutic cloning. *Advanced Drug Delivery Review* **57**, 1904-1917.

IMAMURA, T., CUI, L., TENG, R., JOHKURA, K., OKOUCHI, Y., ASANUMA, K., OGIWARA, N. AND SASAKI, K. (2004) Embryonic stem cell-derived embryoid bodies in three-dimensional culture system form hepatocyte-like cells *in vitro* and *in vivo*. *Tissue Engineering* **10**, 1716-1724.

INGBER, D.E. (1997) Tensegrtity: The Architectural Basis of Cellular Mechanotransduction. *Annual Reviews in Physiology* **59**, 575–99

ITSKOVITZ-ELDOR, J., SCHULDINER, M., KARSENTI, D., EDEN, A., YANUKA, O., AMIT, M., SOREQ, H. AND BENVENISTY, N. (2000) Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers, *Molecular Medicine* **6**, 88-95.

KEHAT, I., KENYAGIN-KARSENTI, D., SNIR, M., SEGEV H., AMIT, M., GEPSTEIN A., LIVNE, E., BINAH, O., ITSKOVITZ-ELDOR, J. AND GEPSTEIN, L. (2001) Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *The Journal of Clinical Investigation* **108**, 407-414.

KHADEMHOSSEINI, A., LANGER, R., BORENSTEIN, J., VACANTI, J.P. (2006) Microscale technologies for tissue engineering and biology. *Proceedings of the National Academy of Sciences of the USA* **103**, 2480-2487.

KHADEMHOSSEINI, A., YEH, J., JON, S.Y., ENG, G., SUH, K.Y., BURDICK, J., LANGER, R. (2004) Molded polyethylene glycol microstructures for docking and shear protecting cells within microfluidic channels, *Lab on a chip* **4**, 425-430.

KHADEMHOSSEINI, A., SUH, K.Y., YANG, J.M., ENG, G., YEH, J., LEVENBERG, S. AND LANGER, R. (2004) Layer-by-layer deposition of hyaluronic acid and poly-L-lysine for patterned cell co-cultures. *Biomaterials* **25,** 3583-3592.

KIM, L., VAHEY, M.D., LEE H.Y. AND VOLDMAN, J. (2006) Microfluidic arrays for logarithmically perfused embryonic stem cell culture. *Lab on a Chip* **6**, 394-406

LAVON, N., YANUKA, O. AND BENVENISTY, N. (2004) Differentiation and isolation of hepatic-like cells from human embryonic stem cells, *Differentiation* **72**, 230-238.

LECLERC, E., SAKAI, Y. AND FUJII, T. (2003) Cell Culture in 3-Dimensional Microfluidic Structure of PDMS, *Biomedical Microdevices* **5**, 109-114.

LECLERC, E., SAKAI, Y. AND FUJII, T. (2003) Fetal Human Hepatocytes and Endothelial Cells co-culture in microfluidic environment, In *Proceeding of 7th International Conference on Miniaturized Chemical and Biochemical Analyst System (u-TAS2003)*, Eds. Norhtrup, M.A., Jensen, K.F., Harrison, D.J., Kluwer Academic Publishers, Squaw Valley, California, USA, 271-274.

LEVENBERG, S., KHADEMHOSSEINI, A., LANGER, R. (2006) Embryonic stem cells and tissue engineering, *In: Handbook of Stem Cells*, Eds. Melton, D., Thomson, J., Gearhart, J., Hogan, B., McKay, R., Pedersen, P., West, M. and Lanza, R., Academic Press, New York, 737-764

LEVENBERG, S., KHADEMHOSSEINI, A., MCDONALD, M., FULLER, J., LANGER, R. (2006) Methods of (human) embryonic stem cell culture. In *Culture of Cells for Tissue Engineering*, Ed. Freshney, I. and Vunjak-Novakovic, G. John Wiley & Sons,New-York ,pp. 61-83.

LEVENBERG, S., BURDICK, J.A., KRAEHENBUEHL, T., LANGER, R. (2005) Neurotrophin-induced differentiation of human embryonic stem cells on three-dimensional polymeric scaffolds. *Tissue Engineering* **11**, 506-512.

LEVENBERG, S., HUANG, N.F., LAVIK, E., ROGERS, A.B., ITSKOVITZ-ELDOR, J., LANGER, R. (2003) Differentiation of human embryonic stem cells on three-dimensional polymer scaffolds. *Proceedings of the National Academy of Sciences of the USA* **100**, 12741-12746.

LEVENBERG, S., GOLUB, J.S., AMIT, M., ITSKOVITZ-ELDOR, J., LANGER, R. (2002) Endothelial cells derived from human embryonic stem cells, *Proceedings of the National Academy of Sciences of the USA* **99**, 4391–4396.

LI, Y.J., BATRA, N.N., YOU, L., MEIER, S.C., COE, I.A., YELLOWLEY, C.E., JACOBS, C.R. (2004) Oscillatory fluid flow affects human marrow stromal cell proliferation and differentiation, *Journal of Orthopaedic Research* **22**, 1283–1289.

LUDWIG T.E., LEVENSTEIN, M.E., JONES, J.M., BERGGREN, W.T., MITCHEN, E.R., FRANE, J.L., CRANDALL1, L.J., DAIGH, C.A., CONARD, K.R., PIEKARCZYK1,M.S.,LLANAS, R.A. AND THOMSON, J.A. (2006) Derivation of human embryonic stem cells in defined conditions. *Nature Biotechnology* **24**, 185-187.

MCBEATH, R., PIRONE, D.M., NELSON, C.M., BHADRIRAJU, K. AND CHEN, C.S. (2004) Cell Shape, Cytoskeletal Tension, and RhoA Regulate Stem Cell Lineage Commitment. *Developmental Cell* **6**, 483–495.

MENENDEZ, P., WANG L., AND BHATIA, M. (2005) Genetic Manipulation of Human Embryonic Stem Cells: A System to Study Early Human Development and Potential Therapeutic Applications. *Current Gene Therapy* **5**, 375-385.

MOORE, J.C., VAN LAAKE, L.W., BRAAM, S.R., XUE, T., TSANG, S.Y., WARD, D., PASSIER, R., TERTOOLEN, L.L., LI, R.A. AND MUMMERY, C.L. (2005) Human embryonic stem cells: genetic manipulation on the way to cardiac cell therapies. *Reproductive Toxicology* **20**, 377-391.

PESCE, M., AND SCHOLER, H. R. (2001) Oct-4: Gatekeeper in the Beginnings of Mammalian Development *Stem Cells* **19,** 271–278.

RICHARDS, M., TAN, S., FONG, C.Y., BISWAS, A., CHAN, W.K. AND BONGSO, A. (2003) Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. *Stem Cells* **21**, 546–556.

RICHARDS, M., FONG, Y.C., CHANG, W.K., WONG, P.C., BONGSO, A. (2002) Human feeder support prolonged undifferentiated growth of human inner cell mass and embryonic stem cells. *Nature Biotechnology* **20**, 933-935.

RIPPON, H.J. AND BISHOP, A.E. (2004) Embryonic stem cells. *Cell Proliferation* **37**, 23-34.

SAHA, S., JI, L., DE PABLO, J.J. AND PALECEK, S.P. (2006) Inhibition of Human Embryonic Stem Cell Differentiation by Mechanical Strain, *Journal of Cellular Physiology* **206**, 126–137.

SCHULDINER, M., EIGES, R., EDEN, A., YANUKA, O., ITSKOVITZ-ELDOR, J., GOLDSTEIN, R.S. AND BENVENISTY, N. (2001) Induced neuronal differentiation of human embryonic stem cells. *Brain Research* **913**, 201-205.

SEGEV, H., FISHMAN, B., ZISKIND, A ,SHULMANB M. AND ITSKOVITZ-ELDOR, J. (2004) Differentiation of human embryonic stem cells into insulin-producing clusters. *Stem Cells* **22**, 265–274.

SHIN, M., MATSUDA, K., ISHII, O., TERAI, H., KAAZEMPUR-MOFRAD, M., BORENSTEIN, J., DETMAR, M. AND VACANTI, J.P. (2004) Endothelialized Networks with a Vascular

Geometry in Microfabricated Poly(dimethyl siloxane). *Biomedical Microdevices* **6**, 269–278.

Soen, Y., Mori, A., Palmer, T.D. and Brown, P.O. (2006) Exploring the regulation of human neural precursor cell differentiation using arrays of signaling microenvironments. *Molecular Systems Biology* 2, 37-51.

Sottile, V., Thomson, A. and McWhir, J. (2003) *In vitro* osteogenic differentiation of human ES cells. *Cloning and Stem Cells* **5**, 149–155.

Stojkovic, M., Lako, M., Strachan, T. and Murdoch, A. (2004) Derivation, growth and applications of human embryonic stem Cells, *Reproduction* **128**, 259–267.

Takayama, S., Ostuni, E., LeDuc, P., Naruse, K., Ingber, D.E. and Whitesides, G.M. (2003) Selective Chemical Treatment of Cellular Microdomains Using Multiple Laminar Streams, *Chemistry & Biology* **10**, 123–130.

Takayama, S., McDonald, J.C., Ostuni, E., Liang, M.N., Kenis, P.J., Ismagilov, R.F. and Whitesides, G.M. (1999) Patterning cells and their environments using multiple laminar fluid flows in capillary networks. *Proceedings of the National Academy of Sciences of the USA* **96**, 5545-5548.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S. and Jones, J.M. (1998) Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147.

Wu, R.Z., Bailey, S.N. and Sabatini, D.M. (2002) Cell-biological applications of transfected-cell microarrays. *Trends in Cell Biology* **12**, 485-488.

Wu, X., Ding, S., Ding, Q., Gray, N.S. and Schultz, P.G. (2004) Small molecules that induce cardiomyogenesis in embryonic stem cells. *Journal of the American Chemical Society* **126**, 1590-1591.

Vogel, G. (2001) Can adult stem cells suffice? *Science* **292**, 1820.

Voldman, J., Gray, M.L. and Schmidt, MA. (1999) Microfabrication in biology and medicine. *Annual Reviews in Biomedical Engineering* **1**, 401–425

Xu, C., Inokuma, M.S., Denham, J., Golds, K., Kundu, P. , Gold, J.D. and Carpenter, M.C. (2001) Feeder-free growth of undifferentiated human embryonic stem cells. *Nature Biotechnology* **19**, 971-974.

Yamamoto, K., Takaaki, S., Watabe, T., Miyazono, K.,Yamashita, J.K., Obi, S., Ohura, N., Matsushita, A., Kamiya, A. and Ando, J. (2005) Fluid shear stress induces differentiation of Flk-1-positive embryonic stem cells into vascular endothelial cells *in vitro*. *American Journal of Physiology Heart and Circulation Physiology* **288**, H1915–H1924.

Yamazoe, H. and Iwata, H. (2005) Cell microarray for screening feeder cells for differentiation of embryonic stem cells. *Journal of Bioscience and Bioengineering* **100**, 292-296.

Zandstra, P.W. and Nagy, A. (2001) Stem cell Bioengineering. *Annual Reviews in Biomedical Engineering* **3**, 275–305.

Ziauddin, J. and Sabatini, D.M. (2001) Microarrays of cells expressing defined cDNAs. *Nature* **411**, 107-110.